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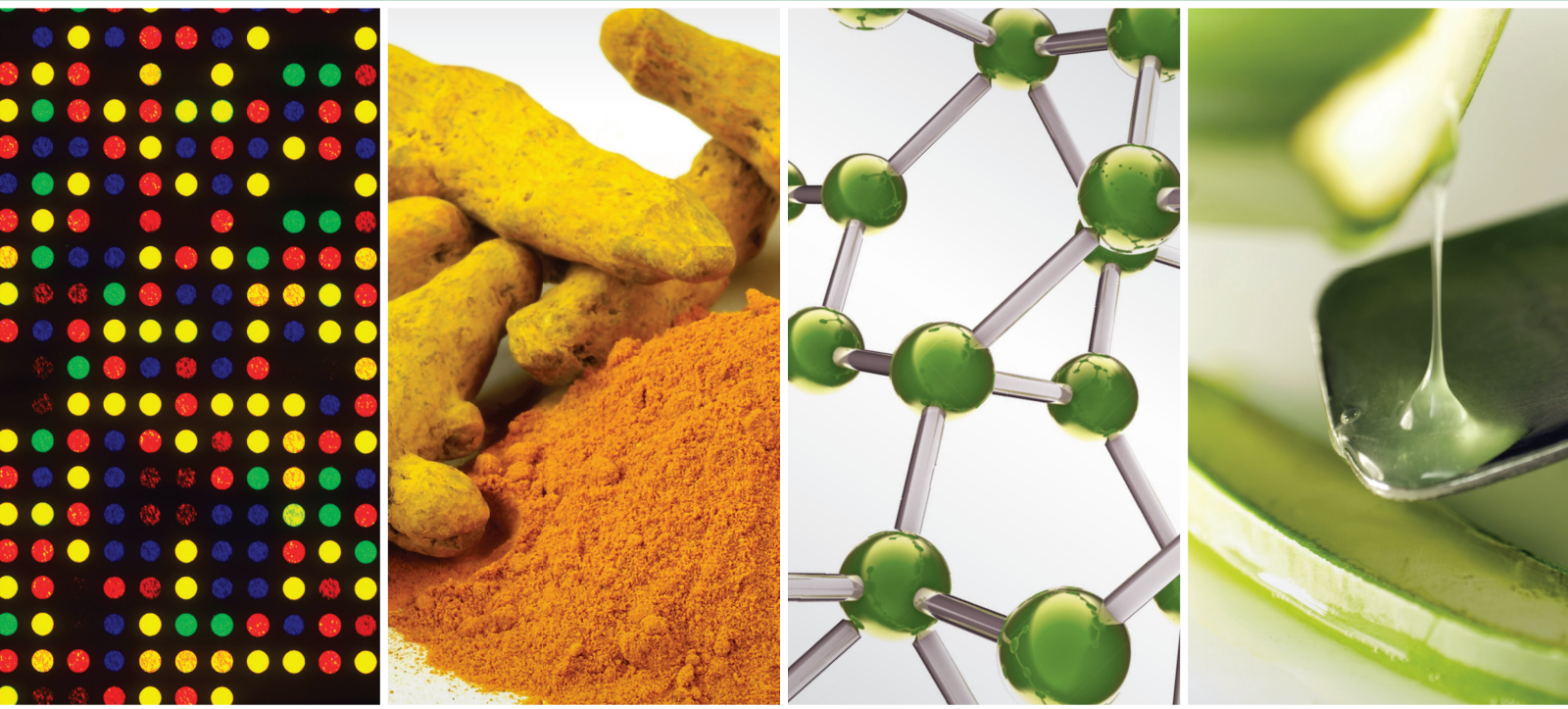
Medicinal Plants and Natural Active Compounds for Cancer Chemoprevention/ Chemotherapy

Guest Editors: Hilal Zaid, Michael Silbermann, Alaa Amash, Dan Gincel, and Claudia Bolognesi



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Editorial Board

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Editorial

Medicinal Plants and Natural Active Compounds for Cancer Chemoprevention/Chemotherapy

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Cancer is a cohort of diseases in which abnormal cells divide without control and are able to invade other tissues (through the blood and lymph systems). Cancer has devastating consequences for the patients' life and indeed is a leading cause of death worldwide. More than 100 different types of cancer are known and are usually named by the organ or type of cell where they start; for example, a cancer that begins in the colon is called colon cancer. The growing incidence of these diseases due to the rising age of the population poses a considerable burden on the public health system. Statistical studies indicate that cancer strikes more than one-third of the population and it is the cause of more than 20% of all deaths. Cancer is caused usually due to abnormalities in the DNA of the affected cells leading to an extra mass of tissue called a tumor. Tumors may be benign (not cancer) or malignant (cancer) [1, 2].

The incidence of cancer might increase in many tissues because of chronic inflammation (e.g., all gastrointestinal). Cancer may develop in cells that are selected for resistance to inflammatory products by virtue of overexpressing anti-apoptotic proteins [3]. Current treatments for cancer, besides surgery, are heavily based on cytotoxic regimens of compounds and radiation that interfere with the cellular replication system and thus aim to primarily target rapidly dividing cells. However, due to low selectivity, these treatments show many side effects and are, more importantly, becoming ineffective due to the development of resistance. A promising and new approach to fight cancer is to develop agents that abrogate the cancer's capability to become resistant. Herbal derived drugs (e.g., polyphenols, brassinosteroids, and taxol) are desired for anticancer treatment, as they are natural and readily available. Currently, a few plant products are being used to treat cancer. However, the molecular mechanism of the anticancer effect of some of those plants products and dissecting new products from those plants await further studies [2].

This special issue provides a comprehensive overview on anticancer traditional herbal medicine. The greater majority of contributions arrived from the Far East (China, Korea, Japan, and Taiwan) and the rest from Africa (Uganda, Algeria) and the Middle East (Palestine). We were highly impressed by both the design of the experiments and their high-class execution. The technologies used are up-to-date and convincing. Most of the studies in this issue applied in vitro (using established cell line) and in vivo (animal models and human patients) tests to evaluate the efficacy of anticancer medicinal plants and phytochemicals activity and their action of mechanism. The current special issue involves a relatively large variety of cancers such as leiomyoma, melanoma, hepatoma, breast, prostate, gastric, thyroid,

colorectal, osteosarcoma, and squamous cell cancer, while addressing features such as apoptosis and growth factors as the indicative factors.

By and large, this new special issue contributes a significant and substantial collection of well documented data to our armamentarium in fighting cancer. An additional issue to be emphasized is the fact that the herbal remedies are not confined to the effort to cure cancer but also to alleviate cancer patients undergoing treatment: chemotherapy. We are convinced that, by increasing the quality of life especially during the last stage of the cancer journey, we are doing an enormous service to the suffering patient and his caring relatives. As physicians, we should never forget that, concomitant with trying to cure the tumor, our obligation is to care for the patient as a human being keeping his self-dignity until his last day and hours. The nonconventional approach of using natural herbal substances provides us with an important tool to minimize pain, suffering, and hopelessness. It is for that reason that we encourage more scientists and clinicians to proceed with their scientific work of characterizing more natural compounds and try to adjust them for clinical use.

Complementary medicine has suffered for many years from lack of solid evidence in order to justify its use in clinical medicine. It appears that we have reached the phase that this claim is less and less valid, as more evidence-based products are allowed to be commercialized and used under the supervision of an authoritative professional (oncologist, palliative care specialist, pain specialist, and others).

For this special issue, the editorial office received forty-six papers and after rigorous peer review process seventeen papers were accepted for publication. The published articles deal with the physiological as well as molecular and biochemical efficacy of medicinal plants and natural active compounds in cancer and tumorigenesis treatment and prevention in vitro and in vivo. Out of the seventeen accepted manuscripts, two articles are very informative high-class review articles. Seven papers used in vivo rat model for their studies; four out of them used clinical trials. In nine publications, the authors used in vitro model utilizing colorectal, hepatoma, squamous carcinoma, leiomyoma, prostate, osteosarcoma, gastric, thyroid, melanoma, and breast cancer cell lines and primary cell culture.

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We are thankful to all contributors of this special issue for their valuable research papers. We are grateful to the reviewers for their constructive criticisms and timely response that made this special issue possible. Our sincere thanks and gratitude go to the editorial board of eCAM for inviting us to edit this special issue. The editorial board hope that readers will enjoy this special issue.

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References

[1] J. Wang, D. L. Yang, Z. Z. Chen, and B. Gou, "Associations of body mass index with cancer incidence among populations, genders, and menopausal status: a systematic review and meta-analysis," *Cancer Epidemiology*, vol. 42, pp. 1–8, 2016.

[2] H. Zaid, M. Silbermann, E. Ben-Arye, and B. Saad, "Greco-Arab and Islamic herbal-derived anticancer modalities: from tradition to molecular mechanisms," *Evidence-Based Complementary and Alternative Medicine*, vol. 2012, Article ID 349040, 13 pages, 2012.

[3] J. Daragmeh, W. Barriah, B. Saad, and Zaid, "Targeting the PI3K pathway in tumorigenesis: an in situ comprehensive study," *Oncology Letters*, vol. 11, pp. 2913–2918, 2016.

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Research Article

Clinical Effects of Xihuang Pill Combined with Chemotherapy in Patients with Advanced Colorectal Cancer

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Objective. To investigate the therapeutic effects of Xihuang pill combined with chemotherapy on advanced colorectal cancer. *Methods*. Sixty-three patients with advanced colorectal cancer were divided into an experimental group ($n = 32$) and control group ($n = 31$). Patients in the experimental group were treated with traditional Chinese medicine combined with Western medicine (i.e., Xihuang pill with FOLFOX or FOLFIRI chemotherapy), and those in the control group were treated with FOLFOX or FOLFIRI chemotherapy alone. Changes in therapeutic efficacy, side effects, blood coagulation function, and quality of life (QOL) were compared between the two groups. *Results*. The response rate was higher in the experimental than control group ($P = 0.011$). The QOL score in the experimental group was significantly lower after treatment than before treatment ($P = 0.003$), while no significant change was found in the control group. In the experimental group, the posttreatment activated partial thromboplastin time and prothrombin time after treatment were 30.05 ± 3.85 and 10.40 ± 1.25 s, respectively, which were prolonged compared with those before treatment (29.12 ± 4.03 and 9.85 ± 1.00 s; $P = 0.010$ and 0.021, respectively). *Conclusion*. In patients with advanced colorectal cancer, Xihuang pill combined with chemotherapy can significantly enhance the therapeutic effects compared with chemotherapy alone and improve patients' QOL and hypercoagulability.

1. Introduction

Colorectal cancer (CRC) is one of the most common tumors worldwide. According to epidemiological statistics, both the incidence and mortality rate of CRC ranked third among all malignant tumors in the United States in 2015 [1]. CRC is diagnosed at an advanced stage in most patients because of its occult symptoms, and the best opportunity for surgical treatment is thus lost. Although FOLFOX and FOFIRI chemotherapy are the international standards for the treatment of advanced CRC, the effect of chemotherapy is poor in most of these patients because of the high tumor load and low sensitivity to treatment as well as the patients' poor quality of life (QOL), intolerance to high-intensity chemotherapy, and hypercoagulable state with resultant predisposition to thromboembolic disease. Therefore, an understanding of the antitumor effects of traditional Chinese medicine is of great clinical significance. Xihuang pill, a classic anticancer Chinese medicine, contains four rare Chinese herbs: bezoar, musk, frankincense, and myrrh. This study was designed to investigate the effects of Xihuang pill combined with chemotherapy on the treatment efficacy, QOL improvement, and incidence of acute thrombosis in patients with advanced CRC.

2. Materials and Methods

2.1. Clinical Data. This study included 63 patients (34 male, 29 female) with advanced metastatic CRC admitted to the Oncology Department of Beijing Chaoyang Hospital from January 2013 to January 2016. The numbers of patients with pulmonary metastasis, liver metastasis, abdominal cavity and retroperitoneal lymph node metastasis, and peritoneal implantation metastasis were 24, 37, 41, and 28, respectively. The patients' ages ranged from 29 to 72 years (Table 1). All patients' diagnoses were confirmed by pathological examination, and none had a history of other tumors.

2.2. Inclusion and Exclusion Criteria. The inclusion criteria were as follows:

(i) Pathological diagnosis of CRC.

Table 1: General situation.

	Experimental group	Control group	P
Gender			0.712
Male	18 (52.9)	16 (47.1)	
Female	14 (48.3)	15 (51.7)	
Age (year)	58.13 ± 10.50	58.45 ± 10.01	0.900
ECOG	1.09 ± 0.73	1.06 ± 0.77	0.878

(ii) Treatment-naïvety with no history of radiotherapy, chemotherapy, or any type of antitumor therapy (for patients who developed relapse after adjuvant chemotherapy following radical resection, the last chemotherapy was completed within 6 months).

(iii) Eastern Cooperative Oncology Group (ECOG) physical status of ≤2 (Table 2).

(iv) Expected survival time of ≥3 months.

(v) Age of 20 to 79 years.

(vi) Measurable lesions (ability to measure at least one diameter line) identifiable by imaging or physical examination; maximum lesion diameter of ≥2 cm under conventional detection conditions or ≥1 cm by computed tomography (CT).

(vii) Neutrophilic granulocyte count of $\geq 1.5 \times 10^9$/L, platelet count of 80×10^9/L, hemoglobin concentration of ≥80 g/L, serum bilirubin concentration of ≤1.5 times the upper limit of normal, and alanine aminotransferase/aspartate aminotransferase concentrations of ≤2.5 times the upper limit of normal (≤5.0 times for patients with liver metastasis).

The exclusion criteria were as follows:

(i) Under treatment with other chemotherapies or radiotherapies.

(ii) Pregnancy, lactation.

(iii) Severe liver and renal impairment.

(iv) History of an uncontrollable mental disorder.

(v) Severe acute cardiac and cerebral vascular diseases.

2.3. Experimental Methods. Using a completely random grouping method, the patients were divided into an experimental group ($n = 32$) and a control group ($n = 31$). There were no significant differences in sex, age, or ECOG physical status between the two groups. Patients in the control group were treated with chemotherapy alone using either FOLFOX (LOHP at 85 mg/m^2 on day 1 + CF at 200 mg/m^2 on days 1 and 2 + 5-FU at 400 mg/m^2 on days 1 and 2 + 5-FU at 1200 mg/m^2 in a continuous intravenous infusion for 44 h every 14 days) or FOLFIRI (CPT-11 at 180 mg/m^2 on day 1 + CF at 200 mg/m^2 on days 1 and 2 + 5-FU at 400 mg/m^2 on days 1 and 2 + 5-FU at 1200 mg/m^2 in a continuous intravenous infusion for 44 h every 14 days). Patients in the experimental group were treated with one of the above chemotherapy regimens plus oral Xihuang pill (3 g/bottle; Beijing Tongrentang Group Co., Ltd.) administered at 3 g twice a day. In both groups, treatment efficacy was determined after four cycles of chemotherapy (one course of treatment was 56 days).

2.4. Clinical Outcome Measures. The clinical outcome measures were as follows:

(i) Routine blood tests, biochemical tests for liver and kidney function, and blood coagulation function testing before and after four cycles of chemotherapy.

(ii) Imaging examinations, such as CT and magnetic resonance imaging, before and after four cycles of chemotherapy.

(iii) Evaluation of ECOG physical status and chemotherapy-related toxicity, such as bone marrow suppression and gastrointestinal reactions. Toxicity evaluation was performed with reference to the Common Terminology Criteria for Adverse Events.

2.5. Response Evaluation Criteria in Solid Tumors (RECIST) Evaluation Criteria (Table 3). The following RECIST criteria were assessed:

(i) Measurable lesions (presence of at least one lesion with a diameter that could be accurately measured; the longest diameter was measured)

Tumorous Lesions

(a) Longest diameter of ≥10 mm by vernier caliper during clinical examination

(b) Longest diameter of ≥20 mm on chest radiograph and ≥10 mm on spiral CT (thinner scan is used if ≤5 mm on spiral CT)

Malignant Lymph Nodes

(a) Shortest lymph node diameter of ≥15 mm on spiral CT (thinner scan is used if ≤5 mm on spiral CT)

(ii) Target lesion selection

Selection of up to five measurable lesions, with up to two for each organ

(iii) Target lesion evaluation

2.6. Statistical Methods. Statistical analysis was performed using SPSS 13.0 software (SPSS Inc., Chicago, IL, USA). Measurement data are presented as mean ± standard deviation and enumeration data as frequency (rate). An independent-sample t-test was used for between-group comparisons, and a paired t-test was used for pre- and posttreatment intragroup comparisons. The data were not consistent with normality, and comparisons between groups were performed with the Mann–Whitney U test. The paired data were compared with the Wilcoxon signed rank test. A P value of <0.05 was considered statistically significant.

Table 2: Performance status by ECOG score standard (ZPS, 5 points).

Grade	Performance status
0	Fully active, able to carry on all predisease performance without restriction
1	Restricted in physically strenuous activity but ambulatory and able to carry out work of a light or sedentary nature, for example, light house work, office work
2	Ambulatory and capable of all self-care but unable to carry out any work activities, up to and about more than 50% of waking hours
3	Capable of only limited self-care; confined to bed or chair more than 50% of waking hours
4	Cannot carry on any self-care, totally confined to bed or chair
5	Dead

Table 3: Evaluation criteria of chemotherapy for solid tumors.

Response assessment	Evaluation criteria
CR	All target lesions have disappeared during the course of treatment and the pathological lymph nodes are reduced to <10 mm
PR	Decreases of at least 30% from base line have been noted in the sum of LD of target lesions
PD	There has been an increase of at least 20% in the sum of the LD of targeted lesions, and it is emphasized that the absolute value of the increased sum of LD is 5 mm, or new lesions appeared
SD	Between PD and PR

Table 4: Comparison of response rates.

Group	PR	SD	PD	RR (%)	*P* value
Experimental group	15	9	8	46.88	0.011
Control group	7	13	11	22.58	

3. Results

3.1. Therapeutic Efficacy. The response rate in the experimental group ($n = 32$) was 46.88% (complete response, $n = 0$; partial response, $n = 15$; stable disease, $n = 19$; progressive disease, $n = 8$). The response rate in the control group ($n = 31$) was 22.58% (complete response, $n = 0$; partial response, $n = 7$; stable disease, $n = 13$; progressive disease, $n = 11$). The difference in the response rates between the two groups was statistically significant (Table 4).

3.2. Tumor Markers. Before treatment, the carcinoembryonic antigen (CEA) concentration in the experimental and control groups was 66.5 and 68.0 ng/ml, respectively, with no significant difference. The CEA concentration was lower after treatment than before treatment in both the experimental and control groups (25.0 and 59.0 ng/ml, respectively); however, the difference was only statistically significant in the experimental group (Table 5).

3.3. Side Effects. No significant differences in side effects, including bone marrow suppression, gastrointestinal reactions, and abnormal liver and renal function, were found between the two groups ($P > 0.05$) (Table 6).

3.4. Coagulation Function. There were no statistically significant differences in the pretreatment activated partial thromboplastin time (APTT), prothrombin time (PT), or D-dimer concentration between the two groups (Table 7). In the experimental group, the APTT and PT were longer after treatment than before treatment, while the D-dimer concentration was lower. These differences were statistically significant. In the control group, however, no significant changes were observed in the APTT, PT, or D-dimer concentration before and after treatment (Table 8).

3.5. QOL. The pretreatment ECOG physical status in the control and experimental groups was 1.06 ± 0.77 and 1.09 ± 0.73, respectively ($P = 0.878$), without statistical significance. That after treatment was 1.16 ± 0.93 in the control group and 0.75 ± 0.57 in the experimental group. The ECOG physical status in the experimental group was significantly lower after treatment than before treatment, while in the control group, there was no significant change in the ECOG physical status before and after treatment (Table 9).

4. Discussion

Xihuang pill, a classic anticancer Chinese medicine, is composed of four rare Chinese herbs: bezoar, musk, frankincense, and myrrh. The combination of these four drugs powerfully clears away heat and toxic material and promotes blood circulation to remove blood stasis. These effects are made possible by the ability of bezoar to clear heat and detoxify, the ability of musk to activate blood stagnation and expel blood stasis, and the ability of frankincense and myrrh to promote blood and vital energy circulation and decrease swelling and pain. The four drugs are combined and made into pills with steamed rhubarb rice, which protects the physiological function of the stomach and eliminates pathogenic factors without injury. Xihuang pill alone or combined with other Western antitumor therapies is applied in the treatment of various malignant tumors and shows preferable antitumor effects on

Table 5: Comparison of CEA.

Groups	n	Pretreatment; ng/ml	Posttreatment; ng/ml	P
Experimental group	32	66.5 (27.3, 462.5)	25.0 (15.5, 117.5)	0.000
Control group	31	68.0 (25.0, 523.0)	59.0 (32.0, 410.0)	0.074
P		0.837	0.033	

Table 6: Side effects ($n = 63$).

Group	Bone marrow suppression	Gastrointestinal reaction	Abnormal liver function	Abnormal renal function
Experimental group	20	28	8	0
Control group	19	29	8	0
P value	0.674	1.000	0.941	—

Table 7: Coagulation function before treatment ($n = 63$).

Group	APTT (seconds)	PT (seconds)	D-dimer (mg/L)
Experimental group	29.12 ± 4.03	9.85 ± 1.00	1.26 ± 0.83
Control group	28.86 ± 3.93	9.61 ± 0.95	1.42 ± 1.39
P value	0.798	0.340	0.578

Table 8: Changes of coagulation function before and after treatment ($n = 63$).

Group		APTT (seconds)	PT (seconds)	D-dimer (mg/L)
Experimental group	Before treatment	29.12 ± 4.03	9.85 ± 1.00	1.26 ± 0.83
	After treatment	30.05 ± 3.85	10.40 ± 1.25	0.85 ± 0.44
	P value	0.010	0.021	0.010
Control group	Before treatment	28.86 ± 3.93	9.61 ± 0.95	1.42 ± 1.39
	After treatment	29.09 ± 3.71	9.75 ± 0.92	1.37 ± 1.20
	P value	0.053	0.155	0.625

Table 9: Changes of ECOG ($n = 63$).

Group	Time	ECOG	P value
Experimental group	Before treatment	1.09 ± 0.73	
	After treatment	0.75 ± 0.57	0.003
Control group	Before treatment	1.06 ± 0.77	
	After treatment	1.16 ± 0.93	0.5

breast cancer, lymphoma, esophageal cancer, ovarian cancer, and primary liver cancer [2–7].

Few clinical studies on the antitumor effects of Xihuang pill in the treatment of CRC have been reported to date. The present study has shown that Xihuang pill combined with chemotherapy (experimental group) has a significantly better response rate than chemotherapy alone (control group) when used as first-line treatment for advanced CRC, indicating that Xihuang pill is a promising adjuvant therapy that enhances the effectiveness of antitumor treatment. The concentration of CEA, a sensitive tumor marker of CRC, decreased in both groups after treatment; however, while the CEA concentration in the experimental group was significantly lower than that before treatment, there was no significant change in the control group. This further proves that Xihuang pill combined with chemotherapy provides better treatment control and reduces the tumor load further than does chemotherapy alone. Several possible reasons for this increased efficacy are as follows. First, Xihuang pill is a compounded Chinese medicine preparation that consists of four herbs: bezoar, musk, frankincense, and myrrh. Frankincense and myrrh contain a substantial amount of volatile oils, mainly a variety of alkenes, including β-, γ-, and δ-elemene. Alkenes exert an antitumor effect through their cytotoxic effects and enhance the immune function of the body [8, 9]. Detailed investigations of the antitumor mechanism of Xihuang pill have been performed in recent years. Duo et al. [10] established a subcutaneously transplanted human colon cancer model in nude mice and found that Xihuang pill inhibited the phosphorylation of extracellular signal-regulated protein kinases 1/2 (ERK1/2) and repressed the mitogen activated protein kinase signaling pathway, thereby inhibiting the proliferation of the human colon cancer xenografts and delaying tumor growth. These results demonstrate that Xihuang pill exerts an inhibitory effect on tumor cell proliferation. In addition, Xihuang pill regulates the expression of E and N cadherin through regulation of the ERK pathway and ZEB1-SCRIB cycle and represses the epithelial-mesenchymal transition of human colon cancer cells, thus inhibiting the invasion and metastasis of tumor cells [11].

In the present study, the ECOG performance status in the experimental group was significantly higher after treatment than before treatment, and the difference was statistically significant. This suggests that Xihuang pill combined with chemotherapy can significantly improve patients' QOL and increase the survival benefit. The main mechanism may involve the potent antitumor effect of Xihuang pill. Patients' QOL may be improved when their tumors are under control and symptoms are relieved. Additionally, Xihuang pill may also improve QOL of patients with advanced cancer by enhancing the body's immune function. Our study findings demonstrate that Xihuang pill plays a regulatory role in the tumor mass and immune microenvironment. In a previous study, ethanol extract of Xihuang pill increased the expression of interleukin-2 and interferon-γ, decreased the expression of interleukin-10, and regulated the ratios of CD3+, CD4+, and CD8+ T lymphocytes in the peripheral blood of rats with tumors [12]. Lan and Peng [13] included Xihuang pill combined with other antitumor therapy in the treatment of postoperative patients with CRC and patients with nasopharyngeal carcinoma undergoing chemotherapy, with an average observation time of 2 years and 7 months. The results showed that long-term use of Xihuang pill after conventional antitumor therapies helps to improve symptoms and prevent the recurrence and metastasis of tumors.

Approximately 90% of patients with malignant tumors are reportedly in a state of hypercoagulability. The application of traditional Chinese medicine that promotes blood circulation to remove blood stasis may improve this hypercoagulability and relieve or prevent its associated adverse events, therefore enhancing patients' QOL. In this study, the effect of Xihuang pill on blood coagulation function was also explored; in the experimental group, the APTT and PT were higher and the D-dimer concentration was lower after treatment than before treatment. The differences were of statistical significance. Therefore, this study has demonstrated that Xihuang pill has a significant curative effect on the hypercoagulative state of patients with CRC. Liu et al. [14] evaluated a subgroup of 80 patients with malignant tumors and found that application of Xihuang pill could safely improve the blood hypercoagulative state and reduce the platelet count and aggregation rate. According to the basic treatment principles of malignant tumors documented in traditional Chinese medicine literature (i.e., reducing phlegm and resolving masses, promoting blood circulation and detoxification, promoting the body's resistance, eliminating pathogenic factors, and purging and tonifying in combination [15]), Xihuang pill can improve patients' hypercoagulative state by its therapeutic effects of heat-clearing and detoxification, activating blood circulation and removing blood stasis, decreasing swelling, and relieving pain.

In summary, the results of this study show that Xihuang pill may improve the response rate, inhibit tumor growth, enhance the QOL, and reverse the hypercoagulative state in patients with CRC.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

References

[1] R. L. Siegel, K. D. Miller, and A. Jemal, "Cancer statistics, 2015," *CA Cancer Journal for Clinicians*, vol. 65, no. 1, pp. 5–29, 2015.

[2] R. Hong, Y.-Q. Wu, and Y. Wu, "Effects of Xihuangwan in assistant treatment of patients with advanced breast cancer," *China Journal of Chinese Materia Medica*, vol. 39, no. 6, pp. 1120–1123, 2014.

[3] G. Pan, W. Wang, L. Wang et al., "Anti-breast cancer effects and mechanisms of Xihuang pill on human breast cancer cell lines," *Journal of Traditional Chinese Medicine*, vol. 33, no. 6, pp. 770–778, 2013.

[4] L. Y. Wang and H. F. Li, "Xihuang Pill with CHOP chemotherapy Treatment of 60 cases of non Hodgkin's lymphoma," *Journal of Shandong University of TCM*, vol. 36, no. 4, pp. 313–315, 2012.

[5] Z. Q. Cheng and W. T. Zhu, "Clinical observation on Xihuang Pill combined with chemotherapy in treating 18 cases of advanced esophageal cancer," *China Journal of Traditional Chinese Medicine and Pharmacy*, vol. 25, no. 8, pp. 1302–1304, 2010.

[6] Y. Q. Guan, J. Q. Liu, and X. F. Wu, "Xihuang Pill combined with TP regimen in the treatment of advanced ovarian cancer after operation and curative effect the effect of immune function," *Hebei Medica Journal*, vol. 37, no. 22, pp. 3429–3431, 2015.

[7] B. Liu, S. Yu, and L. Xing, "Analysis of therapeutical effects of Xihuang Pills with intraarterial intervention chemotherapy on 80 cases of advanced primary hepatic carcinoma," *China Journal of Traditional Chinese Medicine and Pharmacy*, vol. 25, no. 6, pp. 947–948, 2010.

[8] Y.-Y. Cai, Y.-Y. Xia, Y. Gu, C.-X. Liu, and D.-Y. Si, "Analysis on in vivo metabolic pathways of 3-acetyl-11-keto-β-boswellic acid in rats," *Chinese Traditional and Herbal Drugs*, vol. 44, no. 17, pp. 2427–2432, 2013.

[9] N. Xue and H. S. Lin, "Immune editing theory and anti tumor immunity of traditional Chinese medicine," *Journal of Traditional Chinese Medicine*, vol. 53, no. 21, pp. 1801–1804, 2012.

[10] Y. H. Duo, L. N. Sun, S. L. Ying et al., "Effects of Xihuang Pill on the growth of human colorectal cancer cell xenografts in nude mice through the ERK/MAPK pathway," *China Journal of Traditional Chinese Medicine and Pharmacy*, vol. 28, no. 10, pp. 3055–3058, 2013.

[11] M. Wang, J.-Y. Meng, and S.-F. He, "Xihuang Pill (西黄丸) induces mesenchymal-epithelial transition and inhibits loss of apical-basal polarity in colorectal cancer cell through regulating ZEB1-SCRIB Loop," *Chinese Journal of Integrative Medicine*, vol. 20, no. 10, pp. 751–757, 2014.

[12] J. Ma, S. Guan, W. Yang et al., "Experimental study on the effect of Xihuang Pill ethanol extract on immune function of tumor-bearing rats," *Pharmacology and Clinics of Chinese Materia Medica*, vol. 29, no. 4, pp. 24–26, 2013.

[13] L. X. Lan and X. S. Peng, "Xihuang Capsules applied in improving the quality of life of cancer patients," *Contemporary Medicine*, vol. 16, no. 2, pp. 134–135, 2010.

[14] C. Q. Liu, G. H. Li, W. D. Liu et al., "Clinical study of Xihuang Pill in patients with malignant tumors to improve the hypercoagulable state," *Modern Journal of Integrated Traditional Chinese and Western Medicine*, vol. 24, no. 30, pp. 3340–3342, 2015.

[15] Y. Zheng, P. Sun, Q. Dong et al., "Thinking and methods of Traditional Chinese Medicine(TCM) treatment for cancer related hypercoagulation," *China Cancer*, vol. 22, no. 12, pp. 1011–1014, 2013.

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Review Article

Dendrobium officinale Kimura et Migo: A Review on Its Ethnopharmacology, Phytochemistry, Pharmacology, and Industrialization

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Ethnopharmacological Relevance. Dendrobii Officinalis Caulis, the stems of *Dendrobium officinale* Kimura et Migo, as a tonic herb in Chinese materia medica and health food in folk, has been utilized for the treatment of yin-deficiency diseases for decades. *Methods*. Information for analysis of *Dendrobium officinale* Kimura et Migo was obtained from libraries and Internet scientific databases such as PubMed, Web of Science, Google Scholar, ScienceDirect, Wiley InterScience, Ingenta, Embase, CNKI, and PubChem. *Results*. Over the past decades, about 190 compounds have been isolated from *Dendrobium officinale* Kimura et Migo. Its wide modern pharmacological actions in hepatoprotective effect, anticancer effect, hypoglycemic effect, antifatigue effect, gastric ulcer protective effect, and so on were reported. This may mainly attribute to the major and bioactive components: polysaccharides. However, other small molecule components require further study. *Conclusions*. Due to the lack of systematic data of *Dendrobium officinale*, it is important to explore its ingredient-function relationships with modern pharmacology. Recently, studies on the chemical constituents of *Dendrobium officinale* concentrated in crude polysaccharides and its structure-activity relationships remain scant. Further research is required to determine the *Dendrobium officinale* toxicological action and pharmacological mechanisms of other pure ingredients and crude extracts. In addition, investigation is needed for better quality control and novel drug or product development.

1. Introduction

Dendrobium officinale Kimura et Migo spreads in several countries over the world, such as Japan, United States, and Australia, and it distributes more widely in China [1] (Table 1). Modern pharmacology research has confirmed that *Dendrobium officinale* and its polysaccharide fraction possess anticancer, hepatoprotective, hypolipidemic, antifatigue, antioxidant, anticonstipation, hypoglycemic, gastric ulcer protective, and antihypertensive effects, immunoenhancement, and so on [2–4].

Since 1994, polysaccharides of *Dendrobium officinale* have been extracted and analyzed [5], and polysaccharides gradually became research focus of *Dendrobium officinale*. Recently, the chemical compositions and pharmacological effects of *Dendrobium officinale* have attracted more and more attention. In this review, from substantial data about *Dendrobium officinale*, approximately 190 monomer compositions and plenty of pharmacological researches studied in vivo and/or in vitro were summarized. In addition, the development of artificial cultivation of *Dendrobium officinale* accelerates the promotion of its industrialization in China. Patents and health care products were also improved by the combination of production and research mode.

2. Pharmaceutical Botany

Dendrobium, pertaining to the second largest family, Orchidaceae, includes approximately l,400 species worldwide distributed from tropical Asia to Oceania [6]. There are 74 species and 2 variants of genus *Dendrobium* Sw. in China [7]. Some of them can be processed as Dendrobii Caulis

Table 1: The taxonomic classification, names, and distribution of *Dendrobium officinale* Kimura et Migo.

Synonyms	*Dendrobium catenatum* Lindl., Gen. Sp. Orchid. Pl.: 84 (1830).	*Dendrobium stricklandianum* Rchb.f., Gard. Chron., n.s., 7: 749 (1877).	*Callista stricklandiana* (Rchb.f.) Kuntze, Revis. Gen. Pl. 2: 655 (1891).	*Dendrobium tosaense* Makino, J. Bot. 29: 383 (1891).	*Dendrobium pere-fauriei* Hayata, Icon. Pl. Formosan. 6: 70 (1916).	*Dendrobium tosaense var. pere-fauriei* (Hayata) Masam., J. Soc. Trop. Agric. 4: 196 (1933).	*Dendrobium officinale* Kimura & Migo, J. Shanghai Sci. Inst. 3: 122 (1936).
Taxonomic classification	Species 2000 & ITIS Catalogue of Life: Plantae > Tracheophyta > Liliopsida > Asparagales > Orchidaceae > Dendrobium > *Dendrobium catenatum* Lindl.	IUCN Red List: Plantae > Tracheophyta > Liliopsida > Orchidales > Orchidaceae > Dendrobium > *Dendrobium officinale*	NCBI Taxonomy: Eukaryota > Viridiplantae > Streptophyta > Streptophytina > Embryophyta > Tracheophyta > Euphyllophyta > Spermatophyta > Magnoliophyta > Mesangiospermae > Liliopsida > Petrosaviidae > Asparagales > Orchidaceae > Epidendroideae > Epidendroideae incertae sedis > Dendrobiinae > Dendrobium > *Dendrobium catenatum*	Taxonomic Hierarchy of COL-China 2012: Plantae > Angiospermae > Monocotyledoneae > Microspermae > Orchidaceae > Dendrobium > *Dendrobium tosaense*	Tropicos resource: Equisetopsida C. Agardh > Asparagales Link > Orchidaceae Juss. > Callista Lour. > *Callista stricklandiana* Kuntze	Tropicos resource: Equisetopsida C. Agardh > Asparagales Link > Orchidaceae Juss. > Dendrobium Sw. > *Dendrobium officinale* Kimura & Migo	
Distribution	Japan > Kyushu	United States > Missouri > Saint Louis City		Australia > New South Wales		China > Taiwan, Anhui, Zhejiang, Fujian, Guangxi, Sichuan, Yunnan, Xizang, etc.	
Common name	鐵皮石斛 *(tiepishihu)*			黃石斛 *(huangshihu)*			
Reference	http://www.kew.org/ http://www.theplantlist.org/		http://www.catalogueoflife.org/ http://www.tropicos.org/	http://www.gbif.org/ http://www.eol.org/	http://frps.eflora.cn/	[50] [51]	[10]

(a) (b)

Figure 1: (a) *Dendrobium officinale* Kimura et Migo; (b) *tiepifengdou*.

(Chinese: 石斛, *shihu*) of Chinese materia medica [8]. In 2005 Chinese Pharmacopoeia (ChP), *tiepishihu* (Chinese: 鐵皮石斛) was one species of medicinal *shihu* called *Dendrobium candidum* Wall. ex Lindl. [9]; however this Latin name was later disputed and considered the synonym of *Dendrobium moniliforme* (L.) Sw. [10, 11]. In 2010 ChP, *tiepishihu* was renamed as *Dendrobium officinale* Kimura et Migo (aka *Dendrobium officinale*, Figure 1(a)). This Latin name was firstly denominated by two Japanese scholars in 1936 when they found the new species of *Dendrobium* in China [12]. Facilitating the preservation, the fibrous stems of *Dendrobium officinale* can be twisted into a spiral and dried as *tiepifengdou* (Figure 1(b)) or directly cut into sections and dried as well. What is more, in 2010 ChP, *tiepishihu* was initially isolated as one single medicine; its processed stem was called Dendrobii Officinalis Caulis (valid botanical name: *Dendrobium catenatum* Lindl., taxonomic classification and synonyms in Table 1); in the meanwhile, *Dendrobium nobile* Lindl., *Dendrobium chrysotoxum* Lindl., *Dendrobium fimbriatum* Hook., and their similar species were collectively referred to as *shihu* [13].

3. Ethnopharmacological Use

The stems of *Dendrobium officinale* are mainly used as health food in folk. There are a variety of methods to enjoy its fresh stems, for example, chewing, juicing, decocting, and making dishes [14]. In addition, combining with other tonic Chinese herbs is viable, such as "Panacis Quinquefolii Radix" (*xiyangshen*, American Ginseng), "Lycii Fructus" (*gouqizi*, Barbary Wolfberry Fruit), and "Dioscoreae Rhizoma" (*shanyao*, Rhizome of Common Yam) [15]. As reported, the combination of *Dendrobium officinale* and American Ginseng could strengthen cell-mediated immunity, humoral immunity, and monocyte-macrophages functions of mice [16].

The functions and indications of *tiepishihu* and *shihu* are quite similar in traditional Chinese medicine (TCM) [17, 18]. However, TCM physicians use *shihu* more frequently than *tiepishihu*. As precious medicinal materials, maybe the high price limits the application of *tiepishihu*. Because of the output promotion, *Dendrobium officinale* gradually got more applications, especially in recent decades. Research showed that low-grade fever after gastric cancer operations, atrophic gastritis, mouth ulcers, and diabetes were treated by the fresh *Dendrobium officinale* in TCM [19]. Combined with modern medicine, *Dendrobium officinale* can be used to treat many diseases including Sjögren's syndrome, gastric ulcer, alcoholic liver injury, chronic obstructive pulmonary disease, diabetes, obesity, rheumatoid arthritis, hypertensive stroke, cataract, weak constitution, or subhealth [2, 20–22]. However, these functions were from an earlier investigation and need further research to probe.

4. Phytochemistry

From *Dendrobium officinale*, at least 190 compounds by far were isolated, mainly including polysaccharides, phenanthrenes, bibenzyls, saccharides and glycosides, essential oils, alkaloids, and other compounds (Table 2).

4.1. Polysaccharides. Polysaccharides are the main component in dried *Dendrobium officinale*. Extraction and quantitative and qualitative analysis are described as follows, and the pharmacological effects of polysaccharides are in Table 3.

4.1.1. Extraction. Generally, there are three methods that can isolate crude *Dendrobium officinale* polysaccharides (DOP): a solvent method (water extraction by alcohol sedimentation), a biological method (enzyme extraction), and a physical method (ultrasonic extraction and microwave extraction). Among the three, the solvent method is most popular. The physical method could be combined with the biological and solvent methods to accelerate the extraction process and promote the efficiency [23].

Since there are lipids, proteins, pigments, and other impurities after the process of separation of polysaccharides, other separation methods, such as ion-exchange chromatography, gel filtration chromatography, and HPLC, are needed to purify the crude polysaccharides [24]. However, polysaccharides are acknowledged as complex biological macromolecules; the ways and sequences of connections of the monosaccharides determine the difficulty of polysaccharides' analyses [25].

Table 2: Chemical components in *Dendrobium officinale*.

Number	Compounds	PubChem CID	Ref.
Phenanthrenes			
1	2,3,4,7-Tetramethoxyphenanthrene		[52]
2	2,5-Dihydroxy-3,4-dimethoxyphenanthrene		[52]
3	1,5-Dicarboxy-1,2,3,4-tetramethoxyphenanthrene		[52]
4	2,4,7-Trihydroxy-9,10-dihydrophenanthrene	21678577	[41]
5	Denbinobin	10423984	[41]
6	Erianin	356759	[53]
7	1,5,7-Trimethoxyphenanthrene-2,6-diol	11779542	[52]
8	3,4-Dimethoxyphenanthrene-2,7-diol	158975	[52]
9	2,4-Dimethoxyphenanthrene-3,5-diol	44445443	[52]
Bibenzyls			
10	3,4-Dihydroxy-5,4′-dimethoxybibenzyl		[54]
11	Chrysotobibenzyl	3086528	[53]
12	4′,5-Hydroxy-3,3′-dimethoxybenzyl		[55]
13	3,4′-Dihydroxy-5-methoxybibenzyl		[41]
14	Dendrocandin A	102476850	[56]
15	Dendrocandin B	91017475	[56]
16	Dendrocandin C	25208514	[56]
17	Dendrocandin D	25208516	[56]
18	Dendrocandin E	25208515	[56]
19	Dendrocandin F		[56]
20	Dendrocandin G		[56]
21	Dendrocandin H		[56]
22	Dendrocandin I	101481782	[56]
23	Dendrocandin J		[56]
24	Dendrocandin K		[56]
25	Dendrocandin L		[56]
26	Dendrocandin M		[56]
27	Dendrocandin N		[56]
28	Dendrocandin O		[56]
29	Dendrocandin P		[56]
30	Dendrocandin Q		[56]
31	4,4′-Dihydroxy-3,5-dimethoxybibenzyl	442701	[56]
32	4,4′-Dihydroxy-3,3′,5-trimethoxybibenzyl	176096	[56]
33	3,4,4′-Trihydroxy-5-methoxybibenzyl		[57]
34	Dendromoniliside E		[41]
35	Gigantol	3085362	[54]
Phenols			
36	4-(3,5-Dimethoxyphenethyl) phenol		[57]
37	3-(4-Hydroxyphenethyl)-5-methoxyphenol		[57]
38	5-(3-Hydroxyphenethyl)-2-methoxyphenol		[57]
39	4-(4-Hydroxyphenethyl)-2,6-dimethoxyphenol		[57]
40	4-(4-Hydroxy-3-methoxyphenethyl)-2,6-dimethoxyphenol		[57]
Acids			
41	*p*-Hydroxy-coumaric acid	637542	[55]
42	*p*-Hydroxybenzene propanoic acid		[55]
43	Hexadecanoic acid	985	[41]
44	Heptadecanoic acid	10465	[41]
45	Syringic acid	10742	[58]
46	Vanillic acid	8468	[58]
47	*p*-Hydroxy-phenylpropionic acid	10394	[58]

Table 2: Continued.

Number	Compounds	PubChem CID	Ref.
48	Ferulic acid	445858	[58]
49	4-Hydroxybenzoic acid	135	[58]
Esters			
50	*p*-Hydroxyl-trans-cinnamic acid myricyl ester		[41]
51	Trans-ferulic acid octacosyl ester		[41]
52	*p*-Hydroxyl-*cis*-cinnamic acid myricyl ester		[41]
53	Dihydroconiferyl dihydro-*p*-cumarate		[58]
54	Cis-3-(4-Hydroxy-3-methoxy-phenyl)-acrylic acid octacosyl ester		[57]
55	Trans-3-(4-Hydroxy-3-methoxy-phenyl)-acrylic acid octacosyl ester		[57]
56	4-[2-(4-Methoxy-phenyl)-ethyl-6]-oxo-6H-pyran-2-carboxylic acid methyl ester		[57]
Amides			
57	N-Trans-feruloyltyramine	5280537	[55]
58	*cis*-Feruloyl *p*-hydroxyphenethylamine		[55]
59	Trans cinnamyl p-hydroxyphenethylamine		[55]
60	N-*p*-Coumaroyltyramine	5372945	[58]
61	Dihydroferuloyltyramine	90823368	[58]
62	4-Hydroxy-N-[2-(4-hydroxyphenyl)ethyl]benzenepropanamide		[58]
Saccharides and glycosides			
63	4-Allyl-2,6-dimethoxy phenyl glycosidase		[55]
64	Adenosine	60961	[41]
65	Uridine	6029	[41]
66	Vernine	46780355	[41]
67	Apigenin-7-O-β-D-glucoside	5280704	[59]
68	Icariol-A2-4-O-β-D-glucopyranoside	6439218	[59]
69	(+)-Syringaresinol-O-β-D-pyranglucose		[59]
70	Dihydrosyringin	71720642	[58]
71	Vicenin 3	44257698	[55]
72	Isoschaftoside	3084995	[55]
73	Schaftoside	442658	[55]
74	Vicenin 2	442664	[55]
75	Apigenin 6-C-α-L-arabinopyranosyl-8-C-β-D-xylopyranoside		[55]
76	Apigenin 6-C-β-D-xylopyranosyl-8-C-α-L-arabinopyranoside		[55]
77	Vincenin 1	44257662	[55]
78	2-Methoxyphenol-O-β-D-apiofuromosyl-(1→2)-β-D-glucopyranoside		[60]
79	3,4,5-Trimethoxyphenyl-1-O-β-D-apiose-(1→2)-β-D-glucoside		[60]
80	Dictamnoside A	44560015	[61]
81	Leonuriside A	14237626	[60]
82	(1′R)-1′-(4-Hydroxy-3,5-dimethoxylphenyl) propan-1′-ol 4-O-β-D-glucopyranoside		[60]
83	Syringaresinol-4,4′-O-bis-β-D-glucoside		[60]
84	(+)-Syringaresinol-4-β-D-monoglucoside		[60]
85	(+)-Lyoniresinol-3a-O-β-glucopyranoside		[60]
86	3,5-Dimethoxy-4-hydroxylphenyl-1-O-β-D-pyranglucose		[59]
87	7-Methoxycoumarin-6-O-β-D-pyranglucose		[59]
88	Sucrose	5988	[41]
Essential oils			
89	Methyl acetate	6584	[44]
90	Carbon disulfide	6348	[44]
91	Hexane	8058	[44]
92	Ethyl acetate	8857	[44]
93	2-Methyl-propanol	6560	[44]
94	3-Methyl-butanal	11552	[44]

Table 2: Continued.

Number	Compounds	PubChem CID	Ref.
95	2-Methyl-butanal	7284	[44]
96	2-Pentanone	7895	[44]
97	Pentanal	8063	[44]
98	3-Pentanone	7288	[44]
99	3-Methyl-1-butanol	31260	[44]
100	2-Methyl-1-butanol	8723	[44]
101	2-Methyl-3-pentanone	11265	[44]
102	1-Pentanol	6276	[44]
103	(Z)-3-Hexanone		[44]
104	2-Hexanone	11583	[44]
105	Hexanal	6184	[44]
106	(E)-2-Hexenal	5281168	[44]
107	(E)-3-Hexanol		[44]
108	(Z)-3-Hexenol		[44]
109	1-Hexanol	8103	[44]
110	Styrene	7501	[44]
111	(E)-2-Heptenal	5283316	[44]
112	Benzaldehyde	240	[44]
113	1-Heptanol	8129	[44]
114	1-Octen-3-one	61346	[44]
115	1-Octen-3-ol	18827	[44]
116	6-Methyl-5-hepten-2-one	9862	[44]
117	2-Pentyl-furan	19602	[44]
118	Decane	15600	[44]
119	Octanal	454	[44]
120	(Z)-3-Hexenyl acetate	5363388	[44]
121	Hexyl acetate	8908	[44]
122	*p*-Cymene	7463	[44]
123	Limonene	22311	[44]
124	3-Octen-2-one	15475	[44]
125	(E)-β-Ocimene	5281553	[44]
126	(E)-2-Octenal	5283324	[44]
127	Ethyl benzyl ether	10873	[44]
128	1-Phenyl-ethanone	7410	[44]
129	(E)-2-Nonen-1-ol	5364941	[44]
130	1-Octanol	957	[44]
131	Nonanal	31289	[44]
132	2-Heptenyl acetate	85649	[44]
133	(E)-2-Nonenal	5283335	[44]
134	Nonenol	85445514	[44]
135	2-Terpineol		[44]
136	Dodecane	8182	[44]
137	2-Octenyl acetate		[44]
138	Decyl aldehyde	8175	[44]
139	Benzylacetone	17355	[44]
140	(E)-2-Decenal	5283345	[44]
141	1-Dodecanol	8193	[44]
142	Tridecane	12388	[44]
143	α-Cubebene	86609	[44]
144	β-Bourbonene	62566	[44]
145	Tetradecane	12389	[44]

Table 2: Continued.

Number	Compounds	PubChem CID	Ref.
146	α-Cedrene	442348	[44]
147	Zingiberene	92776	[44]
148	Geranyl acetone	19633	[44]
149	2-Methyl tridecane		[44]
150	AR-Curcumene	92139	[44]
151	Pentadecane	12391	[44]
152	β-Bisabolene	403919	[44]
153	(+)-D-Cadinene	441005	[44]
154	5-Phenyl-decane		[44]
155	4-Phenyl-decane		[44]
156	Hexadecane	11006	[44]
157	α-Cedrol	65575	[44]
158	5-phenyl-undecane		[44]
159	4-phenyl-undecane		[44]
160	Heptadecane	12398	[44]
161	Pristane	15979	[44]
162	6-Phenyl-dodecane	17629	[44]
163	5-Phenyl-dodecane	17630	[44]
164	4-Phenyl-dodecane	17631	[44]
165	6,10,14-Trimethyl-2-pentadecanone	10408	[44]
166	Butyl phthalate	3026	[44]
167	Isobutyl phthalate	6782	[44]
168	Sandaracopimaradiene	440909	[44]
169	Hentriacontanol	68345	[62]
170	Citronellol	8842	[55]
171	Citrusin C	3084296	[59]
172	Coniferyl alcohol	1549095	[55]
Others			
173	2,6-Dimethoxycyclohexa-2,5-diene-1,4-dione		[63]
174	(24R)-6-β-Hydroxy-24-ethlcholest-4-en-3-one		[63]
175	Dendroflorin	44418788	[63]
176	Friedelin	91472	[63]
177	3-Ethoxy-5-hydroxy-7-methoxy-1,4-phenanthra-quinone		[63]
178	β-sitosterol	222284	[62]
179	5-Hydroxymethyl furfural	237332	[41]
180	Syringaldehyde	8655	[58]
181	3-O-Methylgigantol	10108163	[54]
182	Syringaresinol	332426	[55]
183	5α,8α-Epidioxy-24(R)-methylcholesta-6,22-dien-3β-ol		[63]
184	(−)-Secoisolariciresinol	65373	[60]
185	Aduncin	101316879	[41]
186	β-Daucosterol	5742590	[62]
187	(−)-Loliolide	12311356	[41]
188	Naringenin	932	[61]
189	3′,5,5′,7-Tetrahydroxyflavanone		[61]
190	Dihydrogen resveratrol		[41]

Table 3: The pharmacological effects of *Dendrobium officinale*.

Effect	Tested material	In vivo/in vitro	Adm.	Conc.	Observations	Ref.
Hepatoprotective effect	Arabinose : mannose : glucose : galactose = 1.26 : 4.05 : 32.05 : 3.67	In vivo	Gavage	50, 100, and 200 mg/kg	Increased the weight in mice	[64]
		In vivo	Gavage	200 mg/kg	Increased liver coefficient in mice	
		In vivo	Gavage	50, 100, and 200 mg/kg	Decreased mice serum ALT, AST, ALP activity, and TBIL levels; increased serum HDL-C; decreased LDL-C levels; accelerated metabolism of serum TG and TC; increased liver ADH, ALDH activities; inhibited mRNA expression of P4502E1, TNF-α, and IL-1β	
	Arabinose : mannose : glucose : galactose = 1.26 : 4.05 : 32.05 : 3.67	In vivo	Gavage	100 and 200 mg/kg	Increased GR activity and GSH-Px activity in mice	[64]
	Original extract solution	In vivo	Gavage	3 g/kg	Reduced chronic alcoholic liver injured mice's serum ALT, AST, and TC levels	[65]
	Original extract solution	In vivo	Gavage	6 g/kg	Reduced serum TC level in mice	
	Tiepifengdou original extract solution	In vivo	Gavage	0.45, 0.9, and 1.35 g/kg	Reduced serum AST levels in mice	
	Original extract solution	In vivo	Gavage	3 g/kg	Increased acute alcoholic hepatic injured mice's SOD of serum and liver and DSG-Px of liver	[66]
	Tiepifengdou original extract solution	In vivo	Gavage	0.45, 0.90, and 1.35 g/kg	Increased acute alcoholic hepatic injured mice's SOD of serum and liver and DSG-Px of liver	
	Original extract solution	In vivo	Gavage	3 and 6 g/kg	Reduced MDA of serum and liver in mice	
	Tiepifengdou original extract solution	In vivo	Gavage	0.45 and 0.90 g/kg	Reduced MDA of serum and liver in mice	
	Original extract solution	In vivo	Gavage	9 g/kg	Reduced serum MDA in mice	
Anticancer effect	Water extraction by alcohol sedimentation, extraction rate (in dry herb): 19.2%	In vivo	Gavage	10 and 20 g/kg	Increased the level of carbon clearance indexes and NK cells activity of LLC mice ($P < 0.05$)	[67]
		In vivo	Gavage	20 g/kg	Improved the LLC mice's spleen lymphocyte transformation and hemolysin levels ($P < 0.05$)	
	Water extraction by alcohol sedimentation	In vitro	—	100, 200, and 400 μg/mL	Inhibited the growth of human hepatoma cells (HepG2), human lung cancer cells (A549), and human teratoma stem cells (NCCIT)	[68]
		In vitro	—	200 and 400 μg/mL	Inhibited murine teratoma stem cells (F9) and promoted their apoptosis	
		In vitro	—	100 μg/mL	Promoted the proliferation of mouse spleen cells	
	Water extraction and alcohol precipitation	In vivo	Gavage	—	Inhibited the growth transplantation tumor (CNE1 and CNE2) of NPC nude mice	[69]
		In vitro	—	128 and 256 mg/L	Inhibited the proliferation and induced the apoptosis of CNE1 and CNE2 cells; activated caspase-3; declined Bcl-xL, Mcl-1 protein levels	

Table 3: Continued.

Effect	Tested material	In vivo/in vitro	Adm.	Conc.	Observations	Ref.
Hypoglycemic effect	DOP: 2-O-acetylglucomannan consisted of Man, Glc, and Ara in the molar ratio of 40.2 : 8.4 : 1.0	In vivo	Gavage	200, 100, and 50 mg/kg	Decreased levels of fasting blood glucose (FBG) and glycosylated serum protein (GSP); increased level of serum insulin in alloxan induced diabetic mice; attenuated the occurrence of oxidative stress in the liver and kidney of alloxan-induced diabetic mice (decreased MDA levels; increased GSH concentrations and antioxidative enzyme activities)	[70]
	Extract, crude drug 1.8 g/g	In vivo	Gavage	0.125 and 0.25 g/kg	Reduced STZ-DM rats' blood glucose and glucagon levels; enhanced the number of islet β cells; declined the number of islet α cells	[71]
		In vivo	Gavage	0.5 and 1.0 g/kg	Decreased blood glucose and increased liver glycogen content in adrenaline induced hyperglycemia rats	
	Total polysaccharide: 43.1%, total flavonoids: 19.6%, crude drug 1 g/mL	In vivo	Gavage	TP (total polysaccharides, 100 mg/kg), TF (total flavonoids, 35 mg/kg), and TE (water extract, 6 g/kg)	Significantly downregulated the phosphorylation of JNK (Thr^{183}/Tyr^{185}) and upregulated the phosphorylation of AKT ser^{473} in rat	[72]
Antifatigue effect	Hot water reflux and cellulase lixiviating extraction	In vivo	Gavage	1.5 and 4.5 g/kg	Increased mice's glycogen store after exercise fatigue	[73]
	Hot water reflux and cellulase lixiviating extraction	In vivo	Gavage	0.75, 1.5, and 4.5 g/kg	Decreased the level of serum urea and lactic acid accumulation; upregulated the expression of CNTF mRNA in mice	
	Hot water extraction then filtration	In vivo	Gavage	0.75 mg/kg	Increased carbon clearance indexes from 0.025 to 0.034 in mice	[74]
		In vivo	Gavage	3 and 6 mg/kg	Extended burden swimming time of mice; reduced serum lactic acids of mice	[74]
Gastric ulcer protective effect	Lyophilization then hot-water extraction, evaporation	In vivo	Gavage	200 mg/kg	Decreased SD rats' gastric secretion, IL-6, and TNF-α cytokine levels; had 76.6% inhibition of gastric injury rate	[75]
	Squeezing then filtration	In vivo	Gavage	0.5 and 2 g/kg	Declined irritable and chemical gastric ulcer model's ulcer indexes in mice	[76]

Table 3: Continued.

Effect	Tested material	In vivo/in vitro	Adm.	Conc.	Observations	Ref.
	Dry powder	In vivo	Gavage	1.5 and 3 g/kg	Reduced ApoE$^{-/-}$ mice's TG, TCHOL, LDL-C levels, and expression of TNF-α and IL-6 in serum; reduced areas of atheromatous plaque in aortic valve and arch in ApoE$^{-/-}$ mice; then decreased expression of TNF-α and IL-6 in aortic arch	[77]
	Aqueous extract	In vivo	Gavage	0.25, 0.5, and 1.0 crude drug g/kg	Extended Stroke-prone spontaneously hypertensive (SHR-sp) rats' blood pressure, living days, and survival rate	[78]
Others	Water extraction by alcohol sedimentation; mannose : glucose : galactose : arabinose : xylose : glucuronic acid = 10 : 0.25 : 1.2 : 4.7 : 1.3 : 1.4	In vivo	—	20 mg/mL	Inhibited Bax/Bal-2 ratio and caspase-3 expression; decreased expression of cytokines (TNF-α, IL-1β, and IL-6) and activity of MMP-9 in mice	[79]
	Water extraction by alcohol sedimentation; mannose : glucose : galactose : arabinose : xylose : glucuronic acid = 10 : 0.25 : 1.2 : 4.7 : 1.3 : 1.4	In vitro	—	0.1, 1.0, and 10 μg/mL	Ameliorated the abnormalities of aquaporin-5 (AQP-5) on A-253 cell	[79]
	Water extract and alcohol precipitate, extraction rate: 29.87%	In vivo	Smear	5.0 g/L	Increased average score and average quality of hair growth of C57BL/6J mice	[80]
		in vitro	—	0.1, 1.0, and 5.0 mg/L	Increased HaCaT cells survival rate and the VEGF mRNA expression level	
	Dry power decoction then concentration: traditional decoction or dry power steep in hot water: ultra-fine powder decoction	In vivo	Gavage	—	Increased Shannon index and Brillouin index in mice with constipation and improved their molecular diversity of intestinal *Lactobacillus*	[81]

4.1.2. Quantitative and Qualitative Analysis. The average content of neutral sugar in desiccated *Dendrobium officinale* is up to 58.3% [26]. Actually, different production origins, processing methods, growing years, and cultivated or wild and different parts of *Dendrobium officinale* are all related to polysaccharide content. Research showed that there were various polysaccharide contents of the same species cultivated in Guangdong with different origins from Yunnan, Zhejiang, and other provinces [27]. After drying each with different methods, DOP content was arranged as follows: hot air drying > vacuum drying > vacuum freeze drying > natural drying [28]. In addition, amounts of polysaccharide were greater in biennial *Dendrobium officinale* than annual or triennial ones [29], while the wild type contained more content compared to the cultivated type [30]. Research showed that polysaccharides distributing in different parts of *Dendrobium officinale* varied: middle stem > upper stem > lower stem > roots [31]. Therefore, accurate determination of polysaccharide content is of great significance.

Fourier Transform Infrared Spectrometer (FT-IR), Gas Chromatography-Mass Spectrometer (GC-MS), and ^{1}H and ^{13}C NMR spectroscopies have been used to analyze the types of monosaccharide residues and the linkage sites of glycosidic bonds [32]. However, the research of polysaccharide's ratio of mannose to glucose, the existence of branches, and the substitution position of o-acetyl groups was inconsistent [33]. What is more, polysaccharides' pharmacological activities are strongly linked to the composition and content of their monosaccharides [26]. Therefore, further methods to explore the advanced structures and structure-activity relationships of *Dendrobium officinale* polysaccharides need to be established. Research showed that O-acetyl-glucomannan, Dendronan® (Figure 2, A), from *Dendrobium officinale* [34] has been isolated and was affirmed immunomodulatory activity in vitro [35] and in vivo [36], as well as improvement in colonic health of mice [37].

4.2. Phenanthrenes. From *Dendrobium officinale*, nine phenanthrenes **(1–9)** were isolated. *Dendrobium officinale* contains a kind of bibenzyl and a kind of phenanthrene identified in *D. chrysotoxum* before: chrysotobibenzyl **(11)** and erianin **(6)**. Both chrysotobibenzyl and erianin have antitumor effects. Erianin significantly inhibited the proliferation of HepG2 and Huh7 (human hepatoma cell lines) in vitro [38] and also prevented angiogenesis in human hepatoma Be17402 and human melanoma A375 in vivo [39]. IC_{50} of P388 murine leukemia cell treated by erianin in MTT assay was 0.11 μM [40].

4.3. Bibenzyls. Bibenzyls **10–35** were isolated from the stems of *Dendrobium officinale*. Among these compounds, 17 bibenzyls named Dendrocandins A–Q **(14–30)** (Figure 2) were extracted by Li et al. from 2008 to 2014. Next, DCET-2 **(31)** could inhibit the proliferation of A2780 (human ovarian cancer cell line) cells, and DCET-12 **(32)** inhibited BGC-823 (human gastric cancer cell lines) and A2780 cells [41]. DCET-18 **(35)** also inhibited the migratory behavior and induced the apoptosis of non-small-cell lung cancer cells (human NCI-H460 cells) [42].

4.4. Saccharides and Glycosides. In the last few decades, at least 26 saccharides and glycosides have been found from *Dendrobium officinale*, (compounds **63–88**). Among them, Dictamnoside A **(80)** showed immunomodulatory activity in mouse splenocyte assessed as stimulation of proliferation at 10 umole/L after 44 hours by MTT assay in the presence of Concanavalin A (ConA) [43].

4.5. Essential Oils. Compounds **89–172**, isolated from *Dendrobium officinale*, comprise essential oils. The content of limonene **(123)** in the stem is 9.15% for the second; limonene is most abundant in the leaves, accounting for 38% [44]. Limonene contains anticancer properties with effects on multiple cellular targets in preclinical models [45]. *Dendrobium officinale* containing a high content of (E)-2-hexenal **(106)** shows bactericidal activity against *Pseudococcus viburni*, *Pseudococcus affinis*, *Bemisia* sp., and *Frankliniella occidentalis* [46].

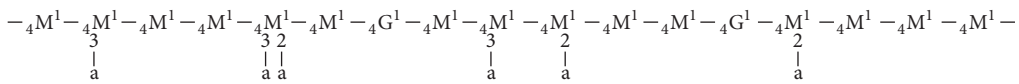
4.6. Alkaloids. Alkaloids are the earliest chemical compounds isolated from *Dendrobium* genus. Dendrobine, a kind of *Dendrobium* alkaloid accounting for 0.52%, was isolated initially in 1932 [47]. It was later proved to be the special content of *D. nobile* [48]. As reported, the alkaloid in *Dendrobium officinale* belongs to the terpenoid indole alkaloid (TIA) class, with its total content measured at approximately 0.02% [49]. However, the isolation and identification of one single kind of alkaloid have been rarely reported.

4.7. Others. Excepting ingredients above, phenols **(36–40)**, acids **(41–49)**, esters **(50–56)**, amides **(57–62)**, and other chemical constituents **(173–190)** were detected in *Dendrobium officinale* (Table 2), while their pharmacological effects remain to be excavated in the future.

5. Pharmacological Effects of *Dendrobium officinale*

Recently, more and more pharmacological actions of *Dendrobium officinale* were reported, such as hepatoprotective effect, anticancer effect, hypoglycemic effect, antifatigue effect, and gastric ulcer protective effect (Table 3).

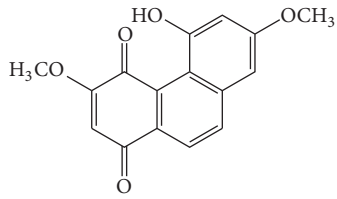
5.1. Hepatoprotective Effect. The hepatoprotective capacity of *Dendrobium officinale* is always related to its antioxidant ability, especially in acute or chronic alcoholic liver injury. Research showed that *Dendrobium officinale* polysaccharides (DOP) accelerated the metabolism of serum TG and TC, while increased liver ADH and ALDH activities, which recovered disorders of lipid metabolism and accelerated excretion of alcohol and its metabolites [64]. In addition, ALT, AST, and TC of *Dendrobium officinale* treated mice models with chronic alcoholic liver injury were elevated compared to the normal groups [65]. Besides, compared to the model group



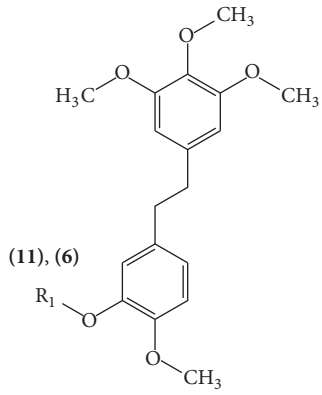
M: β-D-mannopyranose; G: β-D-glucopyranose; a: O-acetyl group

(A) Dendronan®

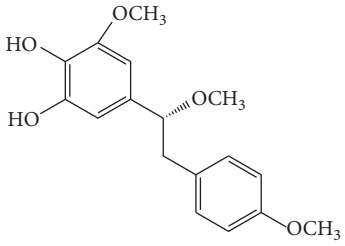
Number	R_1
11	CH_3
6	H
16	OCH_3
17	OCH_2CH_3
18	—
19	OCH_3
20	OH
21	OH
25	—
23	OCH_3
24	OH
27	—
28	OH
29	H
30	H
31	—
32	OCH_3



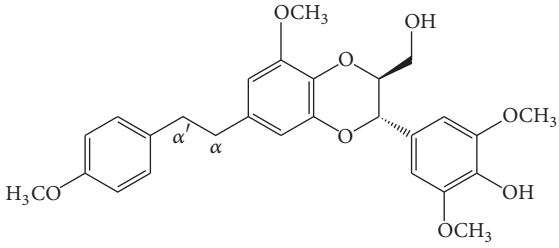
Denbinobin **(5)**



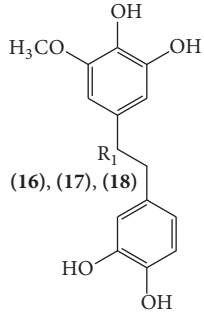
Chrysotobibenzyl **(11)**, erianin **(6)**



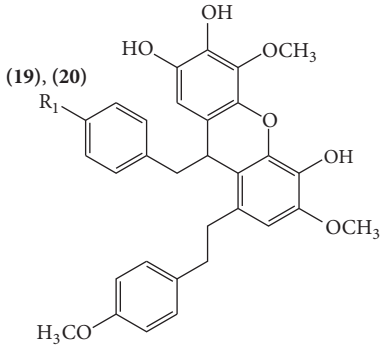
Dendrocandin A **(14)**



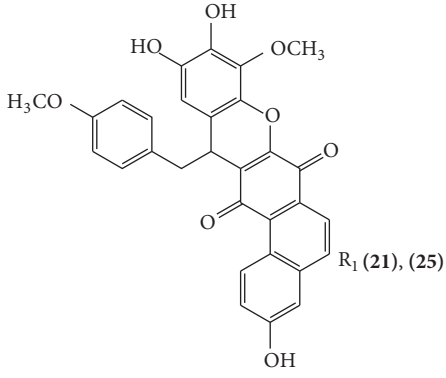
Dendrocandin B **(15)**



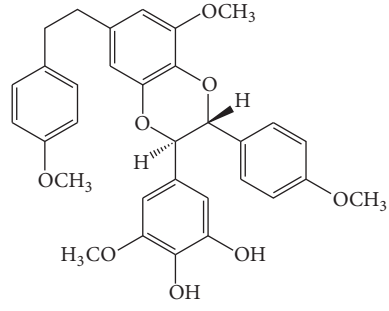
Dendrocandin C **(16)**, D **(17)**, E **(18)**



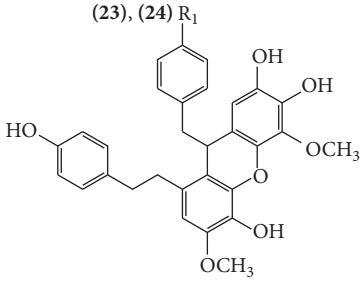
Dendrocandin F **(19)**, G **(20)**



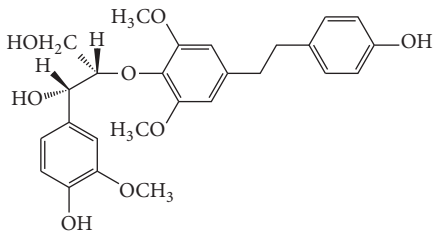
Dendrocandin H **(21)**, L **(25)**



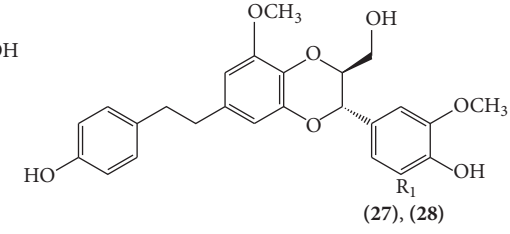
Dendrocandin I **(22)**



Dendrocandin J **(23)**, K **(24)**

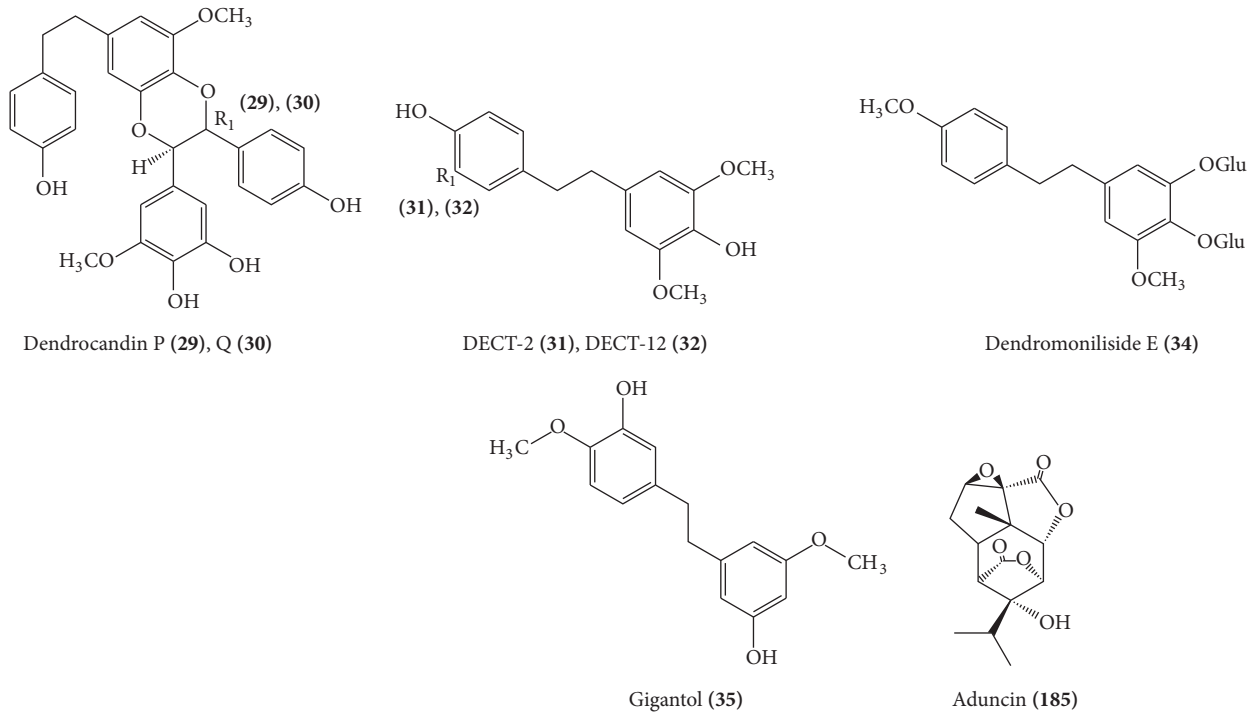


Dendrocandin M **(26)**



Dendrocandin N **(27)**, O **(28)**

Figure 2: Continued.



Dendrocandin P **(29)**, Q **(30)**

DECT-2 **(31)**, DECT-12 **(32)**

Dendromoniliside E **(34)**

Gigantol **(35)**

Aduncin **(185)**

Figure 2: Some chemical structure of compounds isolated form *Dendrobium officinale* which have good potentials.

of mice with acute alcoholic hepatic injury, fresh *Dendrobium officinale* and *tiepifengdou* groups could increase the SOD and reduce MDA of serum and liver tissue [66].

5.2. Anticancer Effect. The anticancer activity of *Dendrobium officinale* extract has been studied and proved, such as anti-HelaS3, anti-HepG2, and anti-HCT-116 in vitro, as well as anti-CNEl and CNE2 in vivo and in vitro [69]. Besides, DOP has manifested anticancer effects in mice with Lewis lung carcinoma (LLC). Its tumor inhibition rate was 8.5%–18.3% ($P > 0.05$); meanwhile, LLC mice spleen lymphocyte transformation and hemolysin levels ($P < 0.05$) were improved [67]. In cellular experiments, Liu's morphological analysis showed that DOP can inhibit the growth of human hepatoma cells (HepG2), human lung cancer cells (A549), human teratoma stem cells (NCCIT), and murine teratoma stem cells (F9) and promoted the proliferation of mouse spleen cells in vitro [68]. Research showed that two DOP fractions, DOP-1 and DOP-2, promoted splenocyte proliferation, enhanced NK cell-mediated cytotoxicity, and increased the phagocytosis and nitric oxide production of macrophages significantly ($P < 0.05$) [82]. Therefore, the anticancer effect of *Dendrobium officinale* may be accompanied by the activity of improving immune system.

5.3. Hypoglycemic Effect. Hypoglycemic activity, another important property of *Dendrobium officinale*, has been studied a lot. Oral administration of DOP decreased levels of fasting blood glucose (FBG) and glycosylated serum protein (GSP) and increased level of serum insulin in alloxan-induced diabetic mice. In addition, DOP attenuated the occurrence of oxidative stress in the liver and kidney of alloxan-induced diabetic mice by decreasing MDA levels, increasing GSH concentrations and antioxidative enzyme activities [70]. Therefore, DOP may regulate blood sugar levels through blood-lipid balance effects and antioxidative damage effects of liver and kidney.

Dendrobium officinale exhibited a significant hypoglycemic effect in adrenaline hyperglycemia mice and streptozotocin-diabetic (STZ-DM) rats [71]. In addition, TP (total polysaccharides, 100 mg/kg), TF (total flavonoids, 35 mg/kg), and TE (water extract, 6 g/kg) groups of *Dendrobium officinale* significantly downregulated the phosphorylation of JNK (Thr183/Tyr185) and upregulated the phosphorylation of AKT ser^{473} compared with the normal control group, which indicates that effective extracts of *Dendrobium officinale* have the effects of inhibiting JNK phosphorylation and promoting AKT ser^{473} phosphorylation [72].

5.4. Antifatigue Effect. The antifatigue effect of *Dendrobium officinale* was illustrated in vivo, when compared with the control groups; *Dendrobium officinale* could increase the glycogen stored in mice after exercise and decrease the level of serum urea and lactic acid accumulation ($P < 0.05$ or $P < 0.01$). In addition, it could upregulate the expression of ciliary neurotrophic factor (CNTF) mRNA ($P < 0.05$ or $P < 0.01$) [73]. In addition, compared to the control groups, *Dendrobium officinale* significantly increased carbon clearance indexes and burden swimming time and reduced the serum lactic acids [74]. In general, antifatigue effect of *Dendrobium officinal* was linked to the TCM use about

enriching consumptive diseases, but its mechanism needs to be further clarified.

5.5. Gastric Ulcer Protective Effect. Research showed that *Dendrobium officinale* had a preventive effect of gastric injury caused by 60% ethanol-hydrochloric acid solution (ethanol was dissolved in 150 mM hydrochloric acid). Intragastric administration of SD rats with 200 mg/kg of *Dendrobium officinale* for two weeks decreased gastric secretion, IL-6 and TNF-α cytokine levels compared with the lower dose groups and the control groups. This concentration (200 mg/kg) had the strongest inhibitory effect of gastric injury (76.6% inhibition of gastric injury rate) [75]. After the successful establishment of the irritable gastric ulcer model (induced by cold water immersion) and chemical gastric ulcer model (indometacin-induced, 40 mg/kg, gavage) the ulcer index was calculated by Guth standard scoring method. Freshly squeezed *Dendrobium officinale* juice (containing crude drugs 0.5, 2 g/kg) showed significant declining irritable and chemical gastric ulcer model ulcer indexes ($P < 0.01$) [76]. However, gastric ulcer is a chronic disease, the security of long-term administration of *Dendrobium officinale* still uncertain.

5.6. Others. It is found that *Dendrobium officinale* had hypolipidemic and hypotensive effects. The fine powder solution of *Dendrobium officinale* (1.5, 3 g/kg) can reduce the levels of TG, TCHOL, and LDL-C in serum significantly and reduce the expression of TNF-α and IL-6 in ApoE$^{-/-}$ mice. In addition, it could reduce areas of atheromatous plaque in aortic valve and arch in ApoE$^{-/-}$ mice and then decrease the expression of TNF-α and IL-6 in aortic arch [77]. Research showed that stroke-prone spontaneously hypertensive (SHR-sp) rats' living days and survival rates were extended by DOP, and hypotensive effect of DOP was significantly better than the nonpolysaccharide ingredients of *Dendrobium officinale* [78].

Moreover, *Dendrobium officinale* was used to treat Sjögren's syndrome (SS) with dry eyes and mouth due to impaired lacrimal and salivary glands [80]. Research showed that DOP could suppress the progressive lymphocytes infiltration and apoptosis and balance the disorders of proinflammatory cytokines in the submandibular gland (SG) in vivo. Further, DOP ameliorated the abnormalities of aquaporin-5 (AQP-5) that was supported by in vitro study on A-253 cell line and maintained its function of saliva secretion [79].

Meanwhile, research showed that the DOP group demonstrated higher average scores and an improved average quality of hair growth of C57BL/6J mice (5.0 g/L, the solution was ultra-pure water, each 0.2 mL, smeared for 21 d), compared with the control group. Besides, DOP significantly increased HaCaT cells survival rate and the VEGF mRNA expression levels compared with the control group [80].

Besides, the *Dendrobium officinale* had an obvious effect on the molecular diversity of intestinal *Lactobacillus* in mice model (irregular diet for 8 d after gavage of folium senna water decoction for 7 d) with constipation resulting from spleen deficiency (TCM syndrome type) [81]. *Dendrobium officinale* also can regulate the digestive function in carbon-induced constipated mice models [83].

6. The Toxicology of *Dendrobium officinale*

After acute toxicity test, genetic toxicity test (Ames test, micronucleus test of bone marrow, and sperm shape abnormality test in mice) and 90-day feeding test in rats, the results showed that protocorms of *Dendrobium officinale* were without toxicity, genetic toxicity and mutagenicity within the scope of the test dose [84]. Furthermore, the 15% decoction of *Dendrobium officinale* was used for mice sperm malformation test, Ames test, and micronucleus test of bone marrow in mice, and all test results were negative. In addition, the acute oral toxicity test showed that the highest dose was 10 g/kg b.w. which belonged to the actual nontoxic category [85]. However, in the pesticide safety aspect, the *Dendrobium officinale* still need more stringent quality control [86].

7. The Industrialization of *Dendrobium officinale*

Due to the special trophic mode, seed germination of *Dendrobium officinale* needs the root symbiotic bacteria [87, 88]; the reproduction of wild *Dendrobium officinale* is limited with low natural reproductive rates. The past three decades witnessed excess herb-gathering of *Dendrobium officinale* destroying much of the wild habitat resource in China [89]. In 1987, *Dendrobium officinale* was on the list of national third-level rare and endangered plants [90]. However, artificial cultivation based on tissue culture technology significantly prompted the yield of *Dendrobium officinale*.

Recently, increased awareness of the tonic therapeutic effect of *Dendrobium officinale* has increased the demand and as a consequence the price [91]. However, the high-profit margin of *Dendrobium officinale* has already led to an increase in the market for counterfeits and adulterants [92] mainly by other confusable species of *Dendrobium* [91]. The appearance of *Dendrobium officinale* and other species of *Dendrobium* is very similar, especially after processing into *"tiepifengdou"*; it is difficult to distinguish them through morphological identification [93]. It is apparent that *Dendrobium officinale* germplasm resources' separation and purification determine its characters and quality [94]. In terms of microscopic identification, *Dendrobium officinale* could be identified by vascular bundle sheath observed under the fluorescence microscopy and the distribution of raphides under normal light microscopy [95]. In addition, the taxonomy, phylogeny, and breeding of *Dendrobium* species have made great progress as the advance of molecular markers in the past two decades [96].

Under such circumstances, pharmacognosy [97], DNA molecule marker technologies including Amplified Fragment Length Polymorphism (AFLP) [98], Inter Simple Sequence Repeat (ISSR) [99], Start codon targeted (SCoT) and target region amplification polymorphism (TRAP) [100], and sequence related amplified polymorphism (SRAP) analysis [101], and so on have been applied to identify *Dendrobium*

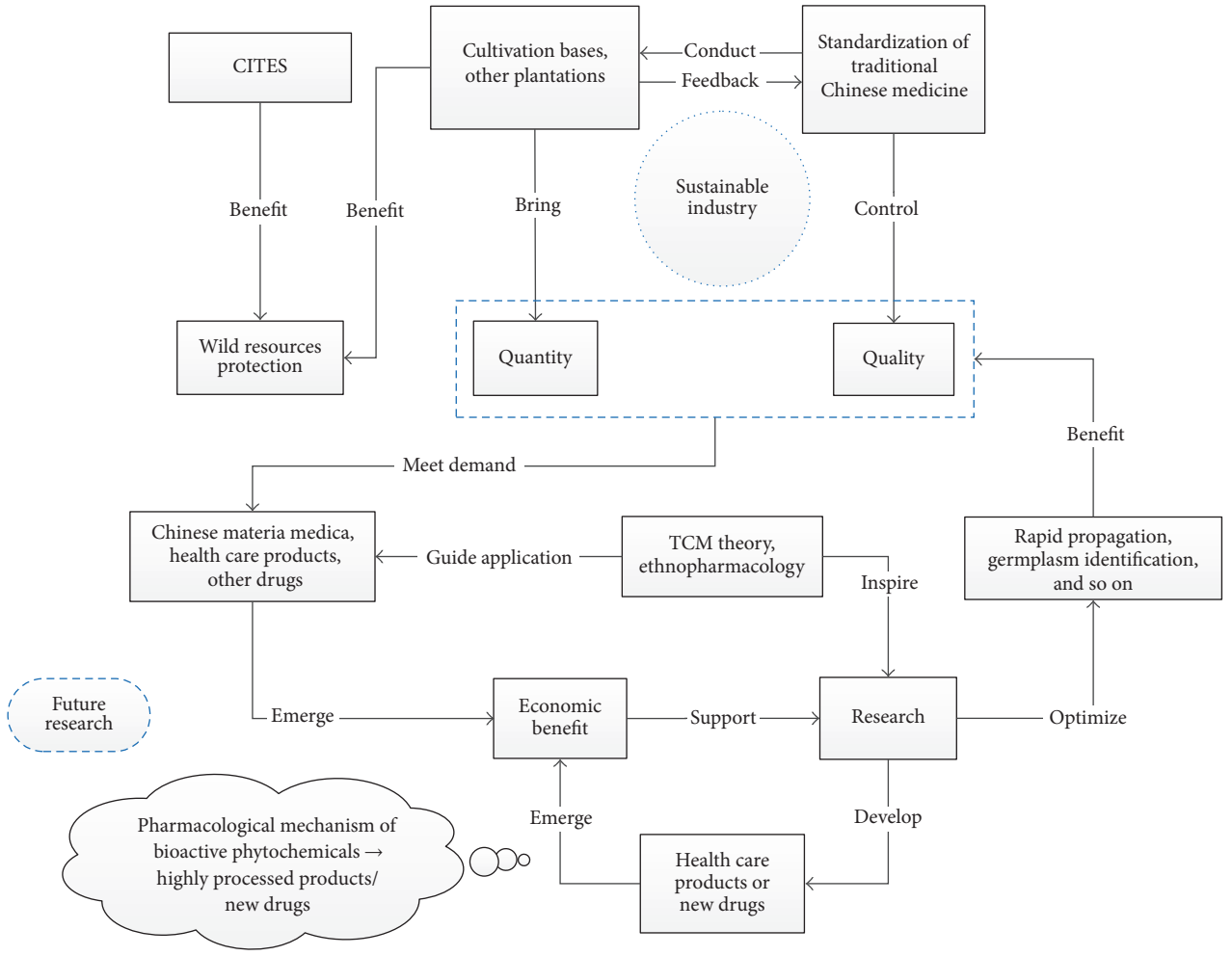


Figure 3: The industry and research *status quo* and future perspectives.

officinale from other Dendrobium species. Further, Yunnan province has completed *Dendrobium officinale* fine gene map and found more than 48,200 protein-coding genes [102]. Therefore, the identification of *Dendrobium officinale* is more precise due to the gene technology, but genetic fingerprints are difficult to evaluate the quality, so the gene technology combining with the physical and chemical identification is necessary.

In recent years, the development of *Dendrobium officinale* health care products is promising [15]. Many related patented products are being registered or have been authorized, including *Dendrobium officinale* ingredients such as antiaging compounds [103], lactobacillus drink [104], antiasthenopia eye ointment [105], immunoenhancement compounds [106], hypotensive extractive [107], and hypoglycemic and hypolipidemic compounds [108]. Some of these patents have been put into production like *"tiepifengdou,"* one of the most famous processed products of *Dendrobium officinale* [101]. However, there are few medicines of *Dendrobium officinale* [109] besides *tiepishihu* of Chinese materia medica in TCM.

In summary, the TCM theory and ethnopharmacology could inspire modern researches committed to yield and quality control of *Dendrobium officinale*, while some standards or laws (such as ISO/TC 249 and CITES) regulated artificial cultivation and protected wildlife resources. Finally, industrialization will drive further researches and is conducive to develop deep-processing products, especially the insufficient new drugs, which will also promote the industrialization and form a virtuous circle simultaneously (Figure 3).

8. Conclusion

Dendrobium officinale, one of the most famous species of *Dendrobium*, has long been regarded as precious herbs and health foods applied in TCM and in folk. In this paper, the ethnopharmacology, phytochemistry, pharmacology, and industrialization of *Dendrobium officinale* were summarized. In recent years, the interest of exploring ethnopharmacology-based bioactive constituents of *Dendrobium officinale* has increased considerably. Rapid propagation technology has gradually matured, so yield is no longer the bottleneck of *Dendrobium officinale* development. In addition, the confusable identification and uneven quality still exist, and

better quality control standards under modern researches are necessary.

Consequently, the following points deserve further investigation. (1) Polysaccharides are the main composition in *Dendrobium officinale* with numerous pharmacological researches, but investigation related to its structure-activity relationships remains scant. (2) The pharmacological effects of *Dendrobium officinale* crude extract and polysaccharide were similar, indicating whether other active ingredients were lost during extraction. (3) There is no enough systemic data about toxicology of *Dendrobium officinale*. (4) How does TCM theory play a role in *Dendrobium officinale* further in-depth development as theoretical guidance and inspiration source.

Over all, further studies at the molecular level are needed to promote the exploration of chemical compositions and pharmacological mechanisms. In addition, the industrialization of *Dendrobium officinale* not only protected the germplasm resources but also attracted a large quantity of researches in China, which makes the innovation of *Dendrobium officinale* novel drug and product a promising prospect.

Abbreviations

ADH:	Antidiuretic hormone
ALDH:	Acetaldehyde dehydrogenase
b.w.:	Body weight
ChP:	Chinese Pharmacopoeia
CITES:	Convention of International Trade in Endangered Species of Wild Fauna and Flora
ConA:	Concanavalin A
D.:	*Dendrobium*
DOP:	*Dendrobium officinale* polysaccharides
GC:	Gas Chromatography
GSH-PX:	Glutathione peroxidase
HDL:	High-density lipoprotein
HPLC:	High Performance Liquid Chromatography
LLC:	Lewis lung carcinoma
MDA:	Malondialdehyde
MMP:	Matrix metalloproteinase
MS:	Mass Spectrometer
MTT:	3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide
NMR:	Nuclear Magnetic Resonance
SOD:	Superoxide dismutase
SS:	Sjögren's syndrome
TC:	Total cholesterol
TCM:	Traditional Chinese medicine
TG:	Triglyceride
VEGF:	Vascular endothelial growth factor.

Competing Interests

The authors declare that they have no competing interests.

Acknowledgments

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References

[1] X. X. Li, *The Phylogenetic of Chinese Dendrobium and Protection and Genetics Research on Dendrobium Officinale*, Nanjing Normal University, Nanjing, China, 2009.

[2] G. Y. Lü, M. Q. Yan, and S. H. Chen, "Review of pharmacological activities of *Dendrobium officinale* based on traditional functions," *China Journal of Chinese Materia Medica*, vol. 38, no. 4, pp. 489–493, 2013.

[3] Q. L. Luo, Z. H. Tang, X. F. Zhang et al., "Chemical properties and antioxidant activity of a water-soluble polysaccharide from *Dendrobium officinale*," *International Journal of Biological Macromolecules*, vol. 89, pp. 219–227, 2016.

[4] T. B. He, Y. P. Huang, L. Yang et al., "Structural characterization and immunomodulating activity of polysaccharide from *Dendrobium officinale*," *International Journal of Biological Macromolecules*, vol. 83, pp. 34–41, 2016.

[5] M. Q. Huang, B. H. Huang, T. Y. Cai, Q. L. Liu, D. Li, and A. Z. Chen, "Extraction, isolation and analysis of Dendrobium officinale's polysaccharides," *Chinese Traditional and Herbal Drugs*, vol. 3, pp. 128–129, 1994.

[6] H. Yu, Y. Qu, and S. Chen, "Efficacy of Golden Dendrobium tablets on clearing and smoothing laryngopharynx," *Yunnan University Natural Sciences*, vol. 27, no. 5, p. 440, 2005.

[7] H. Xu, Z. Wang, X. Ding, K. Zhou, and L. Xu, "Differentiation of Dendrobium species used as "Huangcao Shihu" by rDNA ITS sequence analysis," *Planta Medica*, vol. 72, no. 1, pp. 89–92, 2006.

[8] Y. Lam, T. B. Ng, R. M. Yao et al., "Evaluation of chemical constituents and important mechanism of pharmacological biology in *Dendrobium* plants," *Evidence-Based Complementary and Alternative Medicine*, vol. 2015, Article ID 841752, 25 pages, 2015.

[9] J. Xu, W. M. Zhao, Z. M. Qian, J. Guan, and S. P. Li, "Fast determination of five components of coumarin, alkaloids and bibenzyls in Dendrobium spp. using pressurized liquid extraction and ultra-performance liquid chromatography," *Journal of Separation Science*, vol. 33, no. 11, pp. 1580–1586, 2010.

[10] X. Jin and L. Huang, "(2352) Proposal to conserve the name Dendrobium officinale against D. stricklandianum, D. tosaense, and D. pere-fauriei (Orchidaceae)," *Taxon*, vol. 64, no. 2, pp. 385–386, 2015.

[11] X. G. Xiang, A. Schuiteman, D. Z. Li et al., "Molecular systematics of Dendrobium (Orchidaceae, Dendrobieae) from mainland Asia based on plastid and nuclear sequences," *Molecular Phylogenetics and Evolution*, vol. 69, no. 3, pp. 950–960, 2013.

[12] K. Kimura and H. Migo, *New Species of Dendrobium from the Chinese Drug (Shi-hu)*, Shanghai Science Institute, Shanghai, China, 1936.

[13] P. Hao, L. Guo, L. Xu et al., "Study on HPLC fingerprint in different species of Dendrobii Caulis," *Pharmacy and Clinics of Chinese Materia Medica*, vol. 4, pp. 1–4, 2013.

[14] G. X. Cai, J. Li, S. X. Li, D. Huang, and X. B. Zhao, "Applications of dendrobium officinale in ancient and modern times," *Journal of Traditional Chinese Medicine University of Hunan*, vol. 31, no. 5, pp. 77–81, 2011.

[15] S. H. Chen, M. Q. Yan, G. Y. Lü, and X. Liu, "Development of dendrobium officinale kimura et migo related health foods,"

Chinese Pharmaceutical Journal, vol. 48, no. 19, pp. 1625–1628, 2013.

[16] M. J. Yang, P. Yu, L. D. Lu, Z. M. Lü, and X. X. Chen, "Effects of dendrobium candicum capsules on immune responses in mouse," *Jiangsu Journal of Preventive Medicine*, vol. 19, no. 4, pp. 11–13, 2008.

[17] Chinese Pharmacopoeia Committee, *Chinese Pharmacopoeia*, China Medical Science, Beijing, China, 2005.

[18] Chinese Pharmacopoeia Committee, *Chinese Pharmacopoeia*, China Medical Science Press, Beijing, China, 2010.

[19] J. Wang and B. C. Wang, "Fresh Dendrobium officinale clinic applications," *Zhejiang Journal of Traditional Chinese Medicine*, vol. 47, no. 11, pp. 841–842, 2012.

[20] C. Huang, D. Wang, L. Sun, and L. Wei, "Effects of exogenous salicylic acid on the physiological characteristics of dendrobium officinale under chilling stress," *Plant Growth Regulation*, vol. 79, no. 2, pp. 199–208, 2016.

[21] S. Z. Hou, C. Y. Liang, H. Z. Liu et al., "*Dendrobium officinale* prevents early complications in streptozotocin-induced diabetic rats," *Evidence-Based Complementary and Alternative Medicine*, vol. 2016, Article ID 6385850, 10 pages, 2016.

[22] T. H. Song, X. X. Chen, S. C. W. Tang et al., "Dendrobium officinale polysaccharides ameliorated pulmonary function while inhibiting mucin-5AC and stimulating aquaporin-5 expression," *Journal of Functional Foods*, vol. 21, pp. 359–371, 2016.

[23] Y. Y. Ye, "Ultrasonic extraction research on polysaccharides of Dendrobium officinale," *Journal of Chinese Medicinal Materials*, vol. 32, no. 4, pp. 617–620, 2009.

[24] S. H. Bao, X. Q. Cha, J. Hao, and J. P. Luo, "Studies on antioxidant activity of polysaccharides from dendrobium candidum in vitro," *Hefei University of Technology*, vol. 30, no. 21, pp. 123–127, 2009.

[25] M. Y. Xie and S. P. Nie, "Research progress of natural polysaccharides' structure and function," *Journal of Chinese Institute of Food Science and Technology*, vol. 10, no. 2, pp. 1–11, 2010.

[26] X. Xing, S. W. Cui, S. Nie, G. O. Phillips, H. D. Goff, and Q. Wang, "Study on Dendrobium officinale O-acetyl-glucomannan (Dendronan®): part I. Extraction, purification, and partial structural characterization," *Bioactive Carbohydrates and Dietary Fibre*, vol. 4, no. 1, pp. 74–83, 2014.

[27] Q. F. Lu, P. P. Wang, J. N. Chen, and S. Huang, "Comparative Study on Polysaccharides Content in Stem and Leaf of Iron Stone Dendrobium Fresh Product from difierent producing place," *Journal of Medical Research*, vol. 42, no. 9, pp. 55–58, 2013.

[28] M. Xin, E. Z. Zhang, N. Li et al., "Effects of different drying methods on polysaccharides and dendrobine from Dendrobium candidum," *Journal of Southern Agriculture*, vol. 44, no. 8, pp. 1347–1350, 2013.

[29] Y. Zhu, J. P. Si, B. L. Guo, B. W. He, and A. L. Zhang, "The content variation regularity of cultivated Dendrobium candidum polysaccharides," *China Journal of Chinese Materia Medica*, vol. 35, no. 4, pp. 427–430, 2010.

[30] X. Y. Shang, "Distribution of polysaccharides from different sources and in different parts of Dendrobium officinale," *Chinese Journal of Modern Drug Application*, vol. 4, no. 13, pp. 104–105, 2010.

[31] J. R. Sheng, Z. H. Li, Y. B. Yi, and J. J. Li, "Advances in the study of polysaccharide from Dendrobium officinale," *Journal of Guangxi Academy of Sciences*, vol. 27, no. 4, pp. 338–340, 2011.

[32] Q. Li, Y. Xie, J. Su, Q. Ye, and Z. Jia, "Isolation and structural characterization of a neutral polysaccharide from the stems of Dendrobium densiflorum," *International Journal of Biological Macromolecules*, vol. 50, no. 5, pp. 1207–1211, 2012.

[33] X. Xing, S. W. Cui, S. Nie, G. O. Phillips, H. Douglas Goff, and Q. Wang, "A review of isolation process, structural characteristics, and bioactivities of water-soluble polysaccharides from *Dendrobium* plants," *Bioactive Carbohydrates and Dietary Fibre*, vol. 1, no. 2, pp. 131–147, 2013.

[34] X. Xing, S. W. Cui, S. Nie, G. O. Phillips, H. D. Goff, and Q. Wang, "Study on Dendrobium officinale O-acetyl-glucomannan (Dendronan®): part II. Fine structures of O-acetylated residues," *Carbohydrate polymers*, vol. 117, pp. 422–433, 2015.

[35] H. L. Cai, X. J. Huang, S. P. Nie, M. Y. Xie, G. O. Phillips, and S. W. Cui, "Study on *Dendrobium officinale* O-acetyl-glucomannan (Dendronan®): Part III-Immunomodulatory activity *in vitro*," *Bioactive Carbohydrates and Dietary Fibre*, vol. 5, no. 2, pp. 99–105, 2015.

[36] X. Huang, S. Nie, H. Cai et al., "Study on *Dendrobium officinale* O-acetyl-glucomannan (Dendronan®): part VI. Protective effects against oxidative stress in immunosuppressed mice," *Food Research International*, vol. 72, pp. 168–173, 2015.

[37] G. Y. Zhang, S. P. Nie, X. J. Huang et al., "Study on Dendrobium officinale O-Acetyl-glucomannan (Dendronan). 7. Improving effects on colonic health of mice," *Journal of Agricultural and Food Chemistry*, vol. 64, no. 12, pp. 2485–2491, 2015.

[38] P. Su, *Research on the Molecular Mechanism of Erianin Anti-Hepatoma Effect*, University of Chinese Academy of Sciences, Beijing, China, 2011.

[39] Y. Q. Gong, *Mechanisms of Erianin Anti-Tumor Angiogenesis*, China Pharmaceutical University, Nanjing, China, 2003.

[40] E. G. Barbosa, L. A. S. Bega, A. Beatriz et al., "A diaryl sulfide, sulfoxide, and sulfone bearing structural similarities to combretastatin A-4," *European Journal of Medicinal Chemistry*, vol. 44, no. 6, pp. 2685–2688, 2009.

[41] Y. Li, C. L. Wang, F. F. Wang et al., "Chemical constituents of *Dendrobium candidum*," *China Journal of Chinese Materia Medica*, vol. 35, no. 13, pp. 1715–1719, 2010.

[42] S. Charoenrungruang, P. Chanvorachote, B. Sritularak, and V. Pongrakhananon, "Gigantol, a bibenzyl from Dendrobium draconis, inhibits the migratory behavior of non-small cell lung cancer cells," *Journal of Natural Products*, vol. 77, no. 6, pp. 1359–1366, 2014.

[43] J. Chang, L. J. Xuan, Y. M. Xu, and J. S. Zhang, "Seven new sesquiterpene glycosides from the root bark of Dictamnus dasycarpus," *Journal of Natural Products*, vol. 64, no. 7, pp. 935–938, 2001.

[44] J. M. Shao, D. P. Wang, Y. P. Zhang, H. Y. Tang, Y. T. Tan, and W. Li, "Analysis on chemical constituents of volatile oil from stem and leaf of Dendrobium officinale by GC-MS," *Guizhou Agricultural Sciences*, vol. 42, no. 4, pp. 190–193, 2014.

[45] J. A. Miller, K. Pappan, P. A. Thompson et al., "Plasma metabolomic profiles of breast cancer patients after short-term limonene intervention," *Cancer Prevention Research*, vol. 8, no. 1, pp. 86–93, 2015.

[46] D. G. Hammond, S. Rangel, and I. Kubo, "Volatile aldehydes are promising broad-spectrum postharvest insecticides," *Journal of Agricultural and Food Chemistry*, vol. 48, no. 9, pp. 4410–4417, 2000.

[47] S. K. Suzuki, "Study on Chinese Flickingeria alkaloids (Dendrobium Alkaloids research," *Pharmaceutical Journal*, vol. 52, no. 12, pp. 1049–1060, 1932.

[48] S. Li, C. L. Wang, and S. X. Guo, "Determination of dendrobin in dendrobium nobile by HPLC analysis," *Chinese Pharmaceutical Journal*, vol. 44, no. 4, pp. 252–254, 2009.

[49] X. Guo, Y. Li, C. Li et al., "Analysis of the Dendrobium officinale transcriptome reveals putative alkaloid biosynthetic genes and genetic markers," *Gene*, vol. 527, no. 1, pp. 131–138, 2013.

[50] H. Asahina, J. K. Shinozaki, K. Masuda, Y. Morimitsu, and M. Satake, "Identification of medicinal *Dendrobium* species by phylogenetic analyses using *matK* and *rbcL* sequences," *Journal of Natural Medicines*, vol. 64, no. 2, pp. 133–138, 2010.

[51] Z. Q. Yuan, J. Y. Zhang, and T. Liu, "Phylogenetic relationship of China Dendrobium species based on the sequence of the internal transcribed spacer of ribosomal DNA," *Biologia Plantarum*, vol. 53, no. 1, pp. 155–158, 2009.

[52] R. S. Li, X. Yang, P. He, and N. Gan, "Studies on phenanthrene constituents from stems of *Dendrobium candidum*," *Journal of Chinese Medicinal Materials*, vol. 32, no. 2, pp. 220–223, 2009.

[53] Q. Xu, S. H. Chen, and G. Y. Lü, "Pharmacological research progress of the three constituents of different Dendrobium," *Asia-Pacific Traditional Medicine*, vol. 6, no. 4, pp. 115–118, 2010.

[54] Y. Li, C. L. Wang, S. X. Guo, J. S. Yang, and P. G. Xiao, "Two new compounds from Dendrobium candidum," *Chemical and Pharmaceutical Bulletin*, vol. 56, no. 10, pp. 1477–1479, 2008.

[55] H. J. Guan, *Chemical Composition and Fingerprints of Dendrobium Officinale*, Shenyang Pharmaceutical University, Shenyang, China, 2009.

[56] Y. Li, *Study on the Chemical Constituents of Dendrobium officinale*, Chinese Peking Union Medical College, Beijing, China, 2009.

[57] L. Yang, *The Chemical Composition and Biological Activity of Dendrobium Officinale and Dendrobium Devonianum*, Anhui University of Chinese Medicine, Anhui, China, 2013.

[58] Y. Li, C. L. Wang, F. F. Wang et al., "Phenolic components and flavanones from Dendrobium candidum," *Chinese Pharmaceutical Journal*, vol. 13, pp. 975–979, 2010.

[59] Z. Y. Wei, J. J. Lu, C. S. Jin, and H. Xia, "Chemical constituents from n-butanol extracts of Dendrobium officinale," *Modern Chinese Medicine*, vol. 15, no. 12, pp. 1042–1045, 2013.

[60] F. F. Wang, Y. Li, H. L. Dong, S. X. Guo, C. L. Wang, and J. S. Yang, "A new compound from Dendrobium candidum," *Chinese Pharmaceutical Journal*, vol. 45, no. 12, pp. 898–902, 2010.

[61] J. Li, S. X. Li, D. Huang, X. B. Zhao, and G. X. Cai, "Advances in the of resources, constituents and pharmacological effects of Dendrobium officinale," *Science & Technology Review*, vol. 29, no. 18, pp. 74–79, 2011.

[62] J. W. Kuang, *Studies on the Chemical Constituents from Lycoris Radiata. and Dendrobium candidum*, Central South University, Hunan, 2013.

[63] H. Yang, *Studies on Chemical Components of Dendrobium Chrysotoxum and D. Candidum*, China Pharmaceutical University, Nanjing, China, 2002.

[64] S. L. Li, *Comparison of Intervention Effects of Polysaccharides from Six Dendrobium Species on Alcoholic Liver Injury*, Hefei University of Technology, Anhui, China, 2013.

[65] G. Y. Lü, S. H. Chen, L. D. Zhang et al., "Dendrobium officinale effect on two kinds of serum transaminases and cholesterol of mice model with chronic alcoholic liver injury," *Chinese Journal of Experimental Traditional Medical Formulae*, vol. 16, no. 6, pp. 192–193, 2010.

[66] X. H. Tang, S. H. Chen, G. Y. Lü, J. Su, and M. C. Huang, "Effects of Dendrobium Candidum on SOD, MDA and GSH-Px in mice model of acute alcoholic hepatic injury," *Zhejiang Journal of Traditional Chinese Medicine*, vol. 45, no. 5, pp. 369–370, 2010.

[67] Y. H. Ge, J. Wang, F. Yang, G. H. Dai, and Y. L. Tong, "Effects of fresh Dendrobium officinale polysaccharides on immune function in mice with Lewis lung cancer," *Zhejiang Journal of Traditional Chinese Medicine*, vol. 49, no. 4, pp. 277–279, 2014.

[68] Y. J. Liu, *Effect of Dendrobium officinale Polysaccharides on Mouse Embryonic Stem Cells and Study on its Anti-Tumor and Immune Activity*, Zhejiang University of Technology Zhejiang, 2014.

[69] P. Deng, *Studies on the Curing of Nasopharyngeal Carcinoma (NPC) with Officinal Dendrobium Stem*, Guangxi Medical University, Guangxi, China, 2010.

[70] L. H. Pan, X. F. Li, M. N. Wang et al., "Comparison of hypoglycemic and antioxidative effects of polysaccharides from four different dendrobium species," *International Journal of Biological Macromolecules*, vol. 64, pp. 420–427, 2014.

[71] H. S. Wu, J. H. Xu, L. Z. Chen, and J. J. Sun, "Studies on anti-hyperglycemic effect and its mechanism of Dendrobium candidum," *China Journal of Chinese Materia Medica*, vol. 29, no. 2, pp. 160–163, 2004.

[72] H. L. Chang, "Effect of Dendrobium officinale on JNK, AKT protein phosphorylation expression in islet tissue of type 2 diabetic rats," *Chinese Pharmaceutical Affairs*, vol. 29, no. 1, pp. 54–57, 2015.

[73] H. Q. Tang, H. Chen, Y. Wei, and L. Lu, "Effects of Dendrobium officinale on energy metabolism and expression of CNTF mRNA in athletic fatigue mice," *Chinese Journal of Experimental Traditional Medical Formulae*, vol. 20, no. 15, pp. 164–167, 2014.

[74] Y. Feng, M. K. Huang, J. B. Ye, N. Le, and W. G. Zhong, "The lowest doses of Dendrobium officinale for improving sports ability, anti-fatigue and immune ability of mice," *Journal of Southern Agriculture*, vol. 45, no. 6, pp. 1089–1093, 2014.

[75] X. Feng and X. Zhao, "Prevention effect of Dendrobium officinale aqueous extract to SD rats with gastric injury," *Jiangsu Agricultural Sciences*, vol. 41, no. 7, pp. 294–296, 2013.

[76] C. Y. Liang, H. B. Li, S. Z. Hou, J. Zhang, S. Huang, and X. P. Lai, "Dendrobium officinale hepatic protection and anti-ulcerative effects," *World Science and Technology-Modernization of Traditional Chinese Medicine and Materia Medica*, vol. 15, no. 2, pp. 234–237, 2013.

[77] Y. M. Li, P. Wu, X. J. Xie, H. L. Yao, L. P. Song, and D. F. Liao, "Effects of Dendrobii officinalis caulis on serum lipid, TNF-α and IL-6 in apolipoprotein E-deficient mice," *Chinese Journal of Experimental Traditional Medical Formulae*, vol. 19, no. 18, pp. 270–274, 2013.

[78] R. Z. Wu, B. X. Yang, Y. P. Li et al., "Experimental study of Dendrobium officinale polysaccharides on anti-hypertensive-stroke effects of SHR-sp mice," *Chinese Journal of Traditional Medical Science and Technology*, vol. 18, no. 3, pp. 204–210, 2011.

[79] X. Lin, P. C. Shaw, S. C. W. Sze, Y. Tong, and Y. Zhang, "Dendrobium officinale polysaccharides ameliorate the abnormality of aquaporin 5, pro-inflammatory cytokines and inhibit apoptosis in the experimental Sjögren's syndrome mice," *International Immunopharmacology*, vol. 11, no. 12, pp. 2025–2032, 2011.

[80] J. Chen, H. Qi, J. B. Li et al., "Experimental study on *Dendrobium candidum* polysaccharides on promotion of hair growth," *China Journal of Chinese Materia Medica*, vol. 39, no. 2, pp. 291–295, 2014.

[81] X. B. Zhao, X. J. Xie, W. J. Wu et al., "Effect of ultra-micro powder Dendrobium officinale on the molecular diversity of intestinal Lactobacillus in mice with spleen-deficiency constipation," *Microbiology*, vol. 41, no. 9, pp. 1764–1770, 2014.

[82] L. Xia, X. Liu, H. Guo, H. Zhang, J. Zhu, and F. Ren, "Partial characterization and immunomodulatory activity of polysaccharides from the stem of Dendrobium officinale (Tiepishihu) in vitro," *Journal of Functional Foods*, vol. 4, no. 1, pp. 294–301, 2012.

[83] R. Wang, P. Sun, Y. Zhou, and X. Zhao, "Preventive effect of *Dendrobium candidum* Wall. ex Lindl. on activated carbon-induced constipation in mice," *Experimental and Therapeutic Medicine*, vol. 9, no. 2, pp. 563–568, 2015.

[84] X. Feng, L. Zhao, H. Chen, Y. Zhou, J. J. Tang, and Z. Y. Chu, "Research on toxicological security of Dendrobium candidum protocorms," *Chinese Journal of Health Laboratory Technology*, vol. 3, pp. 355–358, 2014.

[85] J. Y. Fu, Y. Xia, C. J. Xu et al., "Toxicity and Safety Evaluation of Dendrobium officinale's extracting solution," *Zhejiang Journal of Preventive Medicine*, vol. 4, pp. 250–251, 1998.

[86] S. Zheng, H. Wu, Z. Li, J. Wang, H. Zhang, and M. Qian, "Ultrasound/microwave-assisted solid-liquid-solid dispersive extraction with high-performance liquid chromatography coupled to tandem mass spectrometry for the determination of neonicotinoid insecticides in Dendrobium officinale," *Journal of Separation Science*, vol. 38, no. 1, pp. 121–127, 2015.

[87] X. M. Tan, C. L. Wang, X. M. Chen et al., "In vitro seed germination and seedling growth of an endangered epiphytic orchid, Dendrobium officinale, endemic to China using mycorrhizal fungi (Tulasnella sp.)," *Scientia Horticulturae*, vol. 165, pp. 62–68, 2014.

[88] X. B. Xu, X. Y. Ma, H. H. Lei et al., "Proteomic analysis reveals the mechanisms of Mycena dendrobii promoting transplantation survival and growth of tissue culture seedlings of Dendrobium officinale," *Journal of Applied Microbiology*, vol. 118, no. 6, pp. 1444–1455, 2015.

[89] X. Li, Y. Chen, Y. Lai, Q. Yang, H. Hu, and Y. Wang, "Sustainable utilization of traditional chinese medicine resources: systematic evaluation on different production modes," *Evidence-based Complementary and Alternative Medicine*, vol. 2015, Article ID 218901, 10 pages, 2015.

[90] Y. Bai, Y. H. Bao, J. X. Jin, Y. N. Yan, and W. Q. Wang, "Resources Investigation of Medicinal Dendrobium in China," *Chinese Traditional and Herbal Drugs*, vol. 37, no. 9, pp. I0005–I0007, 2006.

[91] J. M. Luo, "Preliminary Inquiry of Dendrobium officinale's industry and market development," *China Journal of Chinese Materia Medica*, vol. 38, no. 4, pp. 472–474, 2013.

[92] L. G. Yao, Z. B. Hu, Z. R. Zheng et al., "Preliminary study on the DNA barcoding in Dendrobium officinale germplasm resources," *Acta Agriculturae Shanghai*, vol. 28, no. 1, pp. 49–54, 2012.

[93] X. M. Chen, C. L. Wang, J. S. Yang, and S. X. Guo, "Research progress on chemical composition and chemical analysis of Dendrobium officinale," *Chinese Pharmaceutical Journal*, vol. 48, no. 19, pp. 1634–1640, 2013.

[94] Z. C. Zhang, J. B. Chen, Z. W. Ma et al., "Discrepancy and correlation analysis on main phenotypic traits of Dendrobium officinale," *Guangdong Agricultural Sciences*, vol. 37, no. 8, pp. 78–80, 2010.

[95] C. Chu, H. Yin, L. Xia, D. Cheng, J. Yan, and L. Zhu, "Discrimination of *Dendrobium officinale* and its common adulterants by combination of normal light and fluorescence microscopy," *Molecules*, vol. 19, no. 3, pp. 3718–3730, 2014.

[96] J. A. Teixeira da Silva, X. Jin, J. Dobránszki et al., "Advances in Dendrobium molecular research: applications in genetic variation, identification and breeding," *Molecular Phylogenetics and Evolution*, vol. 95, pp. 196–216, 2016.

[97] Y. Y. Xiao, J. Y. Weng, and J. S. Fan, "Pharmacognosy identification of Dendrobium officinale and Dendrobium devonianum," *Strait Pharmaceutical Journal*, vol. 23, no. 4, pp. 52–53, 2011.

[98] Y. H. Bao, C. M. Pan, and Y. Bai, "Comprehensive analysis of identification characters of three medicinal dendrobii," *Journal of South China Normal University (Natural Science Edition)*, vol. 46, no. 3, pp. 112–117, 2014.

[99] J. Shen, X. Ding, D. Liu et al., "Intersimple sequence repeats (ISSR) molecular fingerprinting markers for authenticating populations of *Dendrobium officinale* Kimura et MIGO," *Biological and Pharmaceutical Bulletin*, vol. 29, no. 3, pp. 420–422, 2006.

[100] S. Feng, R. He, S. Yang et al., "Start codon targeted (SCoT) and target region amplification polymorphism (TRAP) for evaluating the genetic relationship of Dendrobium species," *Gene*, vol. 567, no. 2, pp. 182–188, 2015.

[101] S. G. Feng, J. J. Lu, L. Gao, J. J. Liu, and H. Z. Wang, "Molecular phylogeny analysis and species identification of Dendrobium (Orchidaceae) in China," *Biochemical Genetics*, vol. 52, no. 3-4, pp. 127–136, 2014.

[102] T. T. Rui, Yunnan has completed Dendrobium officinale's fine gene map, which will push the province's Dendrobium industry. 2013.

[103] S. Z. Hou, S. Huang, Y. M. Liang et al., "The medicinal use of Dendrobium officinale," China Patent CN102552698A, 2012.

[104] B. Liu, Q. Q. Chen, Y. Liu et al., "Preparation method of Dendrobium candidum plant protein lactic acid drink," China Patent CN102940038A, 2013.

[105] Z. H. He and X. P. He, "One kind of anti-asthenopia Dendrobium candidum," China Patent CN103961556A, 2014.

[106] Z. H. He and X. P. He, "One kind of immuno-enhancement Dendrobium officinale health products," China Patent CN103816389A, 2014.

[107] L. Z. Chen, Z. J. Lou, R. Z. Wu et al., "The application of Dendrobium officinale polysaccharides of the medicine preventing and treating hypertension and stroke," China Patent CN101849957B, 2012.

[108] C. G. Cai, W. D. Zhang, and M. R. Zhang, One kind of hypoglycemic and hypolipidemic Dendrobium officinale com-compoundspounds and its preparation method, China Patent CN104126746A, 2014.

[109] China Food Drug Administration, http://www.sfda.gov.cn/WS01/CL0001/.

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Review Article

A Review of the Potential of Phytochemicals from *Prunus africana* (Hook f.) Kalkman Stem Bark for Chemoprevention and Chemotherapy of Prostate Cancer

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Prostate cancer remains one of the major causes of death worldwide. In view of the limited treatment options for patients with prostate cancer, preventive and treatment approaches based on natural compounds can play an integral role in tackling this disease. Recent evidence supports the beneficial effects of plant-derived phytochemicals as chemopreventive and chemotherapeutic agents for various cancers, including prostate cancer. *Prunus africana* has been used for generations in African traditional medicine to treat prostate cancer. This review examined the potential roles of the phytochemicals from *P. africana*, an endangered, sub-Saharan Africa plant in the chemoprevention and chemotherapy of prostate cancer. In vitro and in vivo studies have provided strong pharmacological evidence for antiprostate cancer activities of *P. africana*-derived phytochemicals. Through synergistic interactions between different effective phytochemicals, *P. africana* extracts have been shown to exhibit very strong antiandrogenic and antiangiogenic activities and have the ability to kill tumor cells via apoptotic pathways, prevent the proliferation of prostate cancer cells, and alter the signaling pathways required for the maintenance of prostate cancer cells. However, further preclinical and clinical studies ought to be done to advance and eventually use these promising phytochemicals for the prevention and chemotherapy of human prostate cancer.

1. Introduction

Prostate cancer is one of the most common nonskin cancers in men. It is caused by unregulated prostate cell division, which leads to abnormal growth, with the potential to spread to other parts of the body [1]. These neoplastic cells originate from highly specialized cells through a process of regression to an advanced stage. Unlike the normal parent cells, these cells divide continuously, resulting in a tumor. Approximately, 9–11% of men are at risk of clinically suffering from prostate cancer in their life time [2–5]. Prostate cancer is typically androgen-dependent during its initial stages when the hormone androgen binds to the androgen receptor (AR) and then transactivates target genes [6, 7]. Androgen and AR-mediated signaling are therefore crucial for the development and functioning of both the normal prostate and prostate cancer. The importance of androgen in prostate cancer is further supported by the fact that prostate cancer rarely occurs in men with the deficiency in 5α-reductase, an enzyme that converts testosterone to its active metabolite 5α-dihydrotestosterone (DHT) [8]. Currently, one of the main approaches to the treatment of prostate cancer is downregulation of androgens by antiandrogenic agents [5, 6, 9, 10]. For years, prostate cancer, similar to other forms of cancer, has been managed through the conventional treatment modalities such as surgery, radiation therapy, cryosurgery, and hormone therapies [11]. However, there is still no effective treatment for advanced stages of prostate

cancer. Prostate cancer has been known to progress slowly and it is crucial to prevent its occurrence to reduce the risk of development of the disease. Chemoprevention and chemotherapy, including the administration of one or more naturally occurring antiprostate cancer agents [1, 3, 4, 10] have been identified as approaches by which the prevalence of such diseases as prostate cancer can be reduced, suppressed, or reversed. In the last decades, several plants have been confirmed to contain chemopreventive and therapeutic agents for various cancers including prostate cancer [10, 12–14]. More importantly, over 60% of currently used anticancer agents are estimated to be from natural sources [13]. Among plants with enormous antiprostate cancer potential is *Prunus africana* (African cherry), which belongs to the plant family Rosaceae. This evergreen miraculous plant is only found in sub-Saharan Africa and is highly sought after owing to its unique anticancer phytochemicals [1, 2, 15]. In fact, the use of *P. africana* in African traditional medicine (ATM) to treat prostate cancer and related conditions is not a new phenomenon across various communities in Africa [1]. More importantly, the use of *P. africana* has been patented in France for prostate cancer treatment [16]. In addition to prostate cancer, the bark extract of *P. africana* has for many years been used for the treatment of benign prostatic hyperplasia (BPH). Recent studies by Nyamai et al. [17] and Jena et al. [18] confirmed the effectiveness of the bark extract of *P. africana* in BPH treatment and attributed this to the synergistic effects of pentacyclic triterpenoids, ferulic esters of long-chain fatty alcohols, and phytosterols contained in *P. africana* bark. The phytosterols (including β-Sitosterol) and pentacyclic triterpenoids (including ursolic acids) also have anti-inflammatory effects on the prostate [17]. In ATM, *P. africana* is also used to treat myriad of diseases including but not limited to diarrhea, epilepsy, arthritis, hemorrhage, and hypertension [15, 16, 19–21]. The novel phytochemicals from *P. africana*, suggested for the treatment of prostate cancer are ursolic acid, oleanolic acid, β-amyrin, atraric acid (AA), N-butylbenzene-sulfonamide (NBBS), β-sitosterol, β-sitosterol-3-O-glucoside, ferulic acid, and lauric acid [20, 21]. The use of *P. africana* in cancer chemotherapy and chemoprevention has been discussed in a number of peer reviewed journal articles. This review therefore sought to examine the phytochemicals from *P. africana* that have the potential for prostate cancer chemoprevention and chemotherapy-both in vitro and in vivo with the goal of finding new drugs for prostate cancer.

2. Methods

In this review, we modified the data search process used by Kim et al. [47] and Lin et al. [48] to obtain information from original peer reviewed articles published in scientific journals, with a focus on the botany, distribution, and potential of *P. africana* for cancer chemoprevention and chemotherapy. We carefully searched electronic literature databases including but not limited to PubMed, Scopus, and Google Scholar for relevant records for a period from 1995 to 2016. The following key search terms were used ("*P. africana*" OR "African cherry" OR "*Pygeum africanum*" AND "Prostate cancer" OR "Distribution" OR "Phytochemicals") OR ("Phytochemicals in *P. africana*" AND "Prostate cancer"), OR ("Chemotherapy" OR "Chemoprevention" OR "Treatment" AND "Prostate cancer") OR ("African traditional medicine" AND "Prostate cancer") OR ("*Prunus africana* phytochemicals" AND "Apoptosis" OR "Androgen receptors" OR "Cell proliferation" OR "Anti-prostate cancer properties"). The data obtained were verified independently for their accuracy and any inconsistencies were settled through discussions between the authors. The final data obtained through discussions among the authors were then summarized, analyzed, and compared, and conclusions were made accordingly.

3. *Prunus africana* Botany and Distribution

The genus *Prunus* comprises over 400 species, of which only 98 are of great importance [50]. The African cherry is a species of the genus *Prunus,* with a mature stem diameter of up to 1 m and a height of more than 40 m with open branches (Figure 1(a)); and a blackish-brown bark (Figure 1(b)). Leaves are simple, alternate, oval-shaped, shiny-deep green on the top side and lighter on the underside, with a conspicuous prominent midrib on the underside (Figure 1(c)). Flowers are greenish or white (Figure 1(d)), and fruits are spherical, 7 mm long, 1.3 cm wide, pinkish-brown, and bilobed, with thin, dark red to reddish brown pulp when ripe (Figure 1(e)) [20].

The genus name "*Prunus*" is derived from a Latin word which refers to the plum family, and the scientific name "*Prunus africana*" refers to the species of African origin. This monoecious tree is native to 21 countries in sub-Saharan Africa (Figure 2) [19, 51, 52]. It is a highland forest plant that grows in humid and semihumid conditions at an altitude of about 900–3,400 m above the sea level, with a mean annual rainfall of 890–2,600 mm and a mean annual temperature of 18–26°C [20, 53].

The discovery of the medicinal properties of the *P. africana* bark for a myriad of health conditions initiated a massive harvest of its stem bark for international market needs [54]. To date, there has been an increasing demand for the bark of *P. africana* both locally and internationally, for the production of herbal medicines for the treatment of prostate cancer and related conditions [20]. Unfortunately, in most scenarios, it is the stem bark of the plant that is targeted (Figure 1(b)), which puts the survival of the plant at a great risk, if not done in accordance with the guidelines. In fact, poor harvest practices coupled with overexploitation has severely affected the wild population of *P. africana* [55, 56]. Consequently, *P. africana* has been added to Appendix II of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) list of endangered species, for the regulation of its trade from wild harvest and all exports of *P. africana* are currently subjected to a CITES export permit to protect the plant from extinction [51].

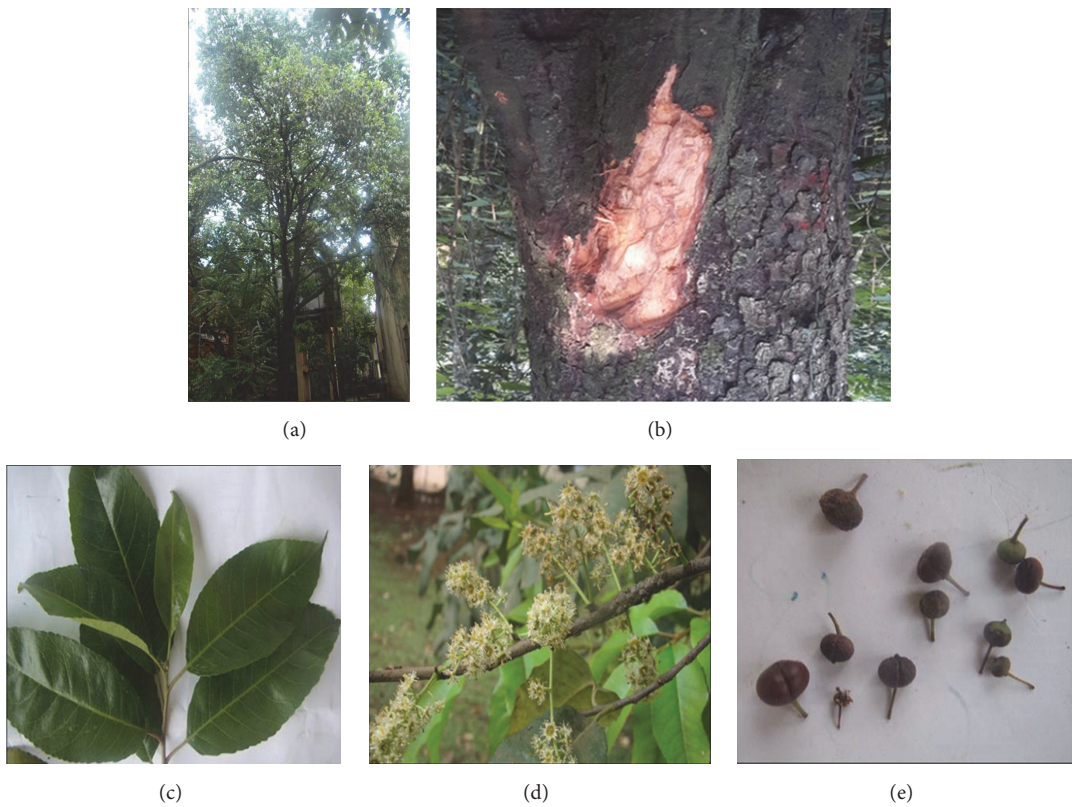


FIGURE 1: The botanical morphology of the main features of *P. africana*. (a) *P. africana* tree growing at backyard of Natural Chemotherapeutics Research Institute, Uganda. (b) Blackish-brown bark of *P. Africana*. (c) Simple, alternate, oval-shaped, leaf of *P. Africa*. (d) Greenish or white flowers of *P. Africana*. (e) Spherical, pinkish-brown, bilobed, fruit of *P. africana*.

4. In Vitro and In Vivo Effects of Ethanolic Stem Bark Extracts of *P. africana* on Prostate Cancer Cells

P. africana is one of the many medicinal plants containing large quantities of bioactive compounds that can be used for prostate cancer management [15, 20, 22, 57]. Previous studies have shown that *P. africana* extracts as an antiprostate cancer treatment targets fast dividing cells by impairing mitosis or by causing target cells to undergo apoptosis [1, 20, 57]. Apoptosis is a biological process that occurs through a series of programmed cell death steps characterized by morphological alterations, including plasma and nuclear membrane blebbing, cell shrinkage, dissolution of the nuclear lamina, and biochemical processes responsible for the activation of apoptosis [58]. In a tissue culture study performed by Shenouda et al. [40], ethanolic extracts of *P. africana* showed growth inhibition of a human prostate cancer cell line (PC-3) and epithelial cells derived from a lymph-node carcinoma of the prostate (LNCaP) by 50% at 2.5 μL/mL and also induced significant apoptosis in both cell lines (PC-3 and LNCaP) at 2.5 μL/mL compared to untreated cells. In an in vivo study using TRAMP (transgenic adenocarcinoma of the mice prostate), a model for the pathogenesis of human prostate cancer, the mice that were fed on *P. africana* extract showed a significant reduction ($p = 0.034$) in the prostate cancer incidence (35%) compared to casein fed mice (62.5%) [40]. In another study by Margalef et al. [59], they observed that *P. africana* ethanolic extract had an antimitogenic effect on prostate cancer cells by inhibiting the mitogenic action of epidermal growth factor which resulted in a decreased number of cells entering the S-phase of the cell cycle. Thus, preclinical findings have shown that *P. africana* has a large potential in the regulation of prostate cancer by inhibiting the growth of prostate cancer cell lines and causing apoptosis both in vitro and in vivo. Hence, *P. africana* phytochemicals can be used as an effective cytotoxic chemotherapy in the treatment of men with prostate cancer and other prostate related conditions.

5. Pharmacological Efficacy of Antiprostate Cancer Phytochemicals from *P. africana*

The antiprostate cancer phytochemicals from *P. africana* can be divided into three major categories based on their targets and pharmacological effects (Table 1): (i) phytochemicals that kill the tumor cells through apoptotic pathways, a common mode of action of chemotherapeutic agents against a wide variety of cancer cells [60], (ii) phytochemicals that alter the signaling pathways required for the maintenance of prostate cancer cells, and (iii) phytochemicals that exhibit strong antiandrogenic and antiangiogenic activities. The details of

Figure 2: Modified map from Hall et al. [49] on distribution of *P. africana*.

the antiprostate cancer effects of each phytochemical are shown in Table 1.

5.1. Ursolic Acid. Ursolic acid (Table 1(a)) is a pentacyclic triterpene compound isolated from many types of medicinal plants and widely present in human diet [23, 28]. Several studies have suggested that ursolic acid is one of the main antiprostate cancer phytochemicals found in both root and stem bark extracts of *P. africana* [2, 22, 24, 25]. This acid has been suggested to suppress inflammation, reduce oxidative stress, regulate cell cycle, inhibit cell proliferation, induce apoptosis, and interact with the tumor microenvironment through modulation of multiple signal transduction pathways [22, 26]. A study has shown that the inhibition of cell viability and the induction of apoptosis in PC-3 and LNCaP cells by ursolic acid were associated with downregulation of the B-Cell Lymphoma 2 (BCL-2) protein [23], a member of the protein cell family that controls apoptosis to prevent prostate cancer progression [27, 28]. This crucially proves the ability of ursolic acid to treat human prostate cancer,

Table 1: In vitro and in vivo effects of *P. africana* phytochemicals on prostate cancer cells.

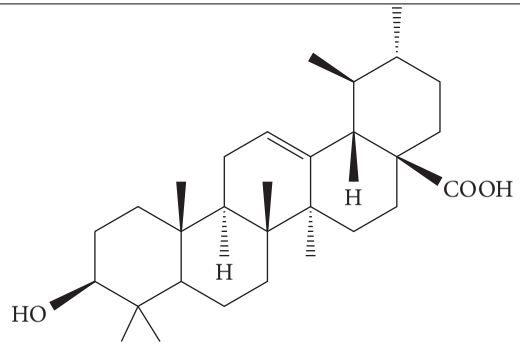
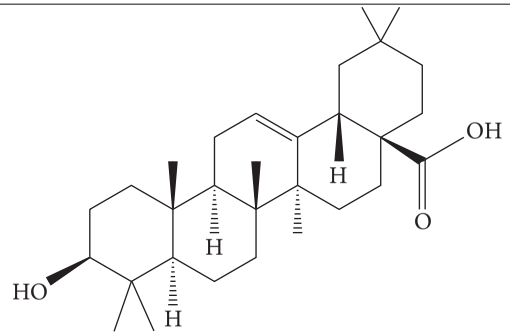
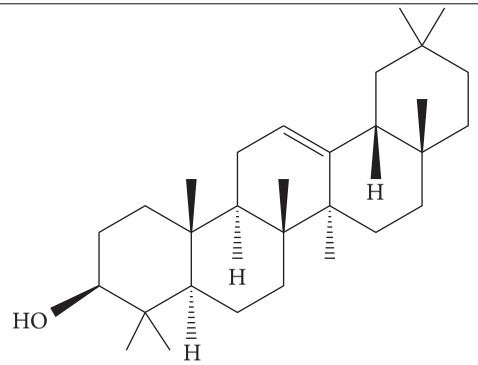
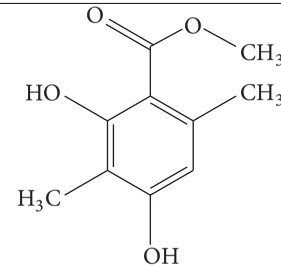
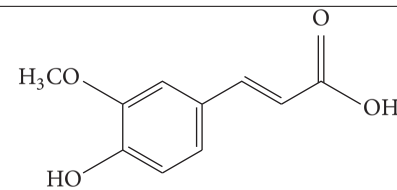
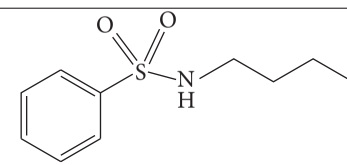
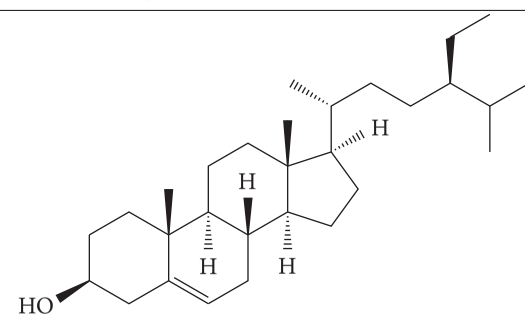
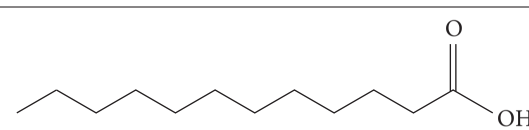
S/No	Class of compound	Phytochemical compounds	Compound structure	Cellular target	Cellular effects	Reference
(a)	Pentacyclic triterpenoid saponins	Ursolic acid		PC-3 LNCaP BCL-2	Inhibited growth of prostate cancer cells; downregulated Bcl-2	[2, 20, 22–28]
(b)	Pentacyclic triterpenoid saponins	Oleanolic acid		PC-3 DU145 LNCaP PI3K/Akt AMPK	Inhibited cell viability, proliferation, promoted cell apoptosis, and arrested G0/G1 phase cell cycle in prostate cancer cells	[2, 22–27, 29, 30]
(c)	Triterpene	β-amyrin		PC-3	Exhibited cytotoxicity and apoptosis on prostate cancer cells	[31]

Table 1: Continued.

S/No	Class	Phytochemical compounds	Chemical structure of compound	Molecular target	Chemopreventive cellular effects	Reference
(d)	Phenol	Atraric acid		PC-3-AR AR	Showed anti-androgenic activity, inhibited AR translocation to the cell nucleus, decreased cell proliferation of PC-3-AR, and inhibited cellular invasion by prostate cancer cells into the extracellular matrix	[6, 9, 32, 33]
(e)	Phenol	Ferulic acid		BCL-2 LNCaP PC-3	Inhibited the angiogenic pathways, inhibited cell proliferation, and promoted apoptosis of PC-3 and LNCaP by downregulating Bcl2 expression	[20, 21, 34–37]
(f)	Phenol	N-butylbenzene-sulfonamide		PC-3-AR AR	Showed antiandrogenic activity, decreased the cell proliferation of PC-3-AR, and inhibited AR translocation to the cell nucleus	[9, 32, 38, 39]
(g)	Sterols	β-sitosterol		LNCaP	Exhibited cytotoxicity and apoptosis and suppressed the production of prostaglandins	[2, 15, 20, 24, 31, 40–43]
(h)	Fatty acid	Lauric acid		LNCaP	Inhibited 5-α-reductase enzyme thus preventing formation of dihydrotestosterone, the modulator of prostate growth	[44–46]

Molecular structure source: https://en.wikipedia.org/.

PC-3: human prostate cancer cell lines. LNCaP: lymph node carcinoma of prostate cell line. BCL-2: B-cell lymphoma 2 protein, which regulates cell death (apoptosis). DU145: a cell line of prostatic cancer derived from brain metastasis. PI3K/Akt: phosphoinositide 3-kinase/protein kinase B, which regulates multiple biological processes including cell survival, proliferation, growth, and glycogen metabolism. AMPK: 5′ adenosine monophosphate-activated protein kinase, an enzyme that plays a role in cellular energy homeostasis. AR: androgen receptor which translocates androgen hormones to the nucleus. PC-3-AR: Human prostate cancer cell lines that expressed the androgen receptor.

a hormone-refractory and androgen-sensitive cancer [23], and to also inhibit the growth of prostate cancer cells [20]. Therefore, downregulation of BCL-2 by ursolic acid results in the apoptosis in human prostate cancer cells making the acid a suitable antiprostate cancer agent.

5.2. Oleanolic Acid. Oleanolic acid (Table 1(b)) is a naturally occurring pentacyclic triterpenoid found in both root and stem bark extracts of *P. africana* [2, 22, 24, 25]. This acid inhibits the survival and proliferation of prostate cancer cells through the induction of apoptosis and interacts with the tumor microenvironment through modulation of multiple signal transduction pathways [22, 26]. These observations were further confirmed by a study by Li et al. [29] who confirmed that oleanolic acid inhibited the cell viability and proliferation and promoted the cell apoptosis and G_0/G_1-phase cell cycle arrest in prostate cancer PC-3, DU145, and LNCaP cells, in a dose-dependent manner. The same study revealed that oleanolic acid exerted anticancer effects in vitro on PC-3 and DU145 cells by repressing the phosphoinositide 3-kinase (PI3K)/protein kinase B (Akt) pathway [29]. In another study, oleanolic acid exerted antitumor activity by interfering with a metabolic pathway in cancer cells through activation of the enzyme 5′-AMP-activated protein kinase (AMPK) [30]. These studies clearly demonstrated the anticancer properties of oleanolic acid for prostate cancer cells, both in vitro and in vivo, and provided the evidence for its use in further preclinical and clinical studies in prostate cancer patients.

5.3. Atraric Acid (AA). Atraric acid is a naturally occurring phenolic compound and ester (Table 1(d)) found in the bark extract of *P. africana* [9, 32]. In vitro studies have shown that it has a very strong antiandrogenic activity [9, 32], which decreased the cell proliferation of PC-3-AR. However, the growth of PC-3 and non-PCa cells lacking the AR expression was not affected by AA, suggesting an AR-dependent growth inhibitory mechanism imposed by AA [32]. In another study which included a reporter gene assay, 10 μM AA repressed the androgen AR-mediated transactivation by about 90% [32]. AA exhibited a 50% inhibition level at a concentration of 1 μM but failed to repress the AR-mediated transactivation at 0.1 μM. These findings show that AA represses androgen AR-mediated transactivation at higher concentrations. AA isolated from *P. africana* was found to exhibit similar inhibition of AR function to that of commercially available AA [6] which further signifies the importance of this phytochemical. AA molecules also inhibited AR translocation to the cell nucleus by binding to the receptor which resulted in downregulation of the androgen level [9]. AA also inhibited the cellular invasion of prostate cancer cells into the extracellular matrix, indicating that it may have a protective role against tumor invasion [33]. Furthermore, a study by Hessenkemper et al. [61] strongly showed that AA led to senescence-associated beta-galactosidase activity, an indication of cellular senescence that resulted into proliferation arrest in PC-3-AR. Therefore, the ability of AA to suppress AR and decrease PC-3 proliferation can be potentially employed in the prostate cancer chemoprevention and chemotherapy.

5.4. Ferulic Acid. Ferulic acid is an abundant phenolic phytochemical (Table 1(e)) found in plant cell wall components of many plants, including *P. africana* [20, 21]. Studies have shown that ferulic acid isolated from the bark extract of *P. africana* inhibited angiogenic pathways [20, 34] and consequently preventing the growth of new blood vessels from preexisting vessels, as well as the growth and spread of prostate cancer [35]. These results were somewhat supported by the data obtained by Mukherji et al. [37], who observed that the inhibition of angiogenic pathways was proven to be an effective strategy for the treatment of several common solid tumors although its definitive role in the management of prostate cancer is yet to be elucidated. In another study conducted by Eroğlu et al. [36], the results revealed that ferulic acid inhibited the cell proliferation and decreased the gene expression of BCL-2 an inhibitor of apoptosis protein 3 (IAP3) in LNCaP cells, resulting in the induction of apoptosis in PC-3 and LNCaP cells. In addition, the authors observed that ferulic acid suppressed the invasion of PC-3 and LNCaP cells [36]. The inhibition of angiogenic pathways and induction of apoptosis by ferulic acid makes it an important therapeutic agent for prostate cancer chemoprevention.

5.5. N-Butylbenzene-Sulfonamide (NBBS). NBBS (Table 1(f)) is one of the compounds naturally found in the bark extract of *P. africana* [9, 32, 38]. It is a sulphur-containing compound that is widely used as a plasticizer in polyacetals and polyamides and it shows high antiandrogenic activity [9]. Although the NBBS phytochemical was found to decrease the cell proliferation of wild-type PC-3-AR cells, the growth of PC-3 and non-PCa cells lacking the AR expression was not affected, suggesting an AR-dependent growth inhibitory mechanism imposed by NBBS [32]. A reporter gene assay that compared AA and NBBS showed that NBBS was less efficacious as an inhibitor of androgen activated AR-mediated transactivation, exhibiting 90% repression only at a higher concentration (100 μM) [9]. NBBS also inhibited both endogenous prostate-specific antigen (PSA) expression and growth of human prostate cancer cells [32]. NBBS molecules also inhibited AR translocation to the cell nucleus by binding to it, thereby downregulating the androgen level [9]. Although the limited available literature reports neurotoxicity of NBBS in rabbits [39], experiments with Sprague Dawley male rats showed no observable effects at a dose of 300 mg/kg/day administered for 27 days [38]. However, deaths of rats were observed at a higher dose of 400 mg/kg/day after 5 days of dosing [38]. These results were confirmed in a review study by Roell and Baniahmad [32] in which the authors revealed that NBBS only had a slight effect on rats at very high doses and a short duration of treatment and that *P. africana* extracts were well tolerated by humans. Therefore, the effects of NBBS on AR and subsequent decrease in PC-3 proliferation can be explored to maximize its potential for the chemoprevention of prostate cancer.

5.6. Beta-Sitosterol. Beta-sitosterol is a plant-derived sterol, also known as a phytosterol (Table 1(g)). The major effects of *P. africana* on prostate cancer have been reported to be due to the presence of this chemical which is present in high concentrations in the plant [24, 40]. Studies have shown that β-sitosterol affects the membrane structure and function of tumor as well as the host tissue signal transduction pathways that regulate tumor growth, leading to an apoptotic condition [40]. Furthermore, this phytosterol exhibited anti-inflammatory effects which suppressed the production of prostaglandins thereby preventing the swelling of the prostate [20]. It also inhibited the human prostate tumor cell invasiveness and reduced the release of matrix metalloproteinases [41]. Studies have also shown that β-sitosterol induced the apoptosis of LNCaP human prostate cancer cells [42, 43]. These antiprostate cancer activities exhibited by β-sitosterol can be further developed for the chemoprevention and chemotherapy of prostate cancer.

5.7. Lauric Acid. Lauric acid is a saturated medium-chain fatty acid with a 12-carbon backbone (Table 1(h)). It is found naturally in various plant and animal fats and oils and is one of the phytochemical components in the stem bark of *P. africana* [20]. It has been found that this acid inhibited the 5-α-reductase enzyme, thus preventing the formation of dihydrotestosterone, the modulator of prostate growth. [20]. Considering that dihydrotestosterone is implicated in the pathogenesis of prostate cancer [45], inhibition of the 5-α-reductase enzyme by lauric acid, resulting in the blockage of testosterone conversion to dihydrotestosterone, plays an integral role in the prevention and treatment of testosterone prostate cancer [44, 45]. The potential of lauric acid for chemoprevention of prostate cancer has been further supported by a study that showed its ability to inhibit the proliferation of LNCaP cells [46].

6. Use of *P. africana* in African Traditional Medicine to Treat Prostate Cancer

Despite the fact that the early detection and commencement of cancer treatment usually increase the chances of survival in developed world, the chances of survival in developing countries are usually much lower since the access to modern cancer diagnostic facilities and effective treatment methods are limited for most people especially for those living in rural areas. In Africa in particular, the herbalists rely on the use of potent herbal medicines to tackle the disease burden. *P. africana* is one of the plants frequently used in many African communities for the treatment of prostate cancer. Thus, according to a study by Ochwang'i et al. [1], the pounded stem bark of *P. africana* is usually mixed with water and drunk as a remedy for Prostate cancer in Kakamega county in Kenya.

7. Conclusion

Prostate cancer is still one of the leading causes of death in men worldwide. Therefore, all possible avenues have to be explored in an attempt to tackle the disease. Easily accessible and affordable options, other than the conventional methods of surgery, radiation therapy, cryosurgery, and hormone therapies especially for the poor need to be explored. This review proves that the use of naturally occurring phytochemicals from *P. africana* can be considered for both chemoprevention and chemotherapy of prostate cancer. This plant has a rich history of use in the treatment and management of prostate cancer in ATM. Scientific studies have proven that phytochemicals from *P. africana* have the ability to affect numerous targets associated with the degradation of the prostate cancer cells. Therefore, these pharmacological effects of *P. africana* phytochemicals can be exploited and utilized for the chemoprevention and chemotherapy of prostate cancer. However, more preclinical and clinical studies need to be done to validate these phytochemicals for possible use in the antiprostate cancer drug development. Unfortunately, despite its significant potential for the treatment of prostate cancer and other diseases, improper methods of stem bark harvesting and illegal logging of *P. africana* have made the plant an endangered species. As a result, there is need for advanced *P. africana* propagation techniques to urgently increase its population and distribution range beyond the current level so as to meet the ever increasing demand for this plant and its constituents.

Additional Points

Limitations. This review has some limitations. First, during our review process we did not include unpublished information and hence our findings could be affected by a publication bias. Second, we might have missed studies or vital information published on sites other than those we focused on. Nevertheless, this review is very significant in that it clearly reveals the potential of *P. africana* for the chemoprevention and chemotherapy of prostate cancer.

Competing Interests

The authors declare that there is no conflict of interests regarding the publication of this review paper.

Authors' Contributions

R. Komakech and Y. Kang equally contributed to this manuscript and should be considered co-first authors.

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References

[1] D. O. Ochwang'i, C. N. Kimwele, J. A. Oduma, P. K. Gathumbi, J. M. Mbaria, and S. G. Kiama, "Medicinal plants used in treatment and management of cancer in Kakamega County, Kenya," *Journal of Ethnopharmacology*, vol. 151, no. 3, pp. 1040–1055, 2014.

[2] V. Steenkamp, "Phytomedicines for the prostate," *Fitoterapia*, vol. 74, no. 6, pp. 545–552, 2003.

[3] N. J. Toyang, H. K. Wabo, E. N. Ateh et al., "In vitro anti-prostate cancer and ex vivo antiangiogenic activity of *Vernonia guineensis* Benth. (Asteraceae) tuber extracts," *Journal of Ethnopharmacology*, vol. 141, no. 3, pp. 866–871, 2012.

[4] H. Ting, G. Deep, C. Agarwal, and R. Agarwal, "The strategies to control prostate cancer by chemoprevention approaches," *Mutation Research*, vol. 760, pp. 1–15, 2014.

[5] P. I. Lorenzo and F. Saatcioglu, "Inhibition of apoptosis in prostate cancer cells by androgens is mediated through down-regulation of c-Jun N-terminal kinase activation," *Neoplasia*, vol. 10, no. 5, pp. 418–428, 2008.

[6] S. Schleich, M. Papaioannou, A. Baniahmad, and R. Matusch, "Activity-guided isolation of an antiandrogenic compound of *Pygeum africanum*," *Planta Medica*, vol. 72, no. 6, pp. 547–551, 2006.

[7] M.-E. Taplin, G. J. Bubley, T. D. Shuster et al., "Mutation of the androgen-receptor gene in metastatic androgen-independent prostate cancer," *New England Journal of Medicine*, vol. 332, no. 21, pp. 1393–1398, 1995.

[8] J. Lü, S.-H. Kim, C. Jiang, H. Lee, and J. Guo, "Oriental herbs as a source of novel anti-androgen and prostate cancer chemopreventive agents," *Acta Pharmacologica Sinica*, vol. 28, no. 9, pp. 1365–1372, 2007.

[9] M. Papaioannou, S. Schleich, D. Roell et al., "NBBS isolated from *Pygeum africanum* bark exhibits androgen antagonistic activity, inhibits AR nuclear translocation and prostate cancer cell growth," *Investigational New Drugs*, vol. 28, no. 6, pp. 729–743, 2010.

[10] T. Gilligan and P. W. Kantoff, "Chemotherapy for prostate cancer," *Urology*, vol. 60, no. 3, pp. 94–100, 2002.

[11] S. Wang, X. Wu, M. Tan et al., "Fighting fire with fire: poisonous Chinese herbal medicine for cancer therapy," *Journal of Ethnopharmacology*, vol. 140, no. 1, pp. 33–45, 2012.

[12] K. R. Landis-Piwowar and N. R. Iyer, "Cancer chemoprevention: current state of the art," *Cancer Growth and Metastasis*, vol. 7, pp. 19–25, 2014.

[13] A. Bhanot, R. Sharma, and M. N. Noolvi, "Natural sources as potential anti-cancer agents: a review," *International Journal of Phytomedicine*, vol. 3, no. 1, pp. 9–26, 2011.

[14] G. M. Cragg, P. G. Grothaus, and D. J. Newman, "Impact of natural products on developing new anti-cancer agents," *Chemical Reviews*, vol. 109, no. 7, pp. 3012–3043, 2009.

[15] C. A. C. Kadu, A. Parich, S. Schueler et al., "Bioactive constituents in *Prunus africana*: geographical variation throughout Africa and associations with environmental and genetic parameters," *Phytochemistry*, vol. 83, pp. 70–78, 2012.

[16] O. M. Grace, H. D. V. Prendergast, A. K. Jäger, and J. Van Staden, "Bark medicines used in traditional healthcare in KwaZulu-Natal, South Africa: an inventory," *South African Journal of Botany*, vol. 69, no. 3, pp. 301–363, 2003.

[17] D. W. Nyamai, W. M. Arika, H. O. Rachuonyo, J. R. Wambani, and M. P. Ngugi, "Herbal management of benign prostatic hyperplasia," *Journal of Cancer Science & Therapy*, vol. 8, no. 5, pp. 130–134, 2016.

[18] A. K. Jena, K. Vasisht, N. Sharma, R. Kaur, M. S. Dhingra, and M. Karan, "Amelioration of testosterone induced benign prostatic hyperplasia by Prunus species," *Journal of Ethnopharmacology*, vol. 190, pp. 33–45, 2016.

[19] L. Jimu, "Threats and conservation strategies for the African Cherry (*Prunus africana*) in its natural range-a review," *Journal of Ecology and Natural Environment*, vol. 3, no. 4, pp. 118–130, 2011.

[20] D. W. Nyamai, A. M. Mawia, F. K. Wanbua, A. Njoroge, and F. Matheri, "Phytochemical profile of *Prunus africana* stem bark from Kenya," *Journal of Pharmacognosy and Natural Products*, vol. 1, p. 110, 2015.

[21] M. C. Ngule, M. H. Ndiku, and F. Ramesh, "Chemical constituents screening and in vitro antibacterial assessment of *Prunus africana* bark hydromethanolic extract," *Journal of Natural Sciences Research*, vol. 4, no. 16, pp. 85–90, 2014.

[22] A. Bishayee, S. Ahmed, N. Brankov, and M. Perloff, "Triterpenoids as potential agents for the chemoprevention and therapy of breast cancer," *Frontiers in Bioscience*, vol. 16, no. 3, pp. 980–996, 2011.

[23] H. Yang and Q. P. Dou, "Targeting apoptosis pathway with natural terpenoids: implications for treatment of breast and prostate cancer," *Current Drug Targets*, vol. 11, no. 6, pp. 733–744, 2010.

[24] M. A. Hass, D. M. Nowak, E. Leonova, R. M. Levin, and P. A. Longhurst, "Identification of components of Prunus africana extract that inhibit lipid peroxidation," *Phytomedicine*, vol. 6, no. 5, pp. 379–388, 1999.

[25] C. Fourneau, R. Hocquemiller, and A. Cavé, "Triterpenes from *Prunus africana* bark," *Phytochemistry*, vol. 42, no. 5, pp. 1387–1389, 1996.

[26] S. H. Safe, P. L. Prather, L. K. Brents, G. Chadalapaka, and I. Jutooru, "Unifying mechanisms of action of the anticancer activities of triterpenoids and synthetic analogs," *Anti-Cancer Agents in Medicinal Chemistry*, vol. 12, no. 10, pp. 1211–1220, 2012.

[27] P. E. Czabotar, G. Lessene, A. Strasser, and J. M. Adams, "Control of apoptosis by the BCL-2 protein family: implications for physiology and therapy," *Nature Reviews Molecular Cell Biology*, vol. 15, no. 1, pp. 49–63, 2014.

[28] V. R. Yadav, S. Prasad, B. Sung, R. Kannappan, and B. B. Aggarwal, "Targeting inflammatory pathways by triterpenoids for prevention and treatment of cancer," *Toxins*, vol. 2, no. 10, pp. 2428–2466, 2010.

[29] X. Li, Y. Song, P. Zhang et al., "Oleanolic acid inhibits cell survival and proliferation of prostate cancer cells in vitro and in vivo through the PI3K/Akt pathway," *Tumor Biology*, vol. 37, no. 6, pp. 7599–7613, 2016.

[30] J. Liu, L. Zheng, N. Wu et al., "Oleanolic acid induces metabolic adaptation in cancer cells by activating the amp-activated protein kinase pathway," *Journal of Agricultural and Food Chemistry*, vol. 62, no. 24, pp. 5528–5537, 2014.

[31] F. Maiyo, R. Moodley, and M. Singh, "Phytochemistry, cytotoxicity and apoptosis studies of β-sitosterol-3-O-Glucoside and β -amyrin from *Prunus africana*," *The African Journal of Traditional, Complementary and Alternative Medicines*, vol. 13, no. 4, 2016.

[32] D. Roell and A. Baniahmad, "The natural compounds atraric acid and N-butylbenzene-sulfonamide as antagonists of the

human androgen receptor and inhibitors of prostate cancer cell growth," *Molecular and Cellular Endocrinology*, vol. 332, no. 1-2, pp. 1–8, 2011.

[33] M. Papaioannoua, A. Söderholma, W. Honge et al., "Computational and functional analysis of the androgen receptor antagonist atraric acid and its derivatives," *Anti-Cancer Agents in Medicinal Chemistry*, vol. 13, no. 5, pp. 801–810, 2013.

[34] G.-W. Yang, J.-S. Jiang, and W.-Q. Lu, "Ferulic acid exerts anti-angiogenic and anti-tumor activity by targeting fibroblast growth factor receptor 1-mediated angiogenesis," *International Journal of Molecular Sciences*, vol. 16, no. 10, pp. 24011–24031, 2015.

[35] B. Nicholson and D. Theodorescu, "Angiogenesis and prostate cancer tumor growth," *Journal of Cellular Biochemistry*, vol. 91, no. 1, pp. 125–150, 2004.

[36] C. Eroğlu, M. Seçme, G. Bağcı, and Y. Dodurga, "Assessment of the anticancer mechanism of ferulic acid via cell cycle and apoptotic pathways in human prostate cancer cell lines," *Tumor Biology*, vol. 36, no. 12, pp. 9437–9446, 2015.

[37] D. Mukherji, S. Temraz, D. Wehbe, and A. Shamseddine, "Angiogenesis and anti-angiogenic therapy in prostate cancer," *Critical Reviews in Oncology/Hematology*, vol. 87, no. 2, pp. 122–131, 2013.

[38] C. V. Rider, K. S. Janardhan, D. Rao, J. P. Morrison, C. A. McPherson, and G. J. Harry, "Evaluation of N-butylbenzenesulfonamide (NBBS) neurotoxicity in Sprague-Dawley male rats following 27-day oral exposure," *NeuroToxicology*, vol. 33, no. 6, pp. 1528–1535, 2012.

[39] M. J. Strong, R. M. Garruto, A. V. Wolff, S. M. Chou, S. D. Fox, and R. Yanagihara, "N-Butyl benzenesulfonamide: a neurotoxic plasticizer inducing a spastic myelopathy in rabbits," *Acta Neuropathologica*, vol. 81, no. 3, pp. 235–241, 1991.

[40] N. Shenouda, M. Sakla, L. Newton et al., "Phytosterol Pygeum africanum regulates prostate cancer in vitro and in vivo," *Endocrine*, vol. 31, no. 1, pp. 72–81, 2007.

[41] F. A. Attiga, P. M. Fernandez, A. T. Weeraratna, M. J. Manyak, and S. R. Patierno, "Inhibitors of prostaglandin synthesis inhibit human prostate tumor cell invasiveness and reduce the release of matrix metalloproteinases," *Cancer Research*, vol. 60, no. 16, pp. 4629–4637, 2000.

[42] R. L. Von Holtz, C. S. Fink, and A. B. Awad, "β-Sitosterol activates the sphingomyelin cycle and induces apoptosis in LNCaP human prostate cancer cells," *Nutrition and Cancer*, vol. 32, no. 1, pp. 8–12, 1998.

[43] A. B. Awad, Y. Gan, and C. S. Fink, "Effect of β-sitosterol, a plant sterol, on growth, protein phosphatase 2A, and phospholipase D in LNCaP cells," *Nutrition and Cancer*, vol. 36, no. 1, pp. 74–78, 2000.

[44] L. Goldenberg, A. So, N. Fleshner, R. Rendon, D. Drachenberg, and M. Elhilali, "The role of 5-alpha reductase inhibitors in prostate pathophysiology: is there an additional advantage to inhibition of type 1 isoenzyme?" *Journal of the Canadian Urological Association*, vol. 3, no. 3, pp. S109–S114, 2009.

[45] H. J. Niederprüm, H. U. Schweikert, J. W. Thüroff, and K. S. Zänker, "Inhibition of steroid 5α−reductase activity by aliphatic fatty acids: candidates for chemoprevention of prostate cancer," *Annals of the New York Academy of Sciences*, vol. 768, no. 1, pp. 227–230, 1995.

[46] J. Liu, K. Shimizu, and R. Kondo, "Anti-androgenic activity of fatty acids," *Chemistry & Biodiversity*, vol. 6, no. 4, pp. 503–512, 2009.

[47] S. Y. Kim, J. L. England, J. A. Sharma, and T. Njoroge, "Gestational diabetes mellitus and risk of childhood overweight and obesity in offspring: a systematic review," *Experimental Diabetes Research*, vol. 2011, Article ID 541308, 9 pages, 2011.

[48] J. Lin, S. Chen, S. Li, M. Lu, Y. Li, and Y. Su, "Efficacy and safety of chinese medicinal herbs for the treatment of hyperuricemia: a systematic review and meta-analysis," *Evidence-Based Complementary and Alternative Medicine*, vol. 2016, Article ID 2146204, 12 pages, 2016.

[49] J. B. Hall, E. M. O'Brian, and F. L. Sinclair, *Prunus africana—A Monograph*, School of Agriculture and Forest Sciences, University of Wales, ICRAF, Bangor, UK, 2000.

[50] D. Biswajit, N. Ahmed, and S. Pushkar, "Prunus diversity-early and present development: a review," *International Journal of Biodiversity and Conservation*, vol. 3, no. 14, pp. 721–734, 2011.

[51] K. Stewart, *Effects of Bark Harvest and Other Human Activity on Populations of the African Cherry (Prunus africana) on Mount Oku, Cameroon*, Elsevier, 2009.

[52] A. B. Cunningham, E. Ayuk, S. Franzel, B. Duguma, and C. Asanga, "An economic evaluation of Medical tree cultivation; *Prunus africana* in Cameroon," in *People and Working Paper 10.*, UNESCO, 2002.

[53] C. Orwa, A. Mutua, R. Kindt, R. Jamnadass, and A. Simons, *Agroforestree Database: A Tree Reference and Selection Guide Version 4.0.*, World Agroforestry Centre, Nairobi, Kenya, 2009.

[54] G. Bodeker, C. Van'T Klooster, and E. Weisbord, "Prunus africana (Hook.f.) Kalkman: the overexploitation of a medicinal plant species and its legal context," *Journal of Alternative and Complementary Medicine*, vol. 20, no. 11, pp. 810–822, 2014.

[55] K. M. Stewart, "The African cherry (*Prunus africana*): can lessons be learned from an over-exploited medicinal tree?" *Journal of Ethnopharmacology*, vol. 89, no. 1, pp. 3–13, 2003.

[56] I. Dawson, J. Were, and A. Lengkeek, "Conservation of *Prunus africana*, an over-exploited African medicinal tree," Forest Genetic Resources 28, FAO, Rome, Italy, 2000.

[57] A. Sofowora, E. Ogunbodede, and A. Onayade, "The role and place of medicinal plants in the strategies for disease prevention," *The African Journal of Traditional, Complementary, and Alternative Medicines*, vol. 10, no. 5, pp. 210–229, 2013.

[58] S.-J. Jeong, W. Koh, B. Kim, and S.-H. Kim, "Are there new therapeutic options for treating lung cancer based on herbal medicines and their metabolites?" *Journal of Ethnopharmacology*, vol. 138, no. 3, pp. 652–661, 2011.

[59] S. M. A. Margalef, P. R. Barzanti, R. J. Puigjaner, R. J. Morote, and T. T. M. Okatsu, "Antimitogenic effect of *Pygeum africanum* extracts on human prostatic cancer cell lines and explants from benign prostatic hyperplasia," *Archivos Españoles de Urología*, vol. 56, no. 4, pp. 369–378, 2003.

[60] W. L. W. Hsiao and L. Liu, "The role of traditional Chinese herbal medicines in cancer therapy from TCM theory to mechanistic insights," *Planta Medica*, vol. 76, no. 11, pp. 1118–1131, 2010.

[61] W. Hessenkemper, J. Roediger, S. Bartsch et al., "A natural androgen receptor antagonist induces cellular senescence in prostate cancer cells," *Molecular Endocrinology*, vol. 28, no. 11, pp. 1831–1840, 2014.

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Research Article

Rosemary Extracts Upregulate Nrf2, Sestrin2, and MRP2 Protein Level in Human Hepatoma HepG2 Cells

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In the past few decades, the incidence of liver cancer has been rapidly rising across the world. Rosemary is known to possess antioxidant activity and is used as natural antioxidant food preservative. It is proposed to have anticancer activity in treating different tumor models. In this study, we try to explore the impact of rosemary extracts on upregulating the level of Nrf2 and Nrf2-regulatory proteins, Sestrin2 and MRP2 in HepG2 cells, and to speculate its potential mechanism. The anticancer activity of rosemary extract, including its polyphenolic diterpenes carnosic acid and carnosol, was evaluated to understand the potential effect on HepG2 cells. Rosemary extract, carnosic acid, and carnosol induced the expression of Sestrin2 and MRP2 associate with enhancement of Nrf2 protein level in HepG2 cells, in which carnosic acid showed most obvious effect. Although the activation pathway of Nrf2/ARE was not exactly assessed, it can be assumed that the enhancement of expression of Sestrin2 and MRP2 may result from upregulation of Nrf2.

1. Introduction

Nrf2/ARE signaling pathway plays significant role in protecting cells from acute and chronic cell injury. Nrf2 regulates the expression of phase II antioxidant enzymes genes by controlling the antioxidant response element (ARE) sequences. Nrf2/ARE pathway is considered as a new way in mediating the antioxidant response in cancer and other chronic degenerative diseases linked to oxidative stress [1, 2].

Many naturally occurring compounds have been shown to have a wide range of pharmacological properties, which can have potent cancer chemopreventive properties, similar to beta-naphtoflavone, tert-butyl hydroquinone, and phenethyl isothiocyanate [3].

Rosemary *(Rosmarinus officinalis)* is an aromatic evergreen herb native to the Mediterranean region, which is an important component of the Mediterranean diet, and has been used in Traditional Chines medicine. Modern pharmacology has demonstrated that rosemary has functions of antiatherogenic [4], antidepressant [5, 6], antioxidant, and anti-inflammatory [7]. Rosemary extracts, especially its diterpenes (e.g., carnosic acid and carnosol) have been proposed to have anticancer activities in treating different tumor models [1, 2, 8]. Rosemary has diverse actions including activation in cell signaling or cell cycle dynamics in hepatoma cells, against oxidative injury through the SIRT1 pathway, and autophagic cell death induction through inhibition of the Akt/mTOR pathway.

Extensively, rosemary can also act as anticancer agent in cell system, which can conduct cytoprotective effects. This hypothesis is based on its antioxidant activity. As Nrf2/ARE pathway is significant cytoprotective medium in cell system, we evaluated the protein level of Nrf2 and Nrf2-regulatory proteins, such as Sestrin2 and MRP2 on HepG2 cells, treated by different concentrations of rosemary extracts to investigate its chemoprevention and anticancer effect on HepG2 cells.

2. Materials and Methods

2.1. Rosemary and Its Compounds, Kits, and Antibodies. Rosemary extract (RE, contained carnosic acid 23.2%, carnosol 12.4%), carnosic acid (CA, above 99% purity), and carnosol (CL, above 99% purity) were obtained from OnRoad Biotech

Table 1: The sequences and Genbank accession number of Nrf2, MRP2, and Sestrin2.

mRNA	Sequences	Genbank accession number	Size
Actin-F	CGTGGACATCCGCAAAGAC	XM_006715764.1	234 bp
Actin-R	TCGTCATACTCCTGCTTGCTG		
NRF2-F	AACCAGTGGATCTGCCAACTACTC	NM_001145413.2	90 bp
NRF2-R	CTGCGCCAAAAGCTGCAT		
MRP2-F	TGAGCAAGTTTGAAACGCACAT	NM_000392.4	78 bp
MRP2-R	AGCTCTTCTCCTGCCGTCTCT		
SESN2-F	GAGAAGACCACCCGAAGAATGT	NM_031459.4	153 bp
SESN2-R	CAGGAGTCAGGTCATGTAGCGG		

Co. (Changsha, China). MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide] was from THEMO cleaved caspase-3. ELISA kit was obtained from Cusabio Biotech Co. Ltd. (Wuhan, China). Antibodies for Western Blot: anti-Nrf2 antibody (ab137550) and anti-MRP2 antibody (ab172630) were from Abcam (USA). Sestrin2 (H-62) antibody was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). BCA protein assay kit was obtained from Beijing ComWin Biotech Co., Ltd. (Beijing, China). One-Step RT-PCR kit was obtained from Life Technologies (Grand Island, NY, USA). RNeasy mini kit and RNase-Free DNase set for RNA extraction were obtained from QIAGEN (Santa Clarita, CA, USA).

2.2. Cell Culture and Treatment. Human hepatoma cell line (HepG2) was obtained from Xiangya Cell Bank (Central South University, Changsha, China), cultured in Dulbecco modified eagle medium (Hyclone, Logan, USA) supplemented with 10% fetal bovine serum (Sijiqing, Hangzhou, China) and 1% penicillin/streptomycin in a 37°C incubator with 5% CO_2. Cells were cultured in mediums supplemented with a range of concentrations of RE, CA, or CL for designed times. RE, CA, and CL were solubilized and delivered in dimethyl sulfoxide (DMSO) and stored at the temperature of −20°C. Drugs were freshly diluted to the indicated concentrations with culture medium before use. The final DMSO concentration in the media for RE was 1% and for CA or CL was 0.1%, respectively. Vehicle controls were employed in all experiments. Treatments started after cells attached for 24 hours.

2.3. Cell Viability (MTT Assay). Cell viability was measured by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) reduction assay. HepG2 cells seeding density was about 2×10^4 in each well. Cells were cultured in mediums which were supplemented with a range of concentrations of RE (0, 10, 20, 30, 40, 50, 75, and 100 μg/mL) or CA and CL (0, 10, 20, 30, 40, 50, 75, and 100 μM), respectively, for 24, 48, or 72 hours. The concentration gradients choice was based on the reference [9]. Then MTT (5 mg/mL) was added to each well (300 μL) and incubated for 3–5 hours, after which the MTT was removed and DMSO (300 μL) was added to each well. After shaking for 10 minutes, 100 μL of each sample was transferred to a 96-well microtiter plate and the absorbance was recorded at 540 nm.

2.4. ELISA. Cleaved caspase-3 levels in same amounts of protein in different cell lysates were detected with ELISA. All procedures were conducted according to the manufacturer's manual (Huamei, Wuhan, China). The seeding density of HepG2 cells was 1×10^6, respectively, in each 10 cm^2 plate. Cells were cultured in mediums which were supplemented with a range of concentrations of RE (0, 30, 50, and 100 μg/mL) or CA and CL (0, 30, 50, and 100 μM), respectively, for 24 hours.

2.5. Western Blots. Whole cell lysates from treated cells were prepared as previously described. Lysates were quantified for protein concentration by using BCA assay according to the manufacturer's manual. A common Western blots protocol was followed [10]. Briefly, similar amounts of protein were loaded to each well of 8% or 10% precast gels (Bio-Rad, Hercules, CA, USA). After transfer, membranes were blocked with 5% skim milk powder solution and incubated with primary antibody (1 : 1000 or 1 : 2000) overnight at the temperature of 4°C, rinsed briefly, and then incubated with secondary antibody (1 : 10000) at room temperature for 1 hour. Membranes were washed, incubated with substrates, and exposed in a FluorChem E imager (Bio-Rad, Hercules, CA, USA). The seeding densities of HepG2 cells were 1×10^6, respectively, in each 10 cm^2 plate. Cells were cultured medium supplemented with a range of concentrations of RE (0, 30, 50, and 100 μg/mL) or CA and CL (0, 30, 50, and 100 μM), respectively, for 24 hours. Western blots were performed with 3 separate lysates at least. Separate lysates were analyzed 3 times by Western Blot to confirm the results.

2.6. RT-PCR. (See Table 1). Primers of Sestrin2 and RT-PCR were synthesized by IDT (Integrated DNA Technologies) (Coralville, IA, USA). HepG2 cells were treated with 40 μg/mL RE or 60 μM CA for 24 hours. Total RNA was extracted from treated and control cells. One microgram of total RNA was used as template for one-step RT-PCR. The manufacturer's protocol was followed. The seeding densities of HepG2 cells were 1×10^6 in each 10 cm^2 plate. Cells were cultured in medium supplemented with a range of

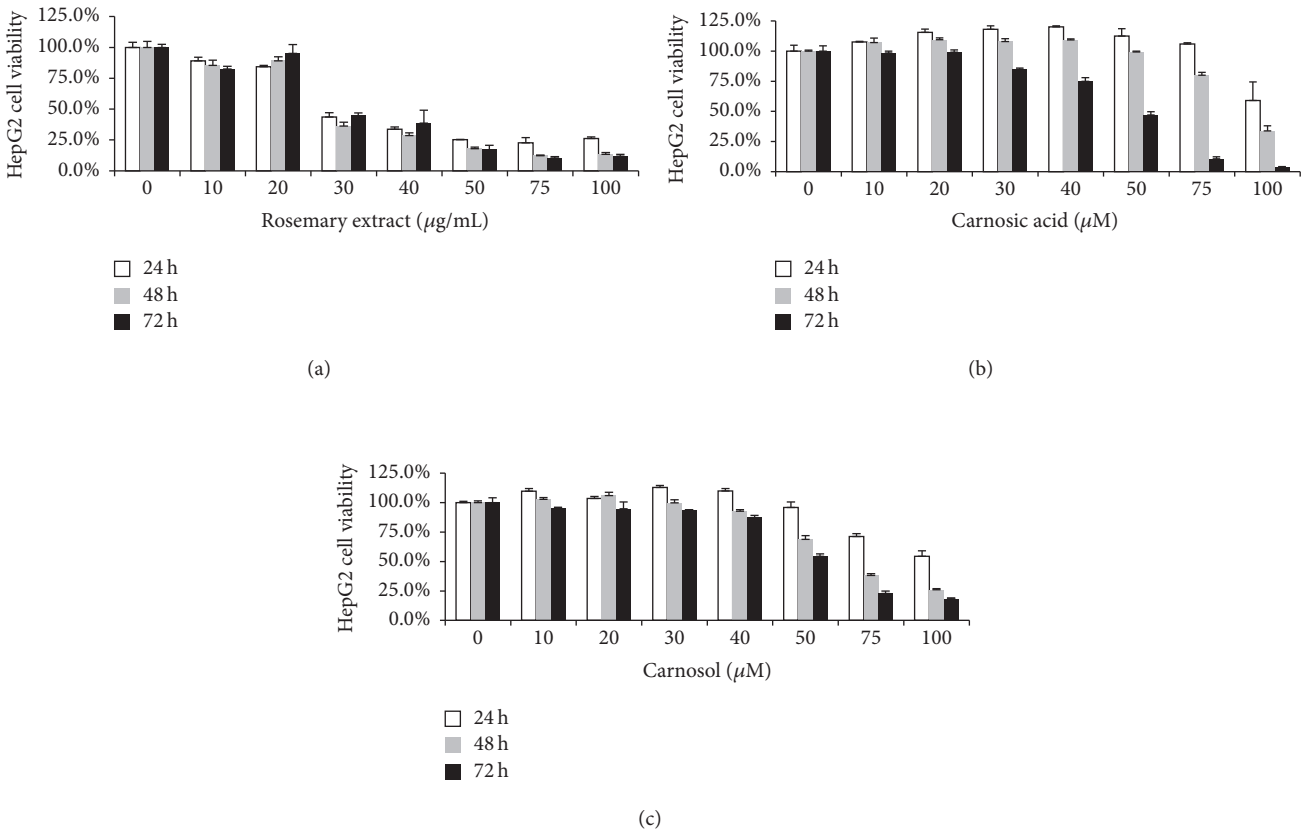

Figure 1: Rosemary extract (RE), carnosic acid (CA), and carnosol (CL) decreased cell viability on HepG2 cells. HepG2 cells (2×10^4 cells per well) were treated with various concentrations of RE (a) (0, 10, 20, 30, 40, 50, 75, and 100 μg/mL), CA (b) (0, 10, 20, 30, 40, 50, 75, and 100 μM), and CL (c) (0, 10, 20, 30, 40, 50, 75, and 100 μM) for 24, 48, and 72 hrs. Results are represented by statistical mean along with standard deviation.

concentrations of RE (0, 30, 50, and 100 μg/mL) or CA and CL (0, 30, 50, and 100 μM), respectively, for 24 hours.

2.7. Statistical Analysis. Results of the experiment were reported as mean ± standard deviation (SD) and conducted with SPSS19.0. All data were analyzed by one-way ANOVA, tested by Tukey's test for multiple comparisons. All statistical tests were two-sided. $P < 0.05$ was considered statistically significant.

3. Results

3.1. RE, CA, and CL Reduced Hepatoma Cell Viability. The impacts of RE, CA, and CL on HepG2 cell viability were determined. HepG2 cells were treated with RE (0, 10, 20, 30, 40, 50, 75, and 100 μg/mL) or CA and CL (0, 10, 20, 30, 40, 50, 75, and 100 μM) for 24, 48, and 72 hours, respectively. MTT results showed that RE, CA, and CL decreased HepG2 cell viability in dose and time-dependent manner (Figure 1). Among them, carnosic acid had the most obvious effect.

3.2. RE, CA, and CL Increased the Degree of Caspase-3 Level in Hepatoma Cancer Cells. Whole cell lysates from HepG2 cells were subjected to ELISA assays of cleaved caspase-3 by using ELISA kit. BCA kit was used to establish standard curve to measure the protein level. ELISA data revealed that the degree of caspase-3 levels in HepG2 cells treated with RE (0, 30, 50, and 100 μg/mL) and CA and CL (0, 30, 50, and 100 μM) was increased in a dose-dependent manner after being treated for 24 hours (Figure 2). Rosemary extract significantly increased cleaved caspase-3 at 50 μg/mL ($P < 0.05$) and 100 μg/mL ($P < 0.01$) after 24 hours. Significant increase of cleavage of caspase-3 from carnosic acid was also observed at 50 μM ($P < 0.05$) and 100 μM ($P < 0.01$) after 24 hours, while carnosol increased cleaved caspase-3 after 24 hours at 100 μM ($P < 0.05$).

3.3. RE, CA, and CL Increased Expression of Sestrin2 and MRP2 Concomitantly with Enhanced Nrf2 mRNA and Protein Levels in HepG2 Cells. In this part, we detected the protein level and mRNA level of Nrf2, Sestrin2, and MRP2 by Western Blot and RT-PCR, respectively, in HepG2 cells. HepG2 cells were treated with RE (0, 30, 50, and 100 μg/mL) and CA and CL (0, 30, 50, and 100 μM) for 24 hours. Western Blot results showed that the protein level of Nrf2 in HepG2 cells was increased by rosemary extract and carnosol in a dose-dependent manner, while Sestrin2 and MRP2 were

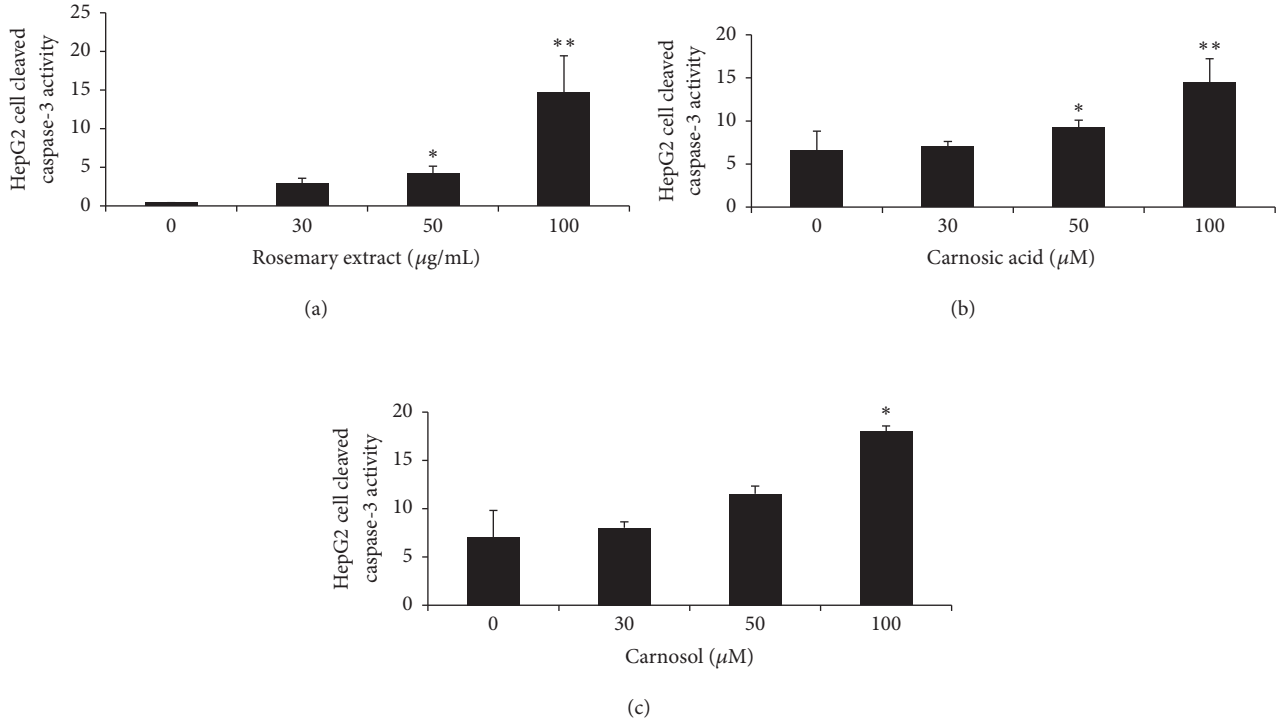

Figure 2: Rosemary extract (RE), carnosic acid (CA), and carnosol (CL) increased the degree of caspase-3 levels in HepG2 cells. HepG2 cells (1×10^6 cells per well) were treated with RE (0, 30, 50, and 100 μg/mL) or CA and CL (0, 30, 50, and 100 μM), respectively, for 24 hrs. (a) Cleaved caspase-3 level in RE treated HepG2 cells after 24 hrs. (b) Cleaved caspase-3 level in CA treated HepG2 cells after 24 hrs. (c) Cleaved caspase-3 level in CL treated HepG2 cells after 24 hrs. Results are represented by statistical mean along with standard deviation, $^{*}P < 0.05$ and $^{**}P < 0.01$.

inconspicuous. Carnosic acid increased the protein level of Nrf2 accompanied with remarkable increase of Sestrin2 and MRP2 (Figure 3). To interpret the results more directly, we also provided the densitometric analysis to make quantitative determination of Western Blot (Figure 3). RT-PCR results showed that the effects of RE, CA, and CL on mRNA were generally consistent with the protein done (Figure 4).

4. Discussion and Conclusion

Rosemary and its polyphenolic diterpene, carnosic acid and carnosol, are known to possess antioxidant activity and are used as natural antioxidant food preservative widely. The most abundant polyphenolic diterpene of rosemary is carnosic acid. It has been thought to be the primary contributor for antioxidant activity [10]. Compared to its antioxidant activity, the anticancer and chemoprevention effects were more intrigued to us.

Sestrin2 is a kind of Nrf2-regulatory protein that may function in the regulation of cell growth and survival and be involved in protecting against oxidative stress. It may be involved in cellular response in different stress conditions [11]. The increase of the expression of Sestrin2 is thought to be cytoprotective. MRP2 is one of the multidrug resistance-associated proteins, which is also regulated by Nrf2. It is thought as a major impediment to the success of cancer chemotherapy. There are many literatures reported that MPRs lead to drug resistance or drug-drug interactions in the process of drug treatment [12]. Normally, MPR2 can also function on cellular detoxification, oxidative stress, inflammation, material transportation, and so on. Chemoprophylactic activity of MRP2 is based on the role of efflux transporter. It can excrete xenobiotics and resist the damage when cells were exposed to external stimulus. The increase of the expression of MRP2 is thought to be chemoprevention. In this study, we detected the protein level of Sestrin2 and MRP2. The results showed that the expression of Sestrin2 and MRP2 was increased after being treated by rosemary extracts. This consequence indirectly probed that the rosemary extracts may possess chemoprevention and anticancer effect on HepG2 cells.

Nrf2/ARE signaling pathway has emerged as novel target for the cancer prevention because of its cytoprotective function. It regulates the expression of phase II antioxidant enzymes genes by controlling the antioxidant response element (ARE) sequences. Sestrin2 and MRP2 are two of the functional proteins regulated by Nrf2. Experimental results show that the expression of Sestrin2 and MRP2 was increased concomitantly with the enhancement of Nrf2 protein levels in HepG2 cells. Although the activation of Nrf2/ARE pathway was not assessed it may be assumed that enhanced expression of Sestrin2 and MRP2 may result from Nrf2 upregulation.

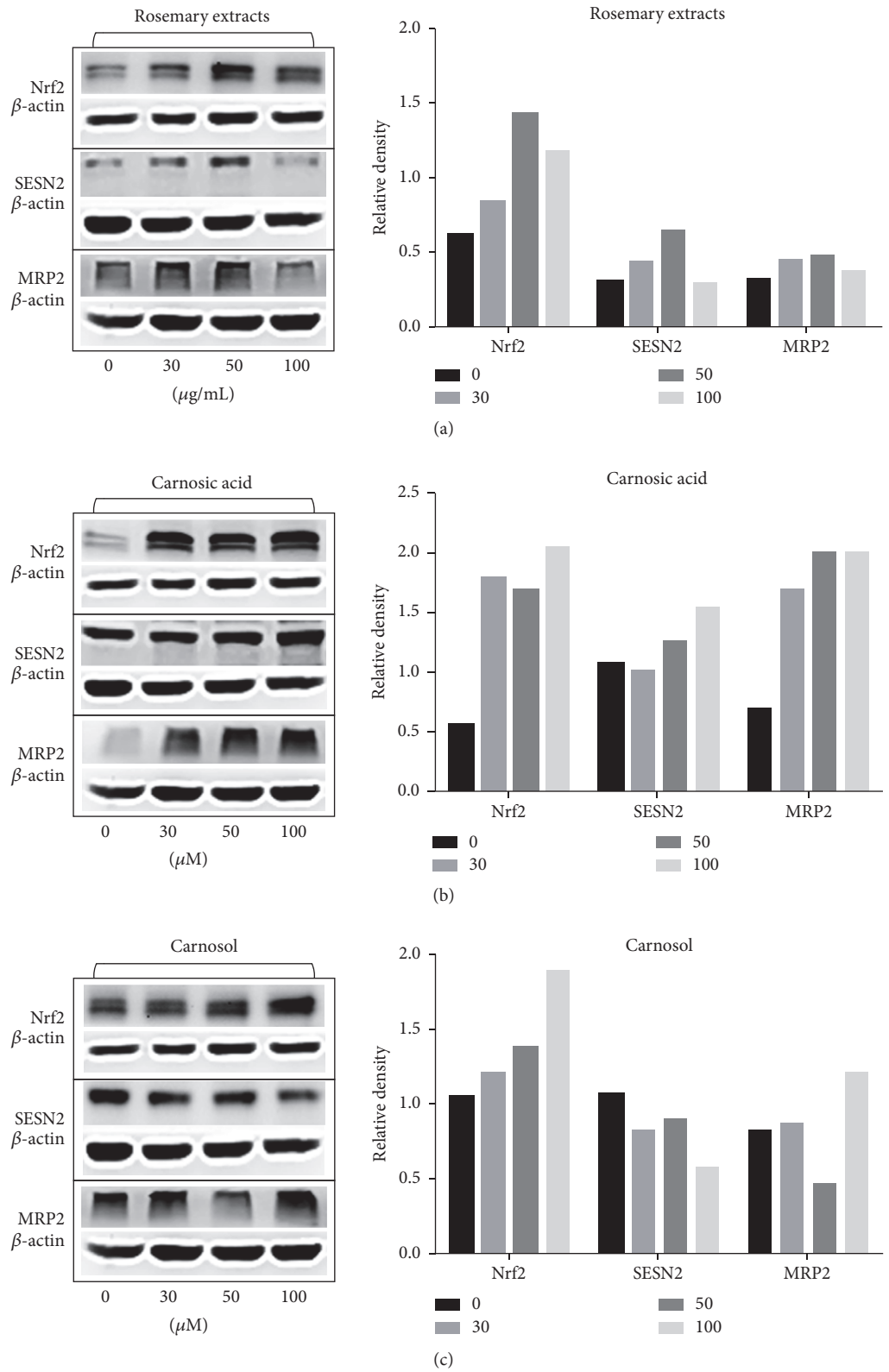

Figure 3: Rosemary extract (RE), carnosic acid (CA), and carnosol (CL) increased the protein levels of Nrf2, Sestrin2, and MRP2 in HepG2 cells. HepG2 cells (1×10^6 cells per well) were treated with RE (0, 30, 50, and 100 μg/mL) or CA and CL (0, 30, 50, and 100 μM), respectively, for 24 hrs. (a) Western Blot analysis of the protein level of Nrf2, MRP2, and Sestrin2 in RE treated HepG2 cells. Left column: protein level in RE treated HepG2 cells after 24 hrs; right column: densitometric analysis of RE Western Blot results. (b) Western Blot analysis of the protein level of Nrf2, MRP2, and Sestrin2 in CA treated HepG2 cells. Left column: protein level in CA treated HepG2 cells after 24 hrs; right column: densitometric analysis of CA Western Blot results. (c) Western Blot analysis of the protein level of Nrf2, MRP2, and Sestrin2 in CL treated HepG2 cells. Left column: protein level in CL treated HepG2 cells after 24 hrs; right column: densitometric analysis of CL Western Blot results.

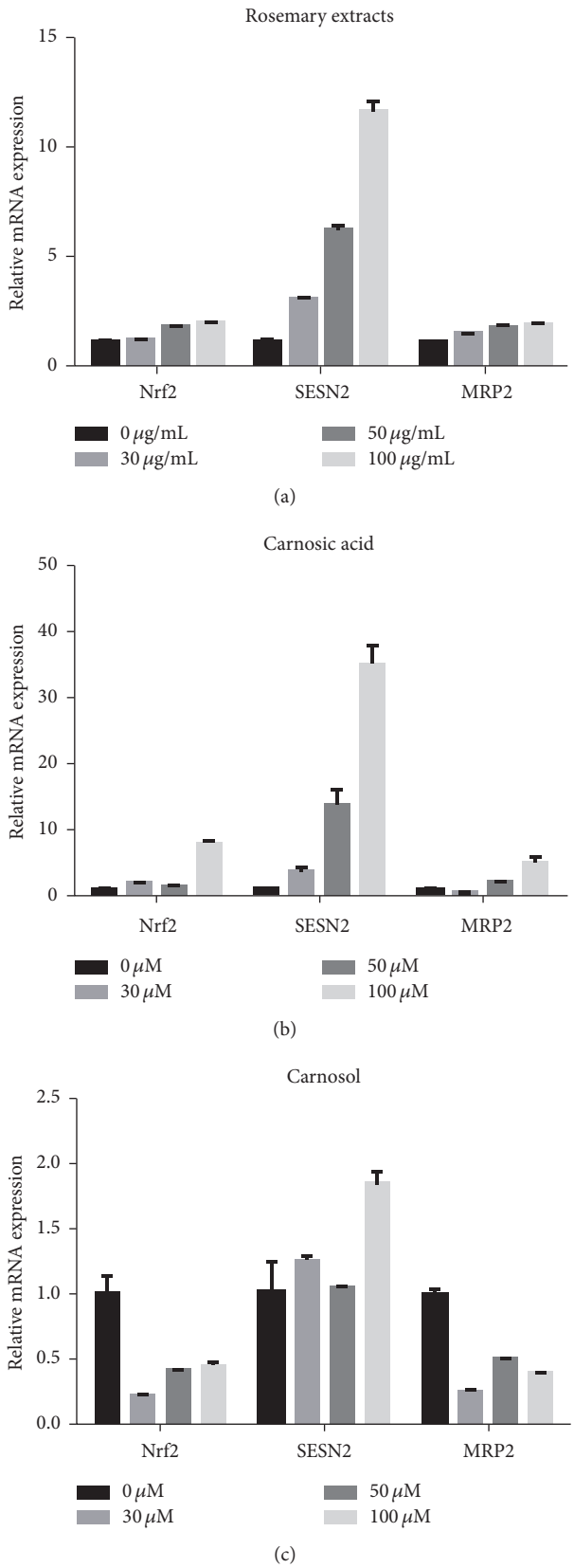

Figure 4: RT-PCR analysis of the mRNA level of Nrf2, MRP2, and Sestrin2 in RE, CA, and CL treated HepG2 cells. (a) mRNA level in RE treated HepG2 cells after 24 hrs; (b) mRNA level in CA treated HepG2 cells after 24 hrs; (c) mRNA level in CL treated HepG2 cells after 24 hrs.

Competing Interests

The authors declare no competing financial interest related to the study.

Authors' Contributions

Xiao-pei Tong and Yan-xia Ma contributed equally to this work.

Acknowledgments

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References

[1] M. V. Barni, M. J. Carlini, E. G. Cafferata, L. Puricelli, and S. Moreno, "Carnosic acid inhibits the proliferation and migration capacity of human colorectal cancer cells," *Oncology Reports*, vol. 27, no. 4, pp. 1041–1048, 2012.

[2] A.-C. Cheng, M.-F. Lee, M.-L. Tsai et al., "Rosmanol potently induces apoptosis through both the mitochondrial apoptotic pathway and death receptor pathway in human colon adenocarcinoma COLO 205 cells," *Food and Chemical Toxicology*, vol. 49, no. 2, pp. 485–493, 2011.

[3] V. Krajka-Kuźniak, J. Paluszczak, H. Szaefer, and W. Baer-Dubowska, "The activation of the Nrf2/ARE pathway in HepG2 hepatoma cells by phytochemicals and subsequent modulation of phase II and antioxidant enzyme expression," *Journal of Physiology and Biochemistry*, vol. 71, no. 2, pp. 227–238, 2015.

[4] S. L. Ullevig, Q. Zhao, D. Zamora, and R. Asmis, "Ursolic acid protects diabetic mice against monocyte dysfunction and accelerated atherosclerosis," *Atherosclerosis*, vol. 219, no. 2, pp. 409–416, 2011.

[5] K. Sasaki, A. El Omri, S. Kondo, J. Han, and H. Isoda, "*Rosmarinus officinalis* polyphenols produce anti-depressant like effect through monoaminergic and cholinergic functions modulation," *Behavioural Brain Research*, vol. 238, no. 1, pp. 86–94, 2013.

[6] A.-V. Ferlemi, A. Katsikoudi, V. G. Kontogianni et al., "Rosemary tea consumption results to anxiolytic- and anti-depressant-like behavior of adult male mice and inhibits all cerebral area and liver cholinesterase activity; phytochemical investigation and in silico studies," *Chemico-Biological Interactions*, vol. 237, pp. 47–57, 2015.

[7] B. Bozin, N. Mimica-Dukic, I. Samojlik, and E. Jovin, "Antimicrobial and antioxidant properties of Rosemary and Sage (Rosmarinus officinalis L. and Salvia officinalis L., Lamiaceae) essential oils," *Journal of Agricultural and Food Chemistry*, vol. 55, no. 19, pp. 7879–7885, 2007.

[8] O. Yesil-Celiktas, C. Sevimli, E. Bedir, and F. Vardar-Sukan, "Inhibitory effects of rosemary extracts, carnosic acid and rosmarinic acid on the growth of various human cancer cell lines," *Plant Foods for Human Nutrition*, vol. 65, no. 2, pp. 158–163, 2010.

[9] M. Yan, G. Li, S. M. Petiwala, E. Householter, and J. J. Johnson, "Standardized rosemary (Rosmarinus officinalis) extract

induces Nrf2/sestrin-2 pathway in colon cancer cells," *Journal of Functional Foods*, vol. 13, pp. 137–147, 2015.

[10] S. M. Petiwala, A. G. Puthenveetil, and J. J. Johnson, "Polyphenols from the Mediterranean herb rosemary (*Rosmarinus officinalis*) for prostate cancer," *Frontiers in Pharmacology*, vol. 4, article 29, 2013.

[11] S. M. Petiwala, S. Berhe, G. Li et al., "Rosemary (Rosmarinus officinalis) extract modulates CHOP/GADD153 to promote androgen receptor degradation and decreases xenograft tumor growth," *PLoS ONE*, vol. 9, no. 3, Article ID e89772, 2014.

[12] R. Januchowski, K. Sterzyńska, K. Zaorska et al., "Analysis of MDR genes expression and cross-resistance in eight drug resistant ovarian cancer cell lines," *Journal of Ovarian Research*, vol. 9, article 65, 2016.

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Research Article

Efficacy of Juzentaihoto for Tumor Immunotherapy in B16 Melanoma Metastasis Model

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Introduction. Medical care for Japanese cancer patients includes Western and Kampo medicines, and treatments with juzentaihoto (JTT) reportedly prevent cancer metastasis and recurrence. In this study, we examined the effects of JTT on natural killer (NK) cell activity and metastasis in combined treatments with anti-PD-1 antibody in a mouse model of melanoma metastasis. *Methods*. C57BL/6 male mice were intravenously injected with B16 melanoma cells (B16 cell) and were given chow containing 3% JTT. In subsequent in vivo experiments, we assessed serum cytokine levels and tumor colony formation in the lungs. Additionally, we assessed NK cell activity in ex vivo experiments. *Results*. JTT significantly suppressed B16 cell metastasis, whereas injection of anti-asialo-GM1 antibody into mice abrogated the inhibitory actions of JTT. JTT significantly increased interleukin- (IL-) 12 and interferon- (IFN-) γ levels in serum and induced NK cell activity. It increased the inhibitory actions of the anti-PD-1 antibody on B16 cell metastasis. *Discussion*. These data suggest that JTT inhibits B16 cell metastasis by inducing NK cell activity. Additionally, combination therapy with JTT and anti-PD-1 antibody increased treatment response rates for B16 melanoma.

1. Introduction

Melanoma affects 12 people per 100,000 and causes 600 deaths per year in Japan [1]. However, in the United States, approximately 60,000 melanomas are diagnosed and cause 9,000 deaths per year. Moreover, metastatic melanoma is known as an aggressive disease with 5-year survival achieved in only 16% of cases and poor responses to most standard chemotherapies [2]. Patients with regional lymph node involvement have high recurrence rates and numbers of deaths per 100,000 persons remained stable between 1992 and 2011 [3].

Complementary and alternative medicine, including Kampo medicine, compensates for the limitations of Western medicine by stimulating self-defense mechanisms. In Japan, clinicians who have studied both Western and Kampo medicines treat cancer patients with combinations of these medical interventions, and increasing numbers of cancer patients receive outpatient chemotherapy. The traditional medicine juzentaihoto (JTT) has been prescribed widely to retain quality of life in Japan [4]. JTT is a Chinese medicinal preparation comprising 10 herbs and is commonly used as a nutritional agent to improve disturbances and imbalances of homoeostasis. As a Kampo medicine, JTT promotes restoration of physical strength after surgery and alleviates adverse effects of anticancer drugs and radiation therapy. JTT has also been reported to prevent cancer metastasis, occurrence, and recurrence [5–9] and may prolong survival [10, 11].

Targeting of natural killer (NK) cells holds potential in patients with minimal disease, such as initial carcinomatous lesions and hematological cancers [12]. However, the efficacy of NK cells varies between cancers. Previously, we showed that JTT obstructs the metastasis and angiogenesis of B16 melanoma cells [13, 14]. Although the functions of dendritic and NK cells remain unknown, we suggested that uptake of interferon-gamma- (IFN-) γ is a common mechanism. Recent studies show that several tumors, including melanoma, have developed the ability to abolish T cell activation and prevent effective T cell antitumor

responses. Checkpoint inhibitors, such as anti-programmed cell death-1 (PD-1) antibody, normally promote antitumor immunity by blocking this key negative regulator of T cell activation [15]. Moreover, PD-1 efficacy may be further increased when used in combination with other immune agents.

Herein, we examined the effects of JTT on NK cell activity and metastasis following combined treatment with anti-PD-1 antibody in a B16 transplantation model of metastasis.

2. Materials and Methods

2.1. Animals. Six-week-old specific pathogen-free C57BL/6 male mice were purchased from Japan CLEA Co. Ltd. (Tokyo, Japan). Animals were maintained in our animal facilities at 25 ± 2°C with 50 ± 2% humidity and a 12 h light/12 h dark cycle. This study was approved by the Showa University Ethics Committee for animal experiments (number 06078).

2.2. Reagents. JTT was provided by Tumura Co. Ltd. (Tokyo, Japan) as a pure, preservative-free powder and was thoroughly mixed with a regular powder diet (CE-2) for rats and mice (Japan CLEA Co., Ltd. Tokyo, Japan) at a concentration of 3.0% [13]. To inhibit NK cell activity, anti-asialo-GM1 mouse antibody (014-09801) and normal rabbit IgG (control mouse antibody: 148-09551) were purchased from Wako Pure Chemical Ind. Ltd. (Tokyo, Japan). The anti-asialo-GM1 mouse monoclonal antibody acts against the glycosphingolipid asialo-GM1 antigen, which is expressed on murine NK cells [16]. PD-1 targeting experiments were performed using an anti-PD-1 mouse antibody (RMP1-14) and isotype control rat IgG (control mouse antibody: 2A3), which were purchased from BioXCell (West Lebanon, NH, USA). NK cell viability was assessed using WST-8 reagent (Cell Counting Kit-8; Dojindo Lab., Kumamoto, Japan). NK cells were separated from spleens using Mouse panNK CD49b Selection Kit (Cat. 18755; StemCell Technologies, Vancouver, BC, Canada).

2.3. Cell Culture. Cells were cultured in Dulbecco's modified eagle medium (DMEM; Sigma-Aldrich Co., St. Louis, MO, USA) or Roswell Park Memorial Institute 1640 medium (RPMI1640; Sigma-Aldrich Co.) supplemented with 10% heat-inactivated fetal calf serum (FCS; Nihon Bio-Supply Center, Tokyo, Japan) and a penicillin-streptomycin-neomycin (PSN) antibiotic mixture containing penicillin and streptomycin at 5 mg/mL and neomycin at 10 mg/mL (15640; Life Technologies, Inc.). Media were sterilized by passing through 0.2 μm pore filters and were stored at 4°C until use.

B16-F10 mouse melanoma cells (ATCC® CRL-6475™; Rockville, MD, USA) were routinely cultured at 37°C in a humidified atmosphere of 5% CO_2 and were maintained in DMEM-FCS-PSN. YAC-1 NK-sensitive murine lymphoma cells (ATCC TIB-160™) were cultured in RPMI-1640-FCS-PSN at 37°C in a humidified atmosphere of 5% CO_2.

2.4. Assay of Tumor Cell Metastasis. B16 cells (2×10^5 cells) were injected intravenously into recipient mice in a volume of 50 μL phosphate-buffered saline (PBS). After 21 or 28 days, mice were euthanized under ether anesthesia and numbers of tumor colonies on lung surfaces were counted using a dissecting microscope (SZ-60; OLYMPUS Co. Ltd., Tokyo, Japan).

2.5. Influence of NK Cell Depression. Mice were intraperitoneally injected with anti-asialo-GM1 mouse antibody or isotype control mouse antibody (200 mg for both) on every 5th day (on days −1, 4, 9, 14, and 19) from the day before B16 cell inoculation [14]. Tumor colony formation was assessed in lungs on the 21st day after B16 cell injection.

2.6. Influence of Combination Therapy with JTT and Anti-PD-1 Antibody. Mice were intraperitoneally injected with anti-PD-1 or isotype control mouse antibodies (200 μg for both) on every 4th day (on days 10, 14, 18, 22, and 26) from the 10th day after B16 cell inoculation [17, 18]. Tumor colony formation in the lung was assessed on the 28th day after B16 cell injections.

2.7. Measurement of Cytokines. Interleukin- (IL-) 12 and IFN-γ levels in serum and culture supernatants were determined using commercially available enzyme-linked immunosorbent assay (ELISA) kits (M1270; MIF00, R&D Systems, Inc., Minneapolis, MN, USA) according to the manufacturer's recommendations. The sensitivity of the IFN-γ assay kit was 2.0 pg/mL and that of the IL-12 assay kit was 2.5 pg/mL. Absorbance at 450 nm was measured using a Multiskan™ GO instrument (Thermo Fisher Scientific Inc. Waltham, MA, USA).

2.8. Separation of the NK Cells from Spleen. NK cells were separated using Mouse panNK (CD49b) Selection Kit according to the manufacturer's instructions [19]. Briefly, spleens from recipient animals were homogenized, and cells were resuspended in medium at 1×10^8 cells/mL. Prior to EasySep separations, spleen cells were incubated for 15 min with a positive selection cocktail containing anti-mouse CD49b antibodies. EasySep magnetic nanoparticles were then added to cell-antibody mixtures and were incubated for 10 min at room temperature. PBS containing 2% FCS and 1 mM EDTA was then added to cell suspension to a final volume of 2.5 mL. Samples were then placed into magnetized chambers and were incubated for 5 min. Magnets and tubes were inverted to remove supernatants without disrupting panNK $CD49b^+$ cell pellets. After repeating the EasySep procedure three times, tubes were removed from the magnet and the remaining cells were resuspended in culture medium. Positively selected cells were then used in assays to determine NK activity.

2.9. Cytotoxicity Assays in NK Cells. NK activities of fresh splenocytes were measured using WST-8 reagent. Briefly, 50 μL aliquots of target YAC-1 cell suspensions (1×10^5 cells/well) were added to 50 μL aliquots of effector cells (fresh non-activated spleen cells) at various effector : target (E : T) ratios (5 : 1, 20 : 1, and 30 : 1) in 96-well flat-bottomed culture plates.

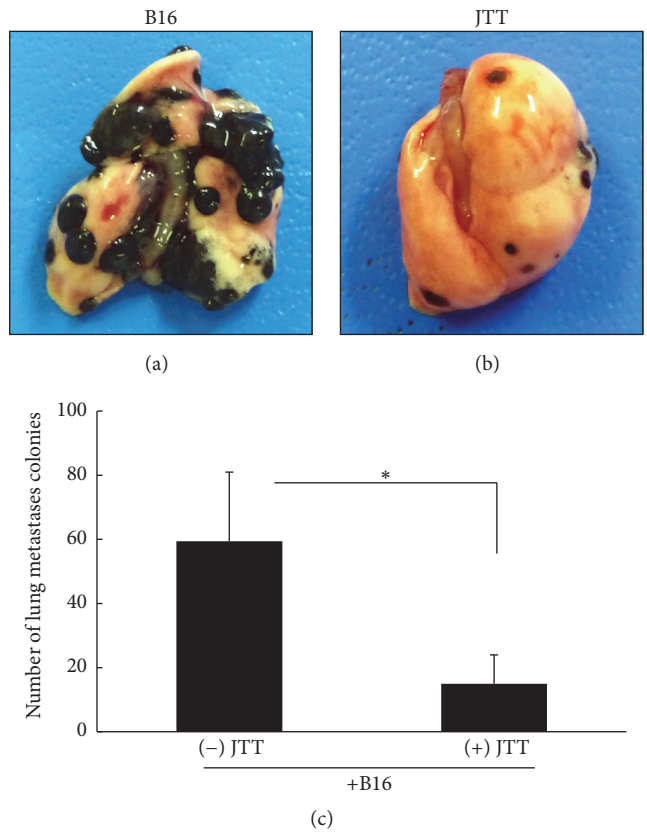

Figure 1: Influence of JTT on B16 melanoma cell metastasis in mice. C57BL/6 mice were orally administered JTT from one week before intravenous injections of 2×10^5 melanoma cells. Mice were sacrificed 3 weeks later, and tumor cell colonies on lung surfaces were counted. (a, b) Image of tumor cell colonies on the lungs; (c) numbers of colonies on the lungs, $^*P < 0.05$ versus control, $n = 10$; data are presented as means ± standard deviations (SD).

The plates were then centrifuged at 1000 rpm for 5 s and incubated in a 5% CO_2 incubator at 37°C. After a 4 h incubation, 10 μL aliquots of WST-8 reagent were added, and the plates were incubated for 1 h. Optical density (OD) was determined using a microplate spectrophotometer at 450 nm (reference 655 nm). Cytotoxic activity at each E : T ratio was calculated as follows: cytotoxicity % = $[(OD_{target} - (OD_{target+effector} - OD_{effector}) - OD_{blank})/(OD_{target} - OD_{blank})] \times 100$ [20].

2.10. Examination of IFN-γ Secretion from NK Cells. Separated NK cells were resuspended at a density of 2×10^5 cells/well in DMEM-FCS-PSN and cultured in triplicate in 24-well plates. Subsequently, B16 cells were added to NK cells at a ratio of 1 : 20 (B16/NK), and supernatants were collected after coculture for 24 h [21] and stored at −80°C until use for ELISA measurements of IFN-γ concentrations.

2.11. Statistical Analysis. Data were expressed as means ± standard deviations (SD). All assays were repeated two times to ensure reproducibility. Differences between control and experimental groups were identified using one-way analysis of variance followed by Scheffe tests and were considered significant when $P < 0.05$.

3. Results

3.1. Suppression of B16 Cell Metastasis by JTT. To examine the influence of JTT on experimental metastasis, we injected 2×10^5 B16 tumor cells intravenously into the caudal vein of mice that were pretreated with 3.0% JTT. Mice were sacrificed 21 days later, and numbers of tumor cell colonies on lung surfaces were counted. As shown in Figure 1, oral administration of 3.0% JTT significantly suppressed B16 cell metastasis.

3.2. Influence of NK Cell Inhibition on Tumor Cell Metastasis in JTT-Treated Mice. To determine the influence of NK cell inhibition on tumor cell metastasis, mice were treated with anti-asialo-GM1 antibody and intravenous injections of B16 cells. After 21 days, the influence of NK cell depletion by anti-asialo-GM1 antibody was assessed by counting numbers of tumor cell colonies on lung surfaces. As shown in Figure 2, injections of anti-asialo-GM1 antibody into mice abrogated the inhibitory actions of JTT on tumor cell metastases. Additionally, numbers of colonies were significantly higher in mice treated with antibody than in control mice.

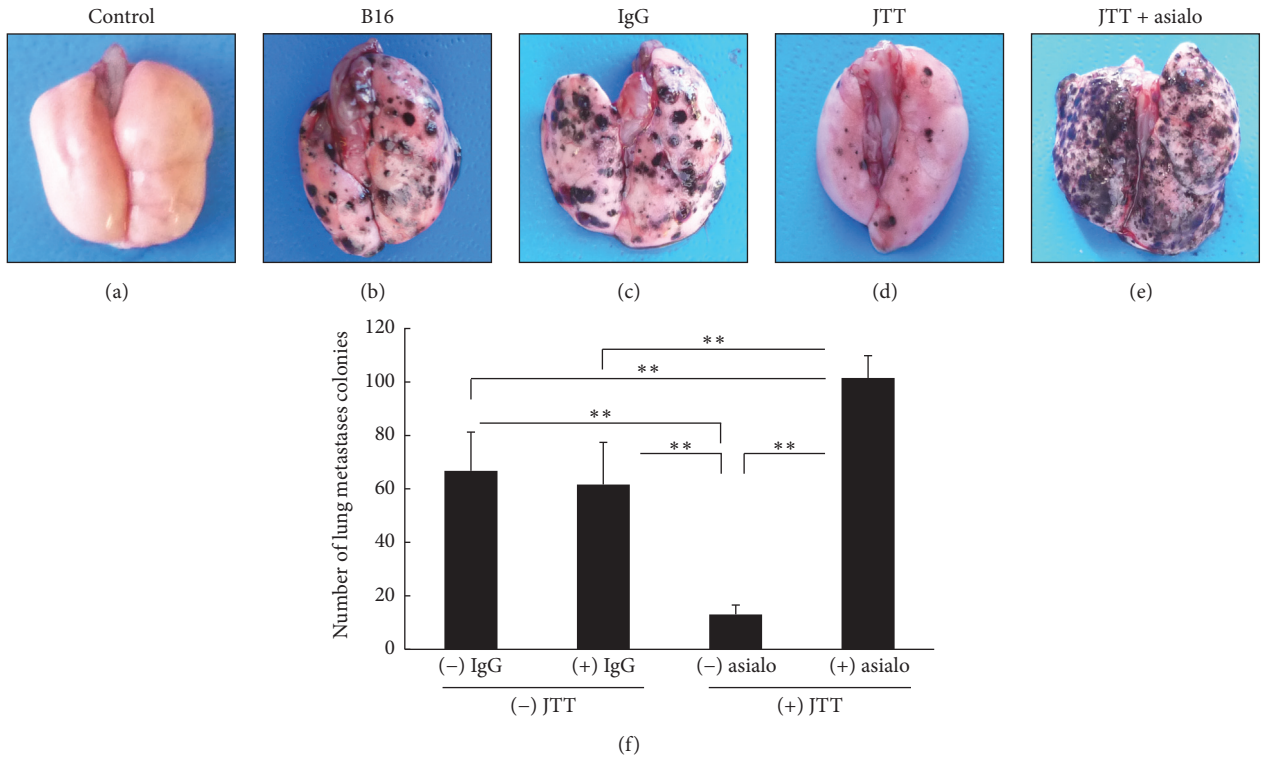

Figure 2: Influence of JTT on IL-12 and IFN-γ production in serum. C57BL/6 mice were orally given JTT from one week before intravenous injections of 2×10^5 melanoma cells. Mice were sacrificed 3 weeks later, and serum IL-12 and IFN-γ levels were determined. (a) Serum IL-12 contents; (b) serum IFN-γ contents; $^{*}P < 0.05$; $^{**}P < 0.01$; $n = 10$; data are expressed as means ± SD.

3.3. NK Cell Activity. Initially, we examined cytotoxicity of subject NK cells for target YAC-1 cells. As shown in Figure 3(a), cytotoxicity of NK cells in JTT-treated B16 transplantation mice was significantly greater than in control mice and untreated B16 transplantation mice. Additionally, cytotoxicity for target cells increased in accordance with NK cell numbers. In further experiments, the influence of IFN-γ production by NK cells was examined in cocultures with ex vivo B16 cells. Subsequently, ELISA experiments (Figure 3(b)) showed that IFN-γ secretion from NK cells was significantly greater in cells from JTT-treated B16 injected mice than in cells from untreated B16 transplantation mice and control mice.

3.4. Serum Cytokine Levels. Cytokine levels in mouse serum were determined using ELISA kits. As shown in Figure 4, serum IL-12 levels were significantly greater in mice with B16 transplantation tumors than in control mice but were not further increased by JTT administration. However, serum IFN-γ levels were significantly greater in mice with B16 transplantation tumors than in control mice and were further elevated by treatments with JTT (Figure 4(b)).

3.5. Influence of Combination Treatments with JTT and Anti-PD-1 Antibody on Tumor Metastases. To examine the influence of JTT administration and anti-PD-1 antibody treatments on tumor cell metastasis, B16 transplantation mice were treated with anti-PD-1 antibody, JTT, or the combination, and numbers of tumor colonies on lung surfaces were counted after 28 days. As shown in Figure 5, JTT administration increased the inhibitory actions of anti-PD-1 antibody against tumor cell metastasis in mice.

4. Discussion

Herbal medicine is frequently and successfully used as a supplemental therapy for various chronic diseases [22]. Moreover, as a cancer treatment, herbal medicines reportedly prevent the progression of colon carcinoma and gastric and breast cancer and can ameliorate cancer metastases to liver, lung, and bone tissues [23]. However, the mechanisms by which herbal medicines improve clinical conditions for cancer patients, including cancer metastasis, remain poorly understood. Herein, we examined anticancer mechanisms of the herbal medicine JTT in mice with 21-day-old B16 cell transplantation tumors.

The current results show that oral administration of JTT inhibits B16 cell colony formation on the lung surface following intravenous injections of tumor cells (Figure 1). The mechanisms that lead to arrest of tumor cell growth and metastasis are diverse and reflect tumor cell death, apoptosis, and immune-mediated cancer regression. Previous studies indicate little chemotherapeutic cytotoxicity of JTT toward B16 cells [14]. However, because NK cell activity contributes to inhibition of B16 cell metastasis, we examined

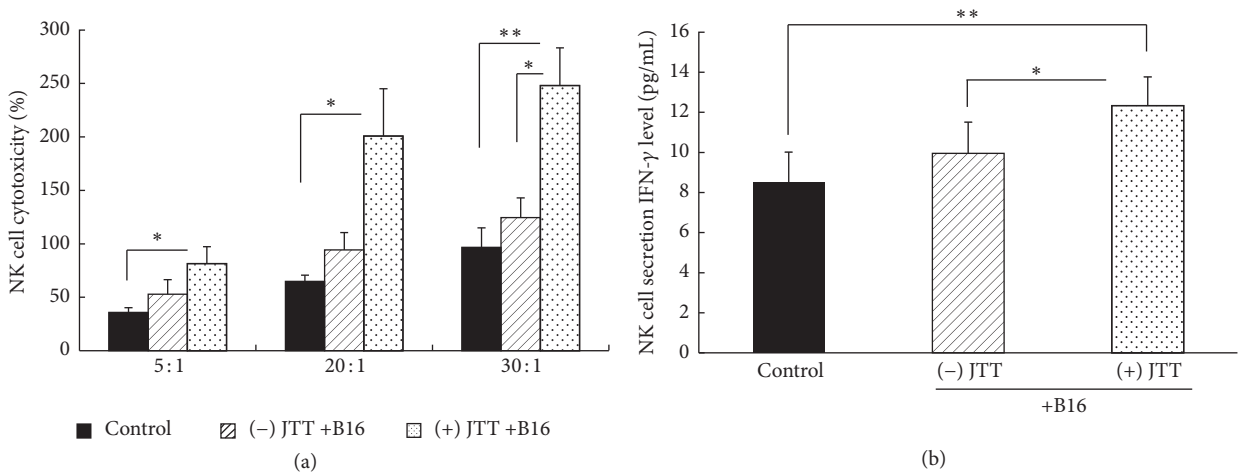

Figure 3: Ex vivo NK cell activity. NK cells were isolated from spleens, and IFN-γ secretions and cytotoxic activities were assayed; B16 cell injections, B16 cell injections with JTT treatment, and control mice. (a) IFN-γ production in NK cells; isolated NK cells were resuspended at a density of 2×10^5 cells/well in DMEM-FCS-PSN and were cultured in 24-well plates in triplicate. B16 cells were then added to NK cells at a 1 : 20 ratio (B16 : NK). After coculture for 24 h, culture supernatants were collected, and IFN-γ levels were measured using enzyme-linked immunosorbent assay (ELISA). (b) Assays of NK cell cytotoxicity toward YCA-1 cells; YAC-1 cells at 1×10^5/well were incubated with fresh spleen cells at fixed E : T ratios for 4 h and NK cell cytotoxicity was measured using a modified WST-8 assay; $^*P < 0.05$; $^{**}P < 0.01$; $n = 10$; data are expressed as means ± SD.

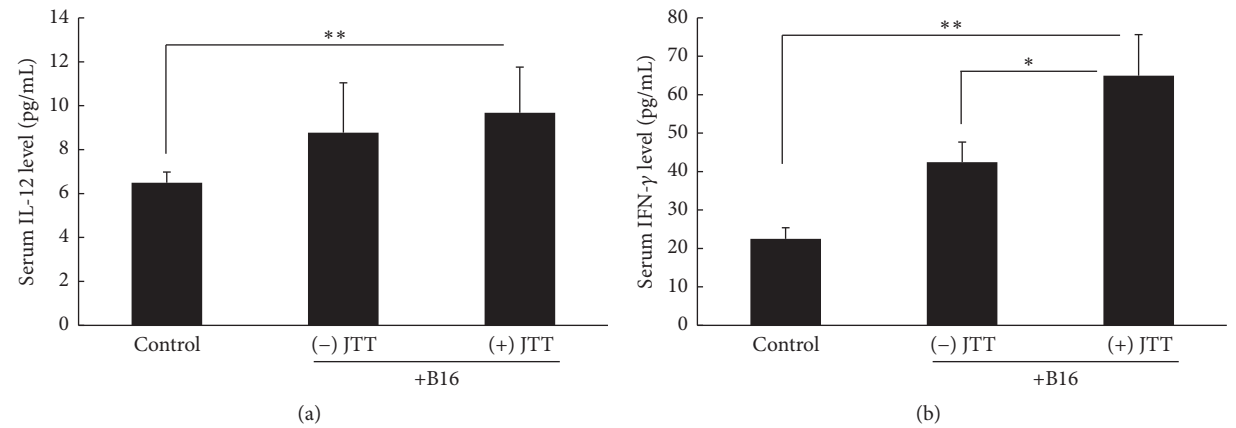

Figure 4: Influence of anti-NK antibody injections on B16 melanoma cell metastasis in mice treated with JTT. C57BL/6 mice were orally given 3.0% JTT for three weeks from one week before injections of 2×10^5 B16 melanoma cells. Anti-asialo-GM1 antibody was injected intraperitoneally and numbers of tumor cell colonies on lungs were counted three weeks later. (a–e) Images of tumor cell colonies on excised lungs; (f) quantification of numbers of colonies on lungs; IgG, normal rabbit IgG; asialo, anti-asialo-GM1 mouse antibody; $^{**}P < 0.01$; $n = 10$; data are presented as means ± SD.

the influences of JTT on NK cell survival and activity. In our initial experiments, we investigated the effects of JTT on B16 cell lung metastases in the presence of the anti-asialo-GM1 antibody, which depletes NK cell numbers in mice. As indicated in Figure 2, JTT administration in NK cell-depleted animals did not inhibit B16 cell metastases. However, upon further examination of the influence of JTT on NK cell cytotoxicity, NK cell after JTT treatments significantly increased the cytotoxicity for YAC-1 cell (Figure 3(a)). Additionally, ex vivo NK cells from JTT-treated B16 transplantation mice had increased IFN-γ secretion when cocultured with B16 cells (Figure 3(b)). NK cells are potent effectors that, unlike T lymphocytes, eliminate target cells spontaneously without prior sensitization. Moreover, NK cells have strong cytolytic activity against virus-infected and tumor cells. Specifically, activated NK cells were shown to exert immunomodulatory effects by producing cytokines such as IFN-γ and tumor necrosis factor and also directly kill target cells by releasing perforins and granzymes [24]. Taken together, these studies suggest that JTT inhibits B16 cell metastasis by inducing NK cell activity. NK cells shape the immune response by interacting with dendritic cells (DC), and crosstalk between DC and NK cells is mediated by secretions of several cytokines, including IL-12 and IFN-γ [25]. Therefore, we examined the roles of these key cytokines in vivo and showed increased serum levels following JTT

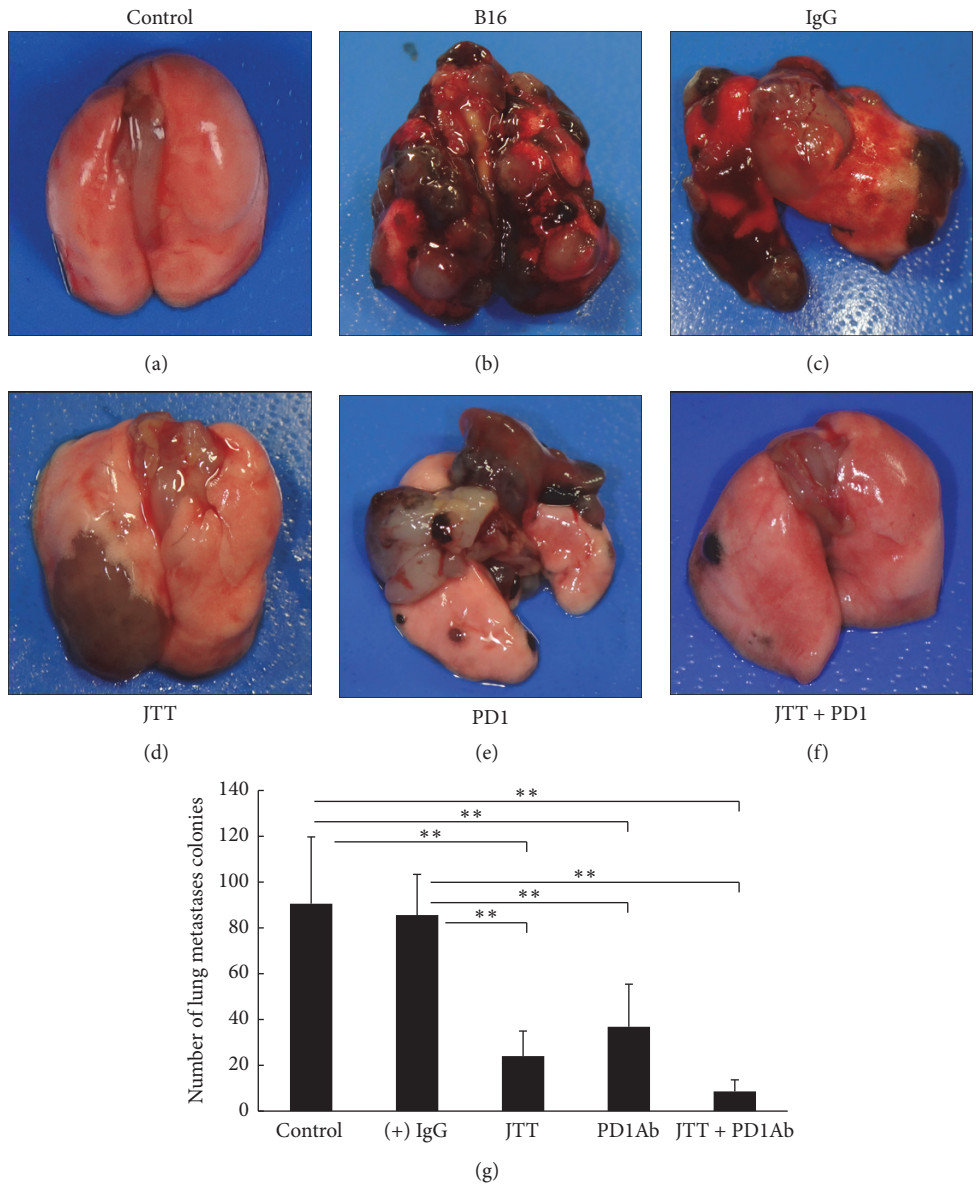

Figure 5: Influence of combined JTT and anti-PD-1 antibody treatments on B16 melanoma cell metastasis in mice. C57BL/6 mice were orally administered with 3.0% JTT for four weeks from the week before 2×10^5 B16 melanoma cells were injected. Anti-PD-1 antibody was injected intraperitoneally and numbers of tumor cell colonies were counted four weeks later. (a–f) Images of tumor cell colonies on lungs; (g) quantification of numbers of colonies on the lungs; IgG, isotype control rat IgG; PD1, anti-PD-1 antibody; $^{**}P < 0.01$; $n = 10$; data are presented as means ± SD.

treatments in B16 cell transplantation mice. NK cells are key mediators in innate responses against tumors, with cytotoxic activity and production of substantial quantities of IFN-γ and other proinflammatory cytokines. Moreover, crosstalk between DC and NK cells involves NKp30 and DNAM-1 and subsequent secretion of cytokines such as IL-12 and IFN-γ [5]. Additionally, NK cells positively and negatively influence anticancer responses by regulating immune crosstalk with DC and T cells [24].

In 1992, Ishida et al. showed that the protein PD-1 induces T cell death [26]. In more recent studies, anti-PD-1 antibodies were used for their strong antitumor effects against several malignancies, including melanoma, and studies of combined effects with various immunotherapies have been conducted widely [27, 28]. Therefore, we examined the effects of combined treatments with JTT and an anti-PD-1 antibody on B16 transplantation mice. These experiments showed that administration of JTT or anti-PD-1 antibody inhibited the formation of B16 metastases on lung surfaces (Figure 5). Although the combination did not result in significant additive effects, there seems to be no possibility that JTT offsets the action of anti-PD-1 antibodies at least. Because JTT likely has no direct effects on the actions of anti-PD-1 antibodies, it may prevent tumor-mediated

inactivation of immune cells such as NK cells or cytotoxic T lymphocytes.

Several cancers, including melanoma, have been shown to inactivate T cells and prevent the ensuing antitumor responses, and inhibitors such as anti-PD-1 antibodies can reverse this immune suppression and restore T cell activation. Accordingly, the present anti-PD-1 antibody has been shown to promote antitumor immunity. NK cells participate in early immune responses against melanoma and also contribute to adaptive immune responses through cytokine secretions and crosstalk with DC [29]. Moreover, IL-18 secretion by tumor cells upregulates PD-1 expression on NK cells and PD-L1 expression on DC and may reduce numbers and actions of mature NK cells [30, 31]. Previous studies show that JTT induces NK cell activity and IFN-γ secretion in the presence of the anti-PD-1 antibody nivolumimab, suggesting that JTT helps to circumvent PD-1 mediated inhibition of NK cells. In a clinical trial of combined JTT and anti-PD-1 antibody treatments, a subset of patients responded to single-agent blockade, while the combined treatment showed the potential to improve response rates. Hence, although combination therapies may also lead to immune related adverse events, its therapies appear to be a means of increasing response rates for cancer treatment.

Previous data suggest that IL-12 from immunocytes such as DC is an important mediator of JTT-induced NK activity. Because various cytokines, such as IL-2, IL-12, IL-15, IL-18, IL-21, and type I IFNs, affect the mobility and cytotoxicity of NK cells [32], future examinations are necessary to determine trends of these cytokines under the present conditions. Furthermore, bidirectional interactions between NK and tumor cells remain poorly characterized.

Lajoie et al. [33] recently showed that NKp46-activating antibodies activate NK cells and lead to degranulation and cytokine secretion, as indicated by CD107a and CD16 expression, and IFN-γ secretion, respectively. Melanoma cells contrive resistance to NK cell-mediated death by increasing the expression of HLA class I molecules [34, 35] and decreasing the expression of MICA and NCR, which are ligands that induce cytotoxic activities of NK cells [36–38]. Additionally, downregulation of DNAM-1-, NKp46-, NKp30-, and NKG2D-activating receptors on NK cells reportedly contributes to the escape of melanoma cells [39–41]. However, in our current study, IFN-γ secretion was increased following JTT treatments of the ex vivo NK cells from B16 transplantation mice (Figure 3(b)). Hence, JTT may not affect antitumor receptors on NK cells in the presence of melanoma, suggesting that JTT circumvents melanoma mediated downregulation of activating receptors on NK cells. However, future examinations are necessary to clarify the effects of JTT on these molecular changes.

5. Conclusion

These present data suggest that JTT inhibits B16 cell metastasis by inducing NK cell activity. Additionally, combination therapy with JTT and anti-PD-1 antibody may increase treatment response rates for B16 melanoma.

Competing Interests

The authors declare no conflict of interests regarding the publication of this paper.

Authors' Contributions

Shintaro Ishikawa and Tadashi Hisamitsu designed the study and helped draft the paper. Shintaro Ishikawa, Takako Ishikawa, Chiaki Tezuka, and Masataka Sunagawa were responsible for data acquisition. Shintaro Ishikawa and Kazuhito Asano performed all statistical analyses and interpreted the data. All authors have read and approved the paper.

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References

[1] H. Uhara, "New treatment of melanoma and breakthrough in cancer immunotherapy," *The Shinshu Medical Journal*, vol. 64, no. 2, pp. 63–73, 2016 (Japanese).

[2] NIH, "SEER Stat Fact Sheets: Melanoma of the Skin," http://seer.cancer.gov/statfacts/html/melan.html.

[3] Medical Research Council Renal Cancer Collaborators, "Interferon-alpha and survival in metastatic renal carcinoma: early results of a randomised controlled trial," *The Lancet*, vol. 353, no. 9146, pp. 14–17, 1999.

[4] Y. Ishiura, Y. Shiba, Y. Terasaki et al., "Effect of Japanese traditional medicine, TJ-48, on the quality of life of patients with non-small cell lung cancer receiving outpatient chemotherapy," *Cancer & Chemotherapy*, vol. 43, no. 3, pp. 331–334, 2016 (Japanese).

[5] K. Tagami, K. Niwa, Z. Lian, J. Gao, H. Mori, and T. Tamaya, "Preventive effect of Juzen-taiho-to on endometrial carcinogenesis in mice is based on Shimotsu-to constituent," *Biological and Pharmaceutical Bulletin*, vol. 27, no. 2, pp. 156–161, 2004.

[6] H. Mori, K. Niwa, Q. Zheng, Y. Yamada, K. Sakata, and N. Yoshimi, "Cell proliferation in cancer prevention; effects of preventive agents on estrogen-related endometrial carcinogenesis model and on an in vitro model in human colorectal cells," *Mutation Research—Fundamental and Molecular Mechanisms of Mutagenesis*, vol. 480-481, pp. 201–207, 2001.

[7] M. Matsuo, T. Tani, and I. Saiki, "Organ selectivity of Juzen-taiho-to and Ninjin-yoei-to in the expression of anti-metastatic efficacy," *Journal of Traditional Medicines*, vol. 19, pp. 93–97, 2002.

[8] Y. Onishi, T. Yamaura, K. Tauchi et al., "Expression of the anti-metastatic effect induced by Juzen-taiho-to is based on the content of Shimotsu-to constituents," *Biological and Pharmaceutical Bulletin*, vol. 21, no. 7, pp. 761–765, 1998.

[9] I. Saiki, "A kampo medicine 'Juzen-taiho-to' : prevention of malignant progression and metastasis of tumor cells and the mechanism of action," *Biological and Pharmaceutical Bulletin*, vol. 23, no. 6, pp. 677–688, 2000.

[10] H.-C. Zheng, A. Noguchi, K. Kikuchi, T. Ando, T. Nakamura, and Y. Takano, "Gene expression profiling of lens tumors, liver and spleen in α-crystallin/SV40 T antigen transgenic mice

treated with Juzen-taiho-to," *Molecular Medicine Reports*, vol. 9, no. 2, pp. 547–552, 2014.

[11] Y. Rino, N. Yukawa, and N. Yamamoto, "Does herbal medicine reduce the risk of hepatocellular carcinoma?" *World Journal of Gastroenterology*, vol. 21, no. 37, pp. 10598–10603, 2015.

[12] E. Vivier, E. Tomasello, M. Baratin, T. Walzer, and S. Ugolini, "Functions of natural killer cells," *Nature Immunology*, vol. 9, no. 5, pp. 503–510, 2008.

[13] S. Ishikawa, T. Ishikawa, K. Asano et al., "Suppressive effect of juzentaihoto on vascularization induced by b16 melanoma cells *in vitro* and *in vivo*," *Evidence-based Complementary and Alternative Medicine*, vol. 2012, Article ID 945714, 9 pages, 2012.

[14] T. Matsuda, K. Maekawa, K. Asano, and T. Hisamitsu, "Suppressive effect of Juzen-Taiho-To on lung metastasis of B16 melanoma cells *in vivo*," *Evidence-Based Complementary and Alternative Medicine*, vol. 2011, Article ID 743153, 5 pages, 2011.

[15] D. B. Johnson, C. Peng, and J. A. Sosman, "Nivolumab in melanoma: latest evidence and clinical potential," *Therapeutic Advances in Medical Oncology*, vol. 7, no. 2, pp. 97–106, 2015.

[16] C. Jackaman, S. Lansley, J. E. Allan, B. W. S. Robinson, and D. J. Nelson, "IL-2/CD40-driven NK cells install and maintain potency in the anti-mesothelioma effector/memory phase," *International Immunology*, vol. 24, no. 6, pp. 357–368, 2012.

[17] C. Vanpouille-Box, J. M. Diamond, K. A. Pilones et al., "TGFβ is a master regulator of radiation therapy-induced antitumor immunity," *Cancer Research*, vol. 75, no. 11, pp. 2232–2242, 2015.

[18] S. Kleffel, C. Posch, S. R. Barthel et al., "Melanoma cell-intrinsic PD-1 receptor functions promote tumor growth," *Cell*, vol. 162, no. 6, pp. 1242–1256, 2015.

[19] M. Olbrich, M. Rieger, S. Reinert, and D. Alexander, "Isolation of osteoprogenitors from human jaw periosteal cells: a comparison of two magnetic separation methods," *PLoS ONE*, vol. 7, no. 10, Article ID e47176, 2012.

[20] H. Kamiyama, S. Takano, E. Ishikawa, K. Tsuboi, and A. Matsumura, "Anti-angiogenic and immunomodulatory effect of the herbal medicine "Juzen-taiho-to" on malignant glioma," *Biological and Pharmaceutical Bulletin*, vol. 28, no. 11, pp. 2111–2116, 2005.

[21] A. L. Rakhmilevich, M. J. Baldeshwiler, T. J. Van De Voort et al., "Tumor-associated myeloid cells can be activated in vitro and in vivo to mediate antitumor effects," *Cancer Immunology, Immunotherapy*, vol. 61, no. 10, pp. 1683–1697, 2012.

[22] M. Utsuyama, H. Seidlar, M. Kitagawa, and K. Hirokawa, "Immunological restoration and anti-tumor effect by Japanese herbal medicine in aged mice," *Mechanisms of Ageing and Development*, vol. 122, no. 3, pp. 341–352, 2001.

[23] K. Handa, N. Kawamura, and S. Takahashi, "Case report," *Kampo Medicine*, vol. 29, pp. 215–220, 2005.

[24] S. Morgado, B. Sanchez-Correa, J. G. Casado et al., "NK cell recognition and killing of melanoma cells is controlled by multiple activating receptor-ligand interactions," *Journal of Innate Immunity*, vol. 3, no. 4, pp. 365–373, 2011.

[25] R. G. Spallanzani, N. I. Torres, D. E. Avila et al., "Regulatory dendritic cells restrain NK cell IFN-γ production through mechanisms involving NKp46, IL-10, and MHC class I-specific inhibitory receptors," *Journal of Immunology*, vol. 195, no. 5, pp. 2141–2148, 2015.

[26] Y. Ishida, Y. Agata, K. Shibahara, and T. Honjo, "Induced expression of PD-1, a novel member of the immunoglobulin gene superfamily, upon programmed cell death," *The EMBO Journal*, vol. 11, no. 11, pp. 3887–3895, 1992.

[27] K. M. Mahoney, G. J. Freeman, and D. F. McDermott, "The next immune-checkpoint inhibitors: Pd-1/pd-l1 blockade in melanoma," *Clinical Therapeutics*, vol. 37, no. 4, pp. 764–782, 2015.

[28] K. K. Tsai and A. I. Daud, "Nivolumab plus ipilimumab in the treatment of advanced melanoma," *Journal of Hematology & Oncology*, vol. 8, no. 1, article 123, 2015.

[29] R. Tarazona, E. Duran, and R. Solana, "Natural killer cell recognition of melanoma: new clues for a more effective immunotherapy," *Frontiers in Immunology*, vol. 6, article 649, 2016.

[30] M. Terme, E. Ullrich, L. Aymeric et al., "IL-18 induces PD-1-dependent immunosuppression in cancer," *Cancer Research*, vol. 71, no. 16, pp. 5393–5399, 2011.

[31] M. Terme, E. Ullrich, L. Aymeric et al., "Cancer-induced immunosuppression: IL-18-elicited immunoablative NK cells," *Cancer Research*, vol. 72, no. 11, pp. 2757–2767, 2012.

[32] C. Guillerey, N. D. Huntington, and M. J. Smyth, "Targeting natural killer cells in cancer immunotherapy," *Nature Immunology*, vol. 17, no. 9, pp. 1025–1036, 2016.

[33] L. Lajoie, N. Congy-Jolivet, A. Bolzec et al., "ADAM17-mediated shedding of FcγRIIIA on human NK cells: identification of the cleavage site and relationship with activation," *Journal of Immunology*, vol. 192, no. 2, pp. 741–751, 2014.

[34] M. Balsamo, G. Pietra, W. Vermi, L. Moretta, M. C. Mingari, and M. Vitale, "Melanoma immunoediting by NK cells," *OncoImmunology*, vol. 1, no. 9, pp. 1607–1609, 2012.

[35] R. Solana, J. G. Casado, E. Delgado et al., "Lymphocyte activation in response to melanoma: interaction of NK-associated receptors and their ligands," *Cancer Immunology, Immunotherapy*, vol. 56, no. 1, pp. 101–109, 2007.

[36] T. Lakshmikanth, S. Burke, T. H. Ali et al., "NCRs and DNAM-1 mediate NK cell recognition and lysis of human and mouse melanoma cell lines in vitro and in vivo," *The Journal of Clinical Investigation*, vol. 119, no. 5, pp. 1251–1263, 2009.

[37] E. Cagnano, O. Hershkovitz, A. Zilka et al., "Expression of ligands to NKp46 in benign and malignant melanocytes," *Journal of Investigative Dermatology*, vol. 128, no. 4, pp. 972–979, 2008.

[38] M. Campoli and S. Ferrone, "HLA antigen and NK cell activating ligand expression in malignant cells: a story of loss or acquisition," *Seminars in Immunopathology*, vol. 33, no. 4, pp. 321–334, 2011.

[39] G. Pietra, M. Vitale, L. Moretta, and M. C. Mingari, "How melanoma cells inactivate NK cells," *OncoImmunology*, vol. 1, no. 6, pp. 974–975, 2012.

[40] G. Pietra, C. Manzini, S. Rivara et al., "Melanoma cells inhibit natural killer cell function by modulating the expression of activating receptors and cytolytic activity," *Cancer Research*, vol. 72, no. 6, pp. 1407–1415, 2012.

[41] K. M. Mirjacic Martinovic, N. L. Babović, R. R. Džodić, V. B. Jurišić, N. T. Tanić, and G. M. Konjević, "Decreased expression of NKG2D, NKp46, DNAM-1 receptors, and intracellular perforin and STAT-1 effector molecules in NK cells and their dim and bright subsets in metastatic melanoma patients," *Melanoma Research*, vol. 24, no. 4, pp. 295–304, 2014.

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Research Article

Potential Antitumor Effect of Harmine in the Treatment of Thyroid Cancer

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Thyroid cancer is one of the most common types of cancer in endocrine system. In latest studies, harmine has been proved to inhibit the growth of several cancers in vitro and in vivo. In the current study, we evaluated the in vitro and in vivo anticancer efficiency of harmine against thyroid cancer cell line TPC-1. The in vitro cytotoxicity of harmine evaluated by XTT assay indicated that harmine suppressed the proliferation of TPC-1 cells in a dose- and time-dependent manner. Moreover, harmine dose-dependently induced apoptosis of TPC-1 cells through regulating the ratio of Bcl-2/Bax. The colony forming ability of TPC-1 cells was also time-dependently inhibited by harmine. The inhibitory effects of harmine on migration and invasion of TPC-1 cells were studied by wound scratching and Transwell assays. In vivo evaluation showed that harmine effectively inhibited the growth of thyroid cancer in a dose-dependent manner in nude mice. Therefore, harmine might be a promising herbal medicine in the therapy of thyroid cancer and further efforts are needed to explore this therapeutic strategy.

1. Introduction

Thyroid cancer is one of the most common kinds of cancer in endocrine system which includes four major types with papillary thyroid cancer (PTC) being the most common one [1]. Though most of PTCs respond well to surgery and chemoradiotherapy, part of them quickly develop resistance to chemotherapy, which generate an urgent demand of novel strategy to improve the efficacy of chemotherapeutics [2].

Recently a growing body of evidences demonstrated the promising antitumor efficiency of herbal medicine [3–5]. Several herbal medicines, such as harmine, resveratrol, and berbamine, have shown substantial tumor inhibitory effect against a series of cancers [6–8]. Among all these herbal medicines, harmine, originally isolated from the seeds of *Peganum harmala*, is a tricyclic compound belonging to the β-carboline alkaloids [9]. It is reported that harmine possesses various pharmacological activities [10]. In latest studies, harmine has been proved to inhibit the growth of several cancers in vitro and in vivo [11, 12]. For example, harmine induces the apoptosis of cancer cells by leading to DNA break as demonstrated in DNA fragmentation and tunnel study in melanoma cells [13]. Possible mechanisms include the block of homologous recombination, one of the major Double Strand Break (DSB) repair pathways and the inhibition of telomerase activity [9, 12]. However, no related reports were found on the antitumor effect of harmine in thyroid cancer.

In the current study, we evaluated the in vitro and in vivo anticancer efficiency of harmine against thyroid cancer. The cell inhibition effect of harmine was measured by XTT assay. Apoptosis of thyroid cancer cell line TPC-1 was measured by DAPI staining. Related apoptotic protein expression was evaluated by western blotting. Influences of harmine on the migration and invasion of TPC-1 cells were also examined by wound scratching and Transwell assays. The in vivo antitumor effect was examined in a xenograft model of thyroid cancer.

2. Materials and Methods

2.1. Materials. Harmine was purchased from Sigma Co., Ltd. (St. Louis, USA). Harmine was dissolved in DMSO to make a stock solution at a concentration of 10 mg/mL. Working solution was prepared by diluting the stock solution into medium with the final concentration of DMSO below 0.1%. Human papillary thyroid cancer cell line TPC-1 was purchased from the Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China). TPC-1 cells were cultured in RPMI 1640 (w L-glutamate) completed by fetal bovine serum (10%) and penicillin/streptomycin (1%) at 37°C in a humidified atmosphere containing 5% CO_2.

Male and female nude mice (nu/nu; 6–8 weeks old and weighing 18–22 g) were purchased from the Animal Center of Nanjing Medical University (Nanjing, China). The animal protocol was reviewed and approved by the Institutional Animal Care and Use Committee of Nanjing Medical University.

2.2. In Vitro Cytotoxicity. TPC-1 cells were seeded in 96-well plates with a density of 10000 cells per well and allowed to grow 24 h before treatment. Cells were then incubated with escalated doses of harmine (2, 4, 8, 16, 32, and 64 μg/mL) for 24, 36, and 48 h. The medium was replaced with 50 μL XTT working solution and incubated for another 18 h. The optical density of each well was measured on a microplate reader at 560 nm (Bio-Rad, Hercules, USA).

2.3. DAPI Staining. Cells were seeded on cover slips in a 24-well plate and allowed to grow 24 h. Cells were then exposed to three kinds of concentration of harmine (4, 8, and 16 μg/mL) for 36 h. After being washed by PBS, cells were fixed by a mixture of cold methanol and acetone (1 : 1) for 5 min. 4 μg/mL 4′,6-diamidine-2′-phenylindole dihydrochloride (DAPI) was added to the well and allowed to incubate for 10 min at room temperature. Cells were imaged under a fluorescent microscope.

2.4. Western Blot. TPC-1 cells were incubated with harmine (4, 8, and 16 μg/mL) for 6 hrs. Cell total proteins were extracted and quantified according to the instruction of the kit. 50 μg of proteins was loaded into each well during the electrophoresis. Protein expression was measured by western blot as reported previously. Primary antibodies (anti-Bax, anti-Bcl-2, and anti-β-actin) were purchased from Sigma Co., Ltd. (St. Louis, MO, USA).

2.5. Clonogenic Assay. The first clonogenic assay was performed to study the inhibition of harmine on the colony forming ability of TPC-1 cells. Cells were treated with harmine (4, 8, and 16 μg/mL) for 24, 36, and 48 h. Another clonogenic assay was performed to evaluate the radiosensitizing effect of harmine. TPC-1 cells were treated with 2 μg/mL harmine and escalated doses of radiation singly or in combination. After each treatment, cells were trypsinized, collected, and set to the calculated concentration. After 10–14 days of incubation in 6-well plates, the 6-well plates were dyed by 0.5% crystal violet and counted as reported previously [14].

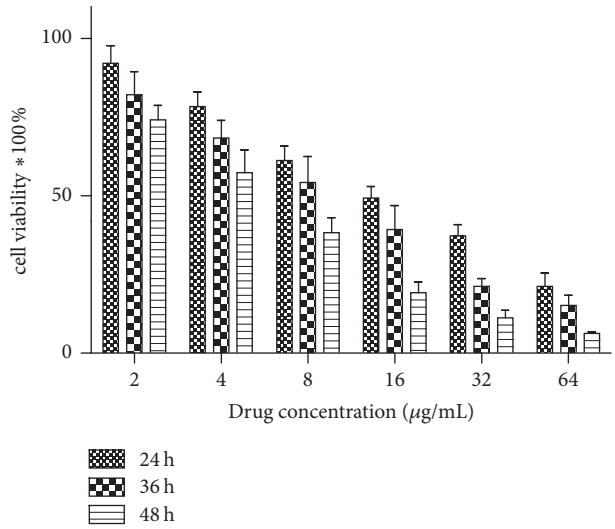

Figure 1: In vitro cytotoxicity of harmine on TPC-1 cells for the incubation of 24, 36, and 48 hrs.

2.6. DNA Fragmentation Assay. TPC-1 cells were seeded in a 24-well plate and allowed to grow for 24 h. Cells were then exposed to three kinds of concentration of harmine (4, 8, and 16 μg/mL) for 36 h. Total DNA was extracted and run by gel electrophoresis as previously reported [15].

2.7. Wound Scratching Assay and Transwell Assay. Cells were seeded in 6-well plate and allowed to grow for 24 h to form a monolayer. Then a tip was utilized to scratch in the middle to generate a clear space (wound). Immediately it is captured by the camera and was designated as 0 h. Then the medium was replaced with serum-free medium containing different concentrations of harmine (4, 8, and 16 μg/mL). After 36 h, another image was captured to measure the width of the wound. The wound healing rate was calculated by the following formula: the average width of wound at 0 h − the average width of wound at 36 h/the average width of wound at 0 h.

Transwell assays were performed in 24-well Transwell chambers (Corning, Acton, MA, USA) as reported previously. Briefly, TPC-1 cells were seeded in the upper chamber in serum-free medium, while the bottom chamber was filled with complete medium as an attractant. 36 h later, the successfully invaded cells on the bottom chamber were stained by crystal violet, counted, and plotted.

2.8. In Vivo Antitumor Efficacy. Briefly, 1.5 million TPC-1 cells were injected subcutaneously in the left axillary space of the mice. 10 days later the mice with the tumor nodule reaching a volume of 70–80 mm^3 were randomly divided into four groups with 6 mice per group. Escalated doses of harmine were administered by tail vein injection.

The mice were then treated with escalated doses of harmine with saline as the control. Tumor volume was measured by a caliper every other day during the whole experiment. The tumor volume (TV) was calculated with the

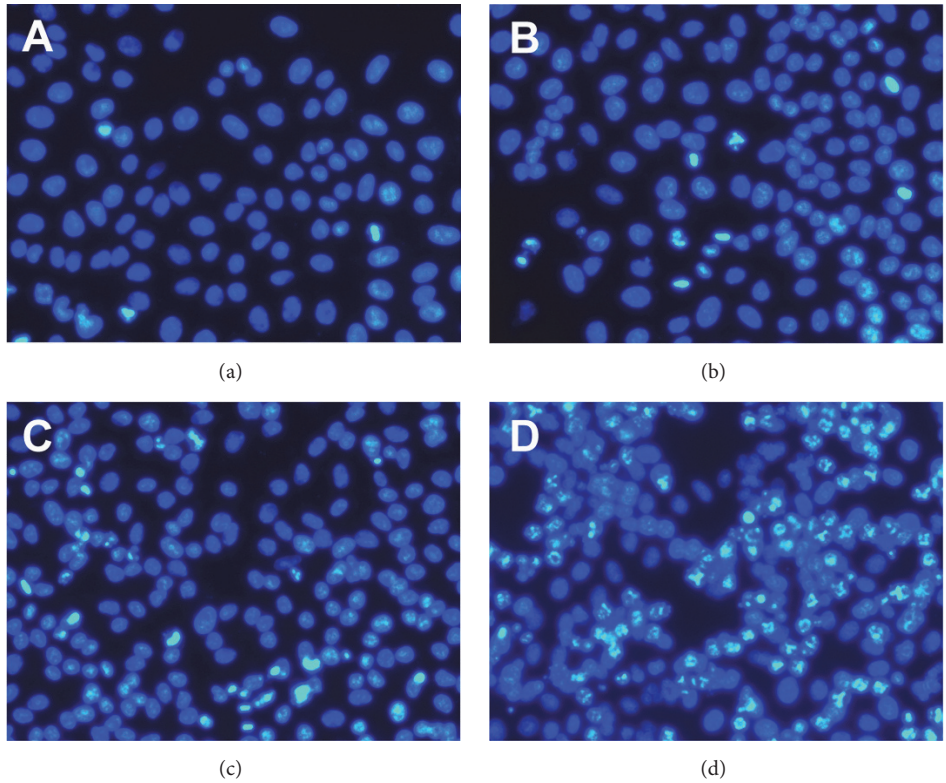

Figure 2: Apoptosis of TPC-1 cells detected by DAPI staining. (a) The nontreated cells. (b) Cells were treated with 4 μg/mL harmine for 36 h. (c) Cells were treated with 8 μg/mL harmine for 36 h. (d) Cells were treated with 16 μg/mL harmine for 36 h.

following formula: TV = $(W^2 * L)/2$, where W is the width of tumor nodule and L is the length of tumor nodule.

2.9. Statistical Analysis. Results were presented as mean ± SD. Statistical analysis was made by Student's t-test or ANOVA. The p value < 0.05 was considered as significant.

3. Results

3.1. Cytotoxicity and Apoptotic Induction of Harmine on TPC-1 Cells. As shown in Figure 1, escalated doses of harmine effectively led to the decrease of cell viability at different incubation times (Figure 1). The IC50 values of harmine against TPC-1 cells at 24, 36, and 48 h were 16.57 ± 1.4, 9.48 ± 1.1, and 5.51 ± 0.7 μg/mL, respectively. It is noted that the IC50 values significantly lowered as the incubation time extended. Therefore, harmine inhibited the proliferation of TPC-1 cells in a time- and dose-dependent manner.

As shown in Figure 2, it is obvious to locate the characterized morphology of apoptotic cells in harmine treated group with brighter DAPI staining with condensed chromatin forming crescent-shaped profiles around the periphery of the nucleus or separate globular structures (apoptotic bodies). Quantitative analysis indicated the significant differences among the three groups treated with elevated doses of harmine, which was in accordance with the results in cytotoxicity tests (Figure 3). Figure 4 indicated that harmine was effective to induce the fragmentation of DNA of TPC-1 cells

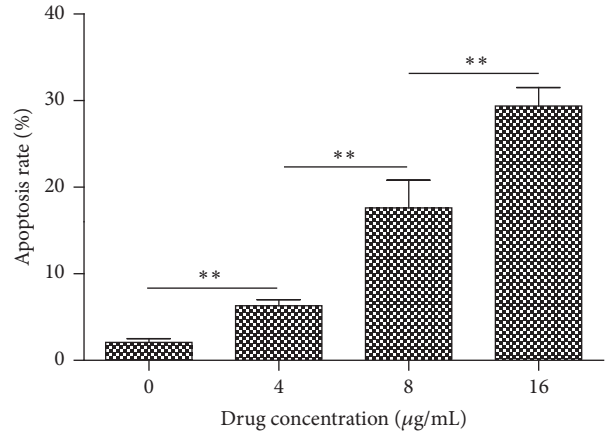

Figure 3: Quantitative analysis of apoptotic rate of cells exposed to different agents. Values are represented as mean ± SD (n = 3). ** means $p < 0.01$.

in a dose-dependent manner, demonstrating the apoptosis inducing effect.

3.2. Effect of Harmine on Apoptotic Proteins and the Activity of Caspase-3. To examine the possible mechanism of apoptotic induction of harmine, the expression of apoptosis related proteins was evaluated by western blot. As shown in Figure 5, treatment of harmine dose-dependently induced the expression of Bax and degraded the existing Bcl-2, leading

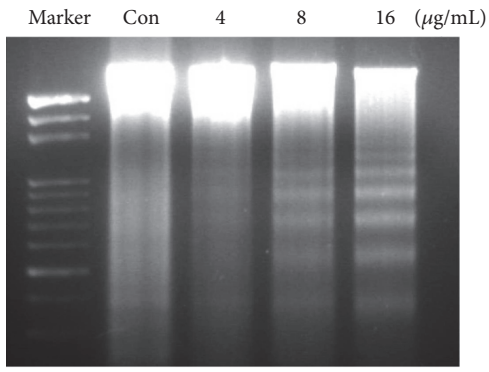

Figure 4: DNA fragmentation assay of TPC-1 cell treated with escalated doses of harmine. Cells were treated with different concentrations of harmine (4, 8, and 16 μg/mL) for 36 h and then total DNA was extracted and electrophoresed.

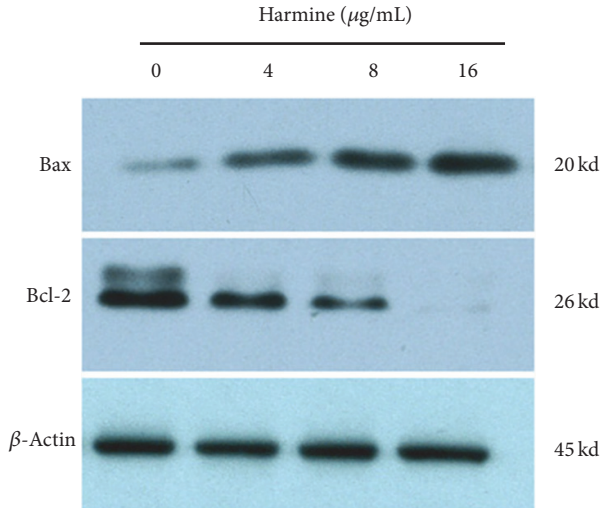

Figure 5: Protein expression of TPC-1 cells treated with different doses of harmine.

to a significant decrease in the ratio of Bcl-2/Bax, which demonstrated the progression of apoptosis.

The activity of Caspase-3 in TPC-1 cells treated with a series of doses of harmine was evaluated by the caspase colorimetric protease assay kit. As one of the crucial mediators of apoptosis, Caspase-3 is considered as an apoptotic marker due to its significant role in catalyzing the specific cleavage of key proteins and generation of apoptotic bodies. Figure 6 showed that harmine was effective in elevating the activity of Caspase-3, which was in accordance with the results of apoptosis detection from Figures 2 and 3.

3.3. Effect of Harmine on the Colony Forming Ability of TPC-1 Cells with or without the Combination of Radiation. Figure 7(a) showed the clonogenic assay of harmine on TPC-1 cells. The colony forming ability of TPC-1 cells was time- and dose-dependently inhibited by harmine. The percent survival of TPC-1 cells decreased as the concentration of harmine increased and incubation time extended, demonstrating that

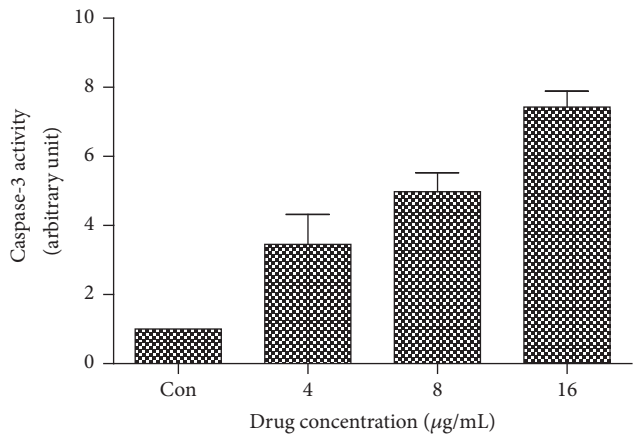

Figure 6: The activity of Caspase-3 in TPC-1 cells treated with different doses of harmine.

the antiproliferative effect of harmine was consistent with the results from cytotoxicity test.

Figure 7(b) indicated that 2 μg/mL harmine effectively enhanced the inhibitory effect of radiation on the colony forming ability of TPC-1 cells. There was a significant difference between the percent survival of radiation alone and radiation plus harmine, which demonstrated the potential radiosensitizing effect of harmine.

3.4. Effects of Harmine on the Migration and Invasion of TPC-1 Cells. Figure 8 indicated the effect of harmine on the wound healing rate of TPC-1 cells. Harmine dose-dependently decreased the wound healing rate of TPC-1 cells, which demonstrated an inhibitory effect of harmine on the migration of thyroid cancer cells. Similarly, harmine inhibited the invasion of TPC-1 cells in a dose-dependent manner as shown in Figure 9. Statistical analysis indicated significant differences among the groups treated with different doses of harmine. Similarly, the number of invading cells decreased significantly as the concentration of harmine increased. Results from wound scratch and Transwell assays demonstrate that harmine is effective in restraining the mobility of TPC-1 cells in a dose-dependent manner, which makes it a potential drug for the reverse of tumor metastasis.

3.5. In Vivo Antitumor Evaluation of Harmine against TPC-1 Xenograft. In vivo antitumor effects of harmine were examined in a xenograft model of TPC-1 cells in nude mice (Figure 10). It is obvious that harmine delayed the growth of thyroid cancer in a dose-dependent manner. However, though the tumor of the mice receiving the lowest dose of harmine grew more slowly than that of the mice in the control group, there was no significant difference between the two groups. On the contrary, the other two relatively high doses of harmine significantly inhibit the growth of tumor when compared with the control group. Among all the three groups, the highest dose of harmine generated the greatest antitumor effect. Moreover, 40 mg/kg harmine restrained the tumor growth more significantly than 20 mg/kg harmine. In

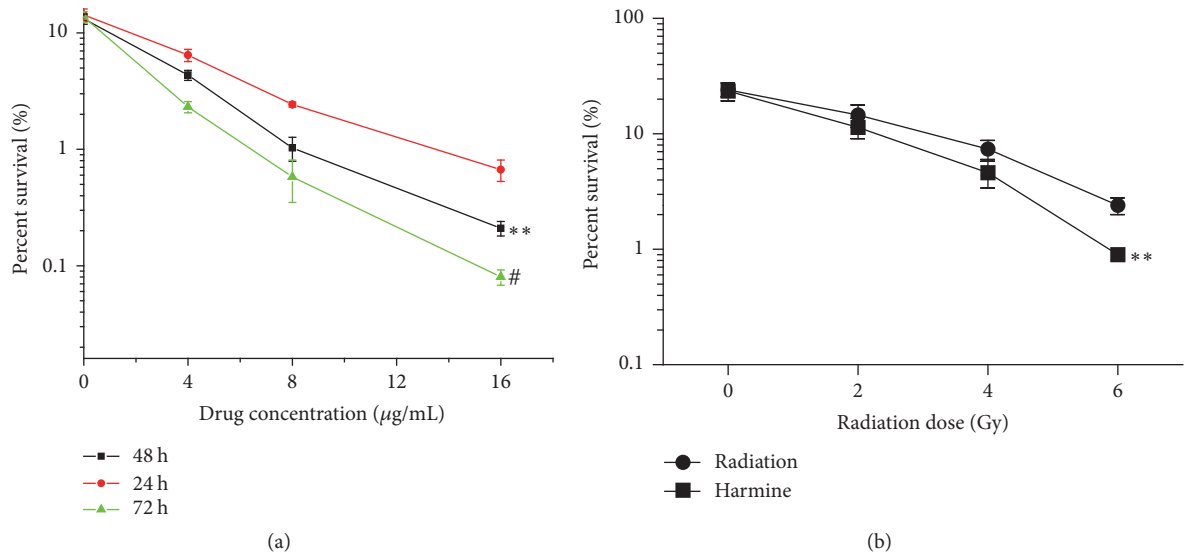

Figure 7: Clonogenic assay of TPC-1 cells treated with harmine and radiation. (a) Inhibitory effect of different doses of harmine on the colony forming ability of TPC-1 cells. ∗∗ represents $p < 0.01$ versus 24 h group. # represents $p < 0.01$ versus 48 h group. (b) Radiosensitization effect of harmine (2 μg/mL) on TPC-1 cells. ∗∗ represents $p < 0.01$ versus radiation group.

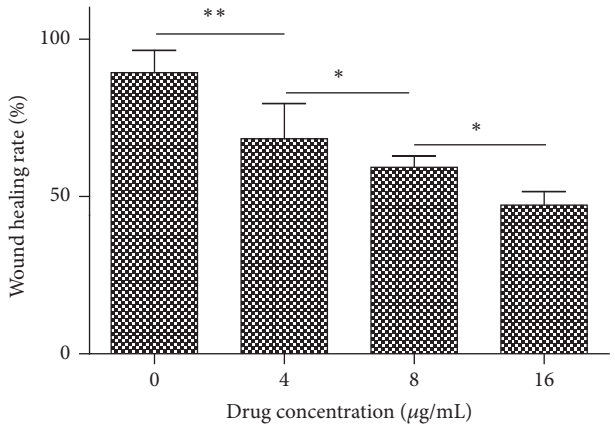

Figure 8: Wound healing ability of TPC-1 cells exposed to a series of doses of harmine. Quantification of cell migration using the monolayer wound scratching assay. ∗ represents $p < 0.05$. ∗∗ represents $p < 0.01$.

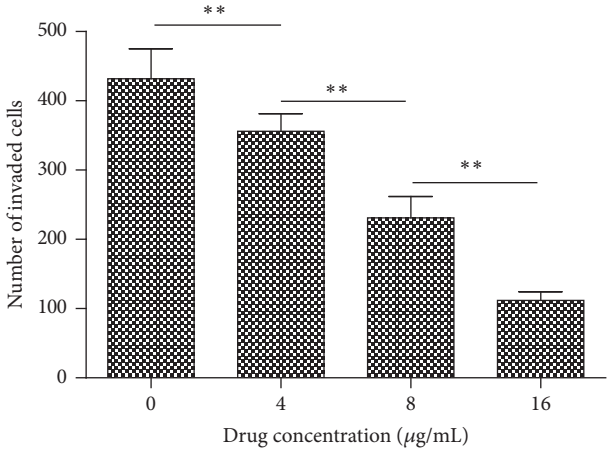

Figure 9: Cell invasive ability of TPC-1 cells exposed to harmine. Quantification of the numbers of invaded cells exposed to a series of doses of harmine. Each data point represents the mean ± SD from three independent experiments. ∗∗ represents $p < 0.01$.

addition, there was no obvious toxicity (such as weight loss or behavior) in all the mice during the whole experiment.

4. Discussion

Here we show the cytotoxic and antiproliferative effect of harmine on the in vitro and in vivo model of thyroid cancer. As reported in previous studies, harmine showed cytotoxic and antiproliferative effect against several kinds of cancers [11, 16]. Several intracellular targets have been identified such as cyclin proteins and interference with DNA [17]. However, no report was about the antitumor effect of harmine in thyroid cancer.

In the current study, we demonstrated a time- and dose-dependent cytotoxicity of harmine against thyroid cancer cell line TPC-1. Both XTT and clonogenic assay showed that the antiproliferative effect of harmine was mainly dependent on the concentration and incubation time of harmine. Several studies have reported the dose-dependent cytotoxicity of harmine against other kinds of cancers. For example, Zhang et al. reported the time- and dose-dependent antiproliferative effect of harmine against two kinds of gastric cancer cells [11]. A recent study from South China also demonstrated the inhibitory effect of harmine derivative on the proliferation of gastric cancer cells [7].

Though most of the studies focused on the antiproliferative effect of harmine, few researchers evaluated the influence of harmine on the colony forming ability of cancer cells as

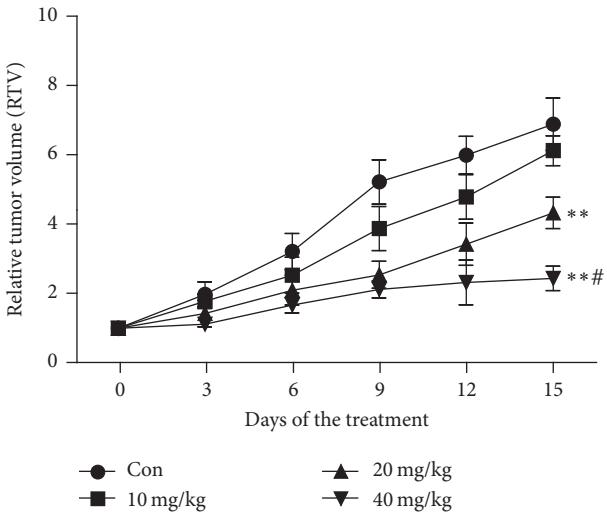

Figure 10: Antitumor effect of harmine in TPC-1 xenograft models. Tumor volume of established TPC-1 xenografts in nude mice during therapy under different treatments. Mice were treated with different protocols on Day 0 as shown in the figure. Saline: vehicle; harmine was administered at the doses of 10, 20, and 40 mg/kg. Different agents were delivered through intravenous pathway when tumor volume measured 100 mm^3. Data are presented as mean ± SD (n = 6). The difference between tumor volumes in the group of saline and either 20 or 40 mg/kg of harmine is significant (** means $p < 0.01$ versus control). Significant difference (# means $p < 0.05$) also is observed between the groups receiving 40 mg/kg and 20 mg/kg harmine.

well as the radiosensitizing effect. As introduced previously, loss of reproductive integrity and the inability to proliferate indefinitely is a key feature to cell death [14, 18]. Cells with integrity but unable to divide and produce a large number of colony are considered dead [19]. Therefore, cells with both the integrity and the ability to proliferate are referred to as "clonogenic," which is pivotal to the proliferation of cancer cells and regarded as a marker of cancer malignancy [20]. Here we showed that harmine exerted a time- and dose-dependent inhibition on the clonogenic ability of TPC-1 cells, which means that harmine could effectively disrupt the reproductive integrity and lead to the inability of proliferation of thyroid cancer cells. Moreover, the current study reported for the first time that harmine showed the potential to be a promising radiosensitizer.

The following DAPI staining of cancer cells indicated that harmine was efficient to induce apoptosis of TPC-1 cells. It is known that apoptosis, also called programmed cell death, is characterized by the specific morphology, such as chromatin condensation, membrane blebbing, internucleosome degradation of DNA, and apoptotic body formation [21]. It is obvious that cells treated with harmine underwent some characteristic phenomenon of apoptosis. It is reported in earlier studies that the Bcl-2 family, including proapoptotic proteins and antiapoptotic proteins, plays an important role in apoptosis [22, 23]. Among all these proteins, the ratio of Bcl-2 and Bax is a key indicator of apoptosis [24]. Figure 4 showed that the treatment by harmine dose-dependently decreased the expression of Bcl-2 while simultaneously increased the expression of Bax, which led to an obvious decline in the ratio of Bcl-2 and Bax. Though the effect of apoptotic induction by harmine was also reported by other groups, different mechanisms underlying the antitumor effect of harmine have been identified such as cell cycle arrest and telomerase activity inhibition [9]. The anticancer effect of harmine was confirmed in TPC-1 xenograft model, which, together with other studies focusing on harmine, proved the efficacy of harmine in the treatment of cancer.

The ability of migration and invasion is the characteristic of malignancy, which often leads to the failure of cancer therapy [25]. The efficient inhibition of tumor migration and invasion is an effective way to control the metastasis of tumor. Since few studies examined the antimetastatic effect of harmine, we evaluate the ability of harmine on cell migration and invasion through wound scratching and Transwell assays. Results from the two experiments demonstrated the dose-dependent inhibitory effect of harmine in vitro. Moreover, animal experiment on metastatic xenograft models in the author's lab is ongoing to further evaluate the in vivo antimetastasis efficacy of harmine.

As mentioned above, evidences from our report not only demonstrate the potential antitumor effect of harmine, but also provide a feasible way to counteract the metastasis of thyroid cancer. Moreover, further studies focusing on the chemosensitization effect of harmine are ongoing in the author's lab to explore more potential application of such alkaloids. It is undoubtedly, however, that the development of herbal medicine, especially alkaloids, warrants more intensive research in order to evaluate the feasibility and advantages of clinical applications.

In summary, the current study demonstrates the antitumor effect of harmine in the treatment of thyroid cancer. In vitro cytotoxicity tests indicate that harmine possesses a dose- and time-dependent cell inhibitory and apoptosis inducing effect on TPC-1 cells. In addition, harmine effectively inhibits the migration and invasion of TPC-1 cells in a dose-dependent manner. Moreover, in vivo evaluation shows that harmine significantly delays the growth of thyroid cancer in a dose-dependent manner in nude mice. Therefore, harmine might be a promising herbal medicine in tumor therapy and further efforts are needed to explore this therapeutic strategy.

Competing Interests

The authors report no conflict of interests in this work.

References

[1] M. E. Cabanillas, D. G. McFadden, and C. Durante, "Thyroid cancer," *The Lancet*, vol. 388, no. 10061, pp. 2783–2795, 2016.

[2] E. Tirrò, M. L. Consoli, M. Massimino et al., "Altered expression of c-IAP1, survivin, and smac contributes to chemotherapy resistance in thyroid cancer cells," *Cancer Research*, vol. 66, no. 8, pp. 4263–4272, 2006.

[3] X. Wei, Z. Chen, X. Yang, and T. Wu, "Chinese herbal medicines for esophageal cancer," *Cochrane Database of Systematic Reviews*, no. 4, Article ID CD004520, 2009.

[4] L. Huang, J. Zhang, T. Song et al., "Antifungal curcumin promotes chitin accumulation associated with decreased virulence of *Sporothrix schenckii*," *International Immunopharmacology*, vol. 34, pp. 263–270, 2016.

[5] Y. Hu, S. Wang, X. Wu et al., "Chinese herbal medicine-derived compounds for cancer therapy: a focus on hepatocellular carcinoma," *Journal of Ethnopharmacology*, vol. 149, no. 3, pp. 601–612, 2013.

[6] L. Tan, W. Wang, G. He et al., "Resveratrol inhibits ovarian tumor growth in an in vivo mouse model," *Cancer*, vol. 122, no. 5, pp. 722–729, 2016.

[7] P. Zhang, C.-R. Huang, W. Wang et al., "Harmine hydrochloride triggers G2 phase arrest and apoptosis in MGC-803 cells and SMMC-7721 cells by upregulating p21, activating caspase-8/Bid, and downregulating ERK/bad pathway," *Phytotherapy Research*, vol. 30, no. 1, pp. 31–40, 2016.

[8] T. Liu, X. Liu, and W. Li, "Tetrandrine, a Chinese plant-derived alkaloid, is a potential candidate for cancer chemotherapy," *Oncotarget*, vol. 7, no. 26, pp. 40800–40815, 2016.

[9] L. Zhao and M. Wink, "The β-carboline alkaloid harmine inhibits telomerase activity of MCF-7 cells by down-regulating hTERT mRNA expression accompanied by an accelerated senescent phenotype," *PeerJ*, vol. 1, article e174, 2013.

[10] T. P. Hamsa and G. Kuttan, "Harmine inhibits tumour specific neo-vessel formation by regulating VEGF, MMP, TIMP and pro-inflammatory mediators both in vivo and in vitro," *European Journal of Pharmacology*, vol. 649, no. 1–3, pp. 64–73, 2010.

[11] H. Zhang, K. Sun, J. Ding et al., "Harmine induces apoptosis and inhibits tumor cell proliferation, migration and invasion through down-regulation of cyclooxygenase-2 expression in gastric cancer," *Phytomedicine*, vol. 21, no. 3, pp. 348–355, 2014.

[12] L. Zhang, F. Zhang, W. Zhang et al., "Harmine suppresses homologous recombination repair and inhibits proliferation of hepatoma cells," *Cancer Biology and Therapy*, vol. 16, no. 11, pp. 1585–1592, 2015.

[13] T. P. Hamsa and G. Kuttan, "Harmine activates intrinsic and extrinsic pathways of apoptosis in B16F-10 melanoma," *Chinese Medicine*, vol. 6, no. 1, article 11, 2011.

[14] H. Rafehi, C. Orlowski, G. T. Georgiadis, K. Ververis, A. El-Osta, and T. C. Karagiannis, "Clonogenic assay: adherent cells," *Journal of Visualized Experiments*, no. 49, 2011.

[15] K. Yuki, A. Takahashi, I. Ota et al., "Sensitization by glycerol for CDDP-therapy against human cultured cancer cells and tumors bearing mutated p53 gene," *Apoptosis*, vol. 9, no. 6, pp. 853–859, 2004.

[16] H. Liu, D. Han, Y. Liu et al., "Harmine hydrochloride inhibits Akt phosphorylation and depletes the pool of cancer stem-like cells of glioblastoma," *Journal of Neuro-Oncology*, vol. 112, no. 1, pp. 39–48, 2013.

[17] Y. Song, D. Kesuma, J. Wang et al., "Specific inhibition of cyclin-dependent kinases and cell proliferation by harmine," *Biochemical and Biophysical Research Communications*, vol. 317, no. 1, pp. 128–132, 2004.

[18] N. A. P. Franken, H. M. Rodermond, J. Stap, J. Haveman, and C. van Bree, "Clonogenic assay of cells in vitro," *Nature Protocols*, vol. 1, no. 5, pp. 2315–2319, 2006.

[19] A. Munshi, M. Hobbs, and R. E. Meyn, "Clonogenic cell survival assay," *Methods in Molecular Medicine*, vol. 110, pp. 21–28, 2005.

[20] J. A. Plumb, "Cell sensitivity assays: clonogenic assay," *Methods in Molecular Medicine*, vol. 88, pp. 159–164, 2004.

[21] S. J. Martin and D. R. Green, "Apoptosis and cancer: the failure of controls on cell death and cell survival," *Critical Reviews in Oncology and Hematology*, vol. 18, no. 2, pp. 137–153, 1995.

[22] P. Gazzaniga, A. Gradilone, R. Vercillo et al., "bcl-2/bax mRNA expression ratio as prognostic factor in low-grade urinary bladder cancer," *International Journal of Cancer*, vol. 69, no. 2, pp. 100–104, 1996.

[23] L. F. Gibson, J. Fortney, G. Magro, S. G. Ericson, J. P. Lynch, and K. S. Landreth, "Regulation of BAX and BCL-2 expression in breast cancer cells by chemotherapy," *Breast Cancer Research and Treatment*, vol. 55, no. 2, pp. 107–117, 1999.

[24] S. Cory and J. M. Adams, "Killing cancer cells by flipping the Bcl-2/Bax switch," *Cancer Cell*, vol. 8, no. 1, pp. 5–6, 2005.

[25] C. E. Henry, E. Llamosas, A. Djordjevic, N. F. Hacker, and C. E. Ford, "Migration and invasion is inhibited by silencing ROR1 and ROR2 in chemoresistant ovarian cancer," *Oncogenesis*, vol. 5, no. 5, article no. e226, 2016.

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Research Article

Antiemetic and Myeloprotective Effects of *Rhus verniciflua Stoke* in a Cisplatin-Induced Rat Model

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Rhus verniciflua Stoke has been commonly used in traditional medicine to treat gastrointestinal (GI) dysfunction diseases. In order to investigate pharmacological properties of *Rhus verniciflua* Stoke water extract (RVX) on cisplatin-induced amnesia, RVX (0, 25, 50, or 100 mg/kg) was orally administrated for five consecutive days after a single intraperitoneal injection of cisplatin (6 mg/kg) to SD rat. Cisplatin injection significantly increased the kaolin intake (emesis) but reduced the normal diet intake (anorexia) whereas the RVX treatment significantly improved these abnormal diet behaviors at both the acute and delayed phase. The serotonin concentration and the related gene expressions (5-HT3 receptors and SERT) in small intestine tissue were abnormally altered by cisplatin injection, which were significantly attenuated by the RVX treatment. Histological findings of gastrointestinal tracts, as well as the proteins level of proinflammatory cytokines (TNF-α, IL-6, and IL-1β), revealed the beneficial effect of RVX on cisplatin-induced gastrointestinal inflammation. In addition, RVX significantly improved cisplatin-induced myelosuppression, as evidenced by the observation of leukopenia and by histological examinations in bone marrow. Our findings collectively indicated *Rhus verniciflua* Stoke improved the resistance of rats to chemotherapy-related adverse effects in the gastrointestinal track and bone marrow.

1. Introduction

A recent study reported that more than 14.1 million people per year are newly diagnosed with various malignancies worldwide [1]. The application of anticancer therapies depends on the status of clinical progression, characteristics of tumors, and patient' conditions [2]. Regarding treatments for patients with cancers, various medical modalities, including surgery, radiotherapy, chemotherapy, and immunotherapy, have been developed [3, 4]. Especially for patients with advanced cancers, chemotherapy is the first choice for more than 8 million patients annually in the United States [5, 6]. Chemotherapy, however, inevitably induces diverse adverse effects including emetic symptoms, leukopenia, gastrointestinal toxicity, hair loss, and fatigue [7]. Generally, 70 to 80% of patients undergoing chemotherapy complain mainly of the emetic symptoms, such as vomiting and nausea [8, 9]. Conventional agents for emesis include 5-hydroxytryptamine 3 (5-HT3) receptor antagonists, neurokinin 1 (NK1) receptor antagonists, glucocorticoids, and metoclopramide [10]. However, these drugs are only effective in either acute emesis or delayed emesis [11], or they evoke additional adverse effects, including insomnia, constipation, diarrhea, and headache [12, 13].

On the other hand, herbal plants or their derived natural compounds have attracted increasing attention in anticancer drug studies particularly regarding antiadverse effects, such as the emesis which was evoked by gastro enteric dysfunctions. *R. verniciflua* Stoke, called the lacquer tree, belonging to the Anacardiaceae family, has traditionally been described for treating digestive troubles [14]. According to the traditional Chinese medical literature, known as *Ben-cao-gang-mu* (本草纲目), the pharmacological efficacies of *R. verniciflua* on

the gastrointestinal tract problems have been well indicated [15].

Several animal studies have reported that *R. verniciflua* exerted beneficial effects on inflammation and several metabolic diseases [16, 17] and protective effects against drug toxicity [18]. The previous studies suggested that *R. verniciflua* would be a potent candidate for drug development to treat chemotherapy-induced side effects, particularly emesis.

The present study therefore aimed to investigate the effects of *R. verniciflua* water extract (RVX) against chemotherapy-related adverse effects, particularly emesis and gastrointestinal inflammation using a model of rats injected with cisplatin.

2. Methods

2.1. Procedure for Fingerprinting and Sequencing Analysis. *R. verniciflua* was obtained from a local specific farm for *R. verniciflua* in Ok-Cheon (Chung-buk, South Korea). After obtaining *R. verniciflua* water extract (RVX), a final yield of 0.70% (w/w), we conducted fingerprint using a HPLC-DAD instrument (YL9100 series, South Korea) as described in Supplementary Information (see Supplementary Material available online at https://doi.org/10.1155/2017/9830342).

Genomic DNA from the leaves of RVX was extracted using a DNeasy Plant Mini kit (Qiagen, Valencia, CA, United States). The internal transcribed spacer (ITS) gene, including 5.8s ribosomal DNA, was amplified by polymerase chain reaction (PCR) using the primers for ITS1 (5′-TAG CGC AGA ACG ACC CGC CAA CCT GTA T-3′) and ITS2 (5′-CAC CTG ACC TGG GGT CGC GAT GCG-3′). Amplification of ITS fragments was conducted with the 2X SG Taq Master Mix (LPS solution, Daejeon, South Korea), and PCR conditions were obtained using an IQ5 PCR Thermal Cycler (Bio-Rad, Hercules, CA, United States) in the following steps: predenaturation at 95°C for 5 min, 35 cycles of 95°C/30 s (denaturation), 55°C/40 s (annealing), 72°C/1 min (extension), and final extension at 72°C for 5 min. The PCR products were analyzed on 1% agarose gel and distinguishable bands were purified using QIAQuick Gel extraction kit (Qiagen). The products were sequenced using an BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems, CA, United States) with an ABI3730XL (Applied Biosystems) automated sequencer. After alignment by ClustalW, the results were analyzed using BioEdit 7.0 version and vector NTI advance software, version 11.

2.2. Chemical Materials. The reagents for the present study were as follows: cisplatin (cis-diammineplatinum (II) dichloride), metoclopramide, protocatechuic acid, fustin, fisetin, sulfuretin, and butein were obtained from Sigma (St. Louis, MO, United States). Arabic gum was purchased from Junsei Chemical Co., Ltd. (Tokyo, Japan). Calci-Clear Rapid was purchased from National Diagnostics (Atlanta, GA, United States). Olive oil was purchased from DC Chemical Co., Ltd. (Seoul, South Korea).

2.3. Kaolin Diet Preparation. Kaolin ($H_2Al_2Si_2O_8 \cdot H_2O$) was prepared according to a previous method [19]. Briefly, pharmacological-grade kaolin (Samchun Pure Chemical Co., Ltd., Pyeongtaek, South Korea) was mixed with 1% acacia or gum Arabic in double-distilled water to form a thick paste. The paste was rolled and cut into small pellets. Pellets were dried completely at room temperature for 3-4 days and were maintained in sterile conditions.

2.4. Animals and Experimental Design. A total of 36 specific pathogen-free Sprague-Dawley male rats (6 weeks old, 160–180 g) were purchased from Dae-Han Bio Link (Chungbuk, South Korea). The rats were housed in a controlled temperature room at 22±2°C, in 55% ± 10% relative humidity with a 12 h : 12 h light-dark cycle, and they were freely fed commercial standard chow (Dae-Han Bio Link) and were provided tap water ad libitum for 7 days. After acclimation, the rats were housed separately in cages and were familiarized with the testing procedures. In addition to routine rat chow and tap water, a measured quantity of kaolin (5 g) pellets was provided in separate containers for 3 days prior to the experiment to allow the rats to adapt to its presence psychologically. After 3 days of habituation, the animals were subjected to pica experiments.

Acclimatized rats were divided into six groups ($n = 6$): normal and control groups, three dosages of RVX treatment groups (25, 50, or 100), and a Met 25 group. At 0 h, the normal group received saline, while all of the other groups received a single intraperitoneal injection of cisplatin in saline (6 mg/kg). Two hours after cisplatin injection, each group was initially treated with oral administration of water (normal and control groups), RVX (25, 50 or 100 mg/kg), or metoclopramide (25 mg/kg), followed by the same administration at 24 h intervals for 96 h. All of the groups were allowed free access to kaolin diets until 120 h. All of the rats were monitored for their consumption of normal and kaolin diets, and their body weights were measured daily. On the last experimental day, all rats were sacrificed by collection of whole blood via the abdominal vein under ether anesthesia.

This animal experiment was approved by the Institutional Animal Care and Use Committee of Daejeon University (DJUARB2015-041) and was conducted in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (Bethesda, MD, United States).

2.5. Hematology and Histopathology. On the final day of the experiment, hematological parameters were measured using a HEMA VET 850 automatic analyzer (CDC Technologies, United States). The white blood cell (WBC), neutrophil, lymphocyte, monocyte, basophil, eosinophil, red blood cell (RBC), hemoglobin, and platelet counts were determined. The spleen and thymus were removed, and their weights were measured. The stomach tissue, small intestine (both proximal and distal), and colon were dissected, washed in ice-cold phosphate buffered saline, and fixed in 10% formalin for 3 days. The paraffin-embedded samples were sectioned (4 μm thickness) and slides stained with H&E or Masson's trichrome

staining. Bone marrow was decalcified with Calci-Clear Rapid (National Diagnostics), then embedded in paraffin, sectioned at 6 μm, and stained with H&E stain. Representative images were obtained using a light microscope (Leica Microsystems, Wetzlar, Germany). The percentage areas of positively stained cells were analyzed using Image J image analysis software (Rasband, Bethesda, MD, United States), version 1.46.

2.6. Quantitative Real-Time PCR Analysis. Total RNA was extracted from small intestine samples using Trizol reagent (Molecular Research Center, Cincinnati, OH, United States). cDNA was synthesized from total RNA (2 μg) in a 20 μL reaction using the High-Capacity cDNA reverse transcription kit (Ambion, Austin, TX, United States). Real-time PCR was performed using SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, United States) with PCR amplification performed in accordance with a standard protocol using the IQ5 PCR Thermal Cycler (Bio-Rad, Hercules, CA, United States). The following primers were used ($5'\rightarrow3'$, forward and reverse): 5-HT3A receptor, GGA CTC CTG AGG ACT TCG ACA A and TTC CCC ACG TCC ACA AAC TC; SERT, CTG TTC ATC ATT TGC AGT TTT CTG A and TCC CTA TGC AGT AGC CCA AGA; and β-actin, AGG CCA ACC GTG AAA AGA TG and CCA GAG GCA TAC AGG GAC AAC.

2.7. Measurement of Serotonin and Proinflammatory Cytokines. Serotonin in the small intestine was measured using a serotonin ELISA kit (LDN, Nordhorn, Germany) and stomach tissue levels of proinflammatory cytokines were analyzed using commercial ELISA kits (TNF-α from BD OptEIA, CA, United States; IL-6 and IL-1β from R&D Systems, MN, United States).

2.8. Statistical Analysis. The results are expressed as the means ± standard deviations (SD). The statistical significance of differences between groups was analyzed by one-way analysis of variance (ANOVA), followed by Fisher's least-significant difference (LSD) test, or Student's t-test. In all of the analyses, values of $p < 0.05$ were considered statistically significant.

3. Results

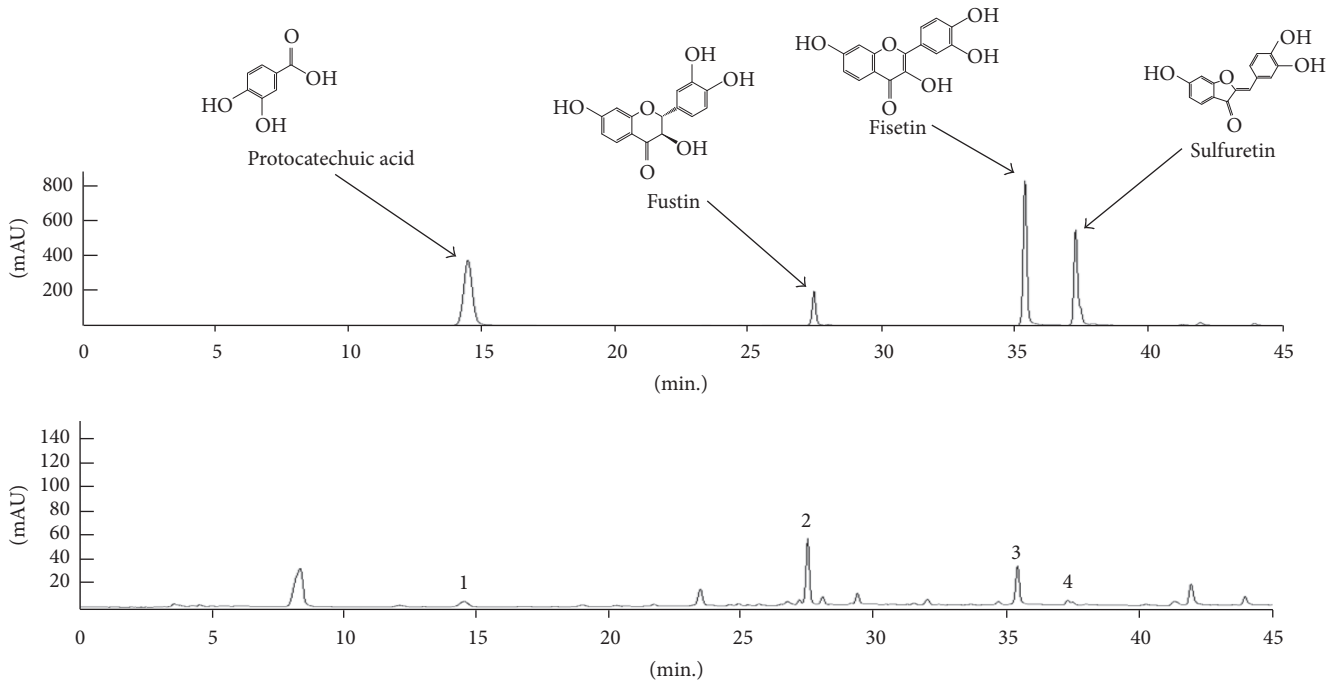
3.1. Fingerprint of RVX and Verification of R. verniciflua. The chemical constitution analysis of RVX was evaluated by performing HPLC-DAD analysis using four reference components: one phenolic compound (protocatechuic acid) and three flavonoid compounds (fustin, fisetin, and sulfuretin), respectively (Figure 1(a)). The fustin was quantified as the prevalent compound (61.61 ± 0.20 μg/mg), and it was followed by fisetin, protocatechuic acid, and sulfuretin, respectively (Figure 1(b)). The gene sequences of the ITS1-5.8s-ITS2 region coincided completely with *R. verniciflua* Stoke (AY510151.1, GenBank) ("*Toxicodendron vernicifluum* internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence," 2004) (Figure 1(c)).

3.2. Changes in Kaolin Diet Intake. Cisplatin injection (6 mg/kg) considerably increased kaolin diet intake by 2.6- to 3.3-fold, between 24 h and 120 h, compared with the normal group. Kaolin diet intake was significantly decreased by RVX 25 mg/kg (at 72 h and 120 h, $p < 0.01$), RVX 50 mg/kg (from 48 h to 72 h, $p < 0.05$ or $p < 0.001$), and RVX 100 mg/kg (from 24 h to 96 h, $p < 0.05$ or $p < 0.01$, Figure 2(a)). Metoclopramide suppressed kaolin intake to a substantial extent from 72 h to 96 h. When the analysis was conducted on total kaolin intake over 0–120 h, the cisplatin-induced increase in total kaolin intake (3.0-fold) was significantly attenuated by RVX treatment ($p < 0.05$ for 50 mg/kg; $p < 0.01$ for 100 mg/kg, Figure 2(b)).

3.3. Changes of Normal Diet Intake and Body Weight. Cisplatin injection significantly reduced normal diet intake by 0.5- to 0.7-fold, between 24 and 120 h, compared with the normal group, while it was significantly attenuated by administration of RVX 50 mg/kg (from 48 to 96 h, $p < 0.001$) and RVX 100 mg/kg (from 24 to 96 h, $p < 0.01$ or $p < 0.001$, Figure 2(c)). Body weight was also significantly reduced by 47.14 g, compared with normal group (at 120 h, $p < 0.001$). Treatment with RVX (50 and 100 mg/kg), however, significantly ameliorated this weight loss compared with the control group (Figure 2(c)). Metoclopramide showed similar effects to RVX (50 mg/kg) in both normal diet intake and recovery of weight loss.

3.4. Histopathological Findings for Gastrointestinal Damage. Hematoxylin and Eosin (H&E) staining revealed that cisplatin injection induced severe degenerative changes, especially in the small intestine and colon, as characterized by disruption of the epithelial architecture and dilated intercellular spaces. Treatment with RVX remarkably reversed these pathological alterations, compared with control group. In stomach tissue, no marked damage but intensive blue staining was observed, indicating migration of gastric chief cells to the outer layer of the stomach lining, in only control and metoclopramide groups but not in the RVX-treated groups (Figure 3(a)). Masson's trichrome staining confirmed loss of collagen at the submucosal region in the small intestine and colon of the control group, whereas RVX treatments notably reduced these collagen losses (Figure 3(b)).

3.5. Serotonin Concentrations and Related Gene Expressions in Small Intestine Tissue. The concentration of serotonin in small intestine tissue was elevated by 1.8-fold by cisplatin injection compared with the normal group, while RVX treatment (especially 50 mg/kg) significantly attenuated this compared with the control group ($p < 0.05$, Figure 4(a)). Cisplatin injection significantly upregulated mRNA expression of 5-HT3A receptor (2.1-fold) but downregulated serotonin transporter (SERT, 0.5-fold) compared with the normal group in small intestine tissue ($p < 0.001$). Treatment with RVX significantly modulated these alterations of 5-HT3A receptor



(a)

Peak number	Reference compounds	Retention time (min)	Mean ± SD (μg/mg)
1	Protocatechuic acid	14.4	0.60 ± 0.01
2	Fustin	27.4	61.61 ± 0.20
3	Fisetin	35.3	7.66 ± 0.18
4	Sulfuretin	37.2	0.26 ± 0.03

(b)

Species	GenBank number	5.8s ribosomal DNA	Similarity (%)	G + C contents (%)
Rhus verniciflua Stoke	AY510151.1	ATAATGACTCTCGGCAACGGATATCTCGGTTCTCGCATCCATGAAG AATGTAGCGAAATGCGATGCTTGCTGTGAATTGCAGAATCCCGTGA ACCATGGAGTCTTTGAATGCAAGTTGCGCCCTCAACCATTAGGCCG AGGGCACGTCTACTTGGGTGTCACGC	—	60
RVW	—	ATAATGACTCTCGGCAACGGATATCTCGGTTCTCGCATCCATGAAG AATGTAGCGAAATGCGATGCTTGCTGTGAATTGCAGAATCCCGTGA ACCATGGAGTCTTTGAATGCAAGTTGCGCCCTCAACCATTAGGCCG AGGGCACGTCTACTTGGGTGTCACGC	100%	60

(c)

Figure 1: HPLC fingerprint and sequencing analysis. RVX and its standard compounds were subjected to HPLC. Chromatogram of reference compounds mixture and RVX (a). The quantitative analysis of each component in RVX (b). The comparative 5.8s rDNA sequences between *R. verniciflua* Stoke and RVX were exhibited (c).

($p < 0.01$ for 50 mg/kg, $p < 0.001$ for 100 mg/kg) and SERT ($p < 0.01$ for 50 mg/kg, $p < 0.05$ for 100 mg/kg) compared with the control group, respectively (Figure 4(b)). Metoclopramide showed similar effects of RVX (50 mg/kg) on the expression of 5-HT3A receptor, but not on the serotonin concentration or SERT gene expression.

3.6. Protein Levels of Proinflammatory Cytokines in Stomach Tissue. Cisplatin injection considerably elevated the protein levels of proinflammatory cytokines, including TNF-α (1.5-fold), IL-6 (3.4-fold), and IL-1β (3.4-fold) in stomach tissue, compared with the normal group. These abnormal elevations of the three cytokines were significantly attenuated by RVX treatment compared with the control group ($p < 0.001$, Figure 4(c)). Metoclopramide showed similar effects to RVX treatment.

3.7. Changes in Hematological Parameters. On the final day of the experiment, the control group showed the leukopenia in approximately 57% of the normal group; in particular, lymphocyte counts were decreased in half that of the normal group. These alterations were significantly improved to near

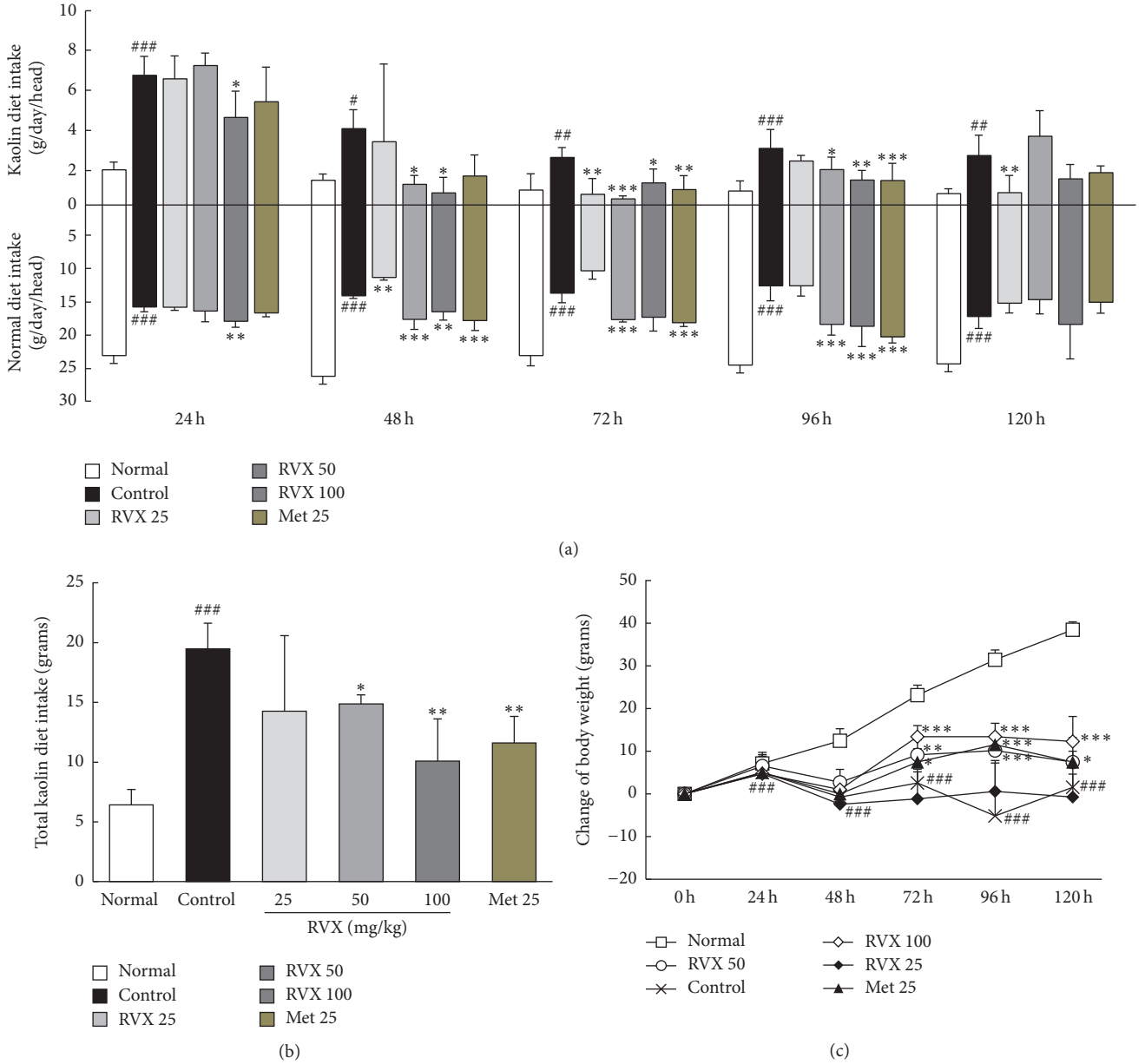


FIGURE 2: Kaolin consumption, normal diet consumption, and body weight change. The consumption of kaolin and a normal diet at different time points (a), total kaolin consumption (b), and body weight change (c) were monitored. Data are expressed as the mean ± SD ($n = 6$). $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, and $^{\#\#\#}p < 0.001$, compared with the normal group; $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$, compared with the control group.

to the normal group by RVX treatment (especially 100 mg/kg, $p < 0.001$, Table 1). The counts of red blood cells and platelets and hemoglobin concentration were not notably affected by cisplatin injection. The lymphocyte count was improved by moderate level in the metoclopramide group.

3.8. Histopathological Findings for Bone Marrow Damage. H&E staining revealed that cisplatin injection considerably injured bone marrow tissue, as evidenced by loss of bone marrow cells and thinning of trabecular bone, compared with the normal group. These alterations (especially vacant spaces in bone marrow) notably attenuated by RVX treatment compared with the control group ($p < 0.01$ or 0.001, Figures 5(a) and 5(b)). Metoclopramide also improved those pathological alterations of bone marrow compared with the control group.

3.9. Changes of Spleen and Thymus Weights. Cisplatin injection considerably decreased the weights of both the thymus (0.5-fold) and spleen (0.6-fold), compared with the normal group; however, treatment with RVX (50 and 100 mg/kg) significantly attenuated these changes in organ weights

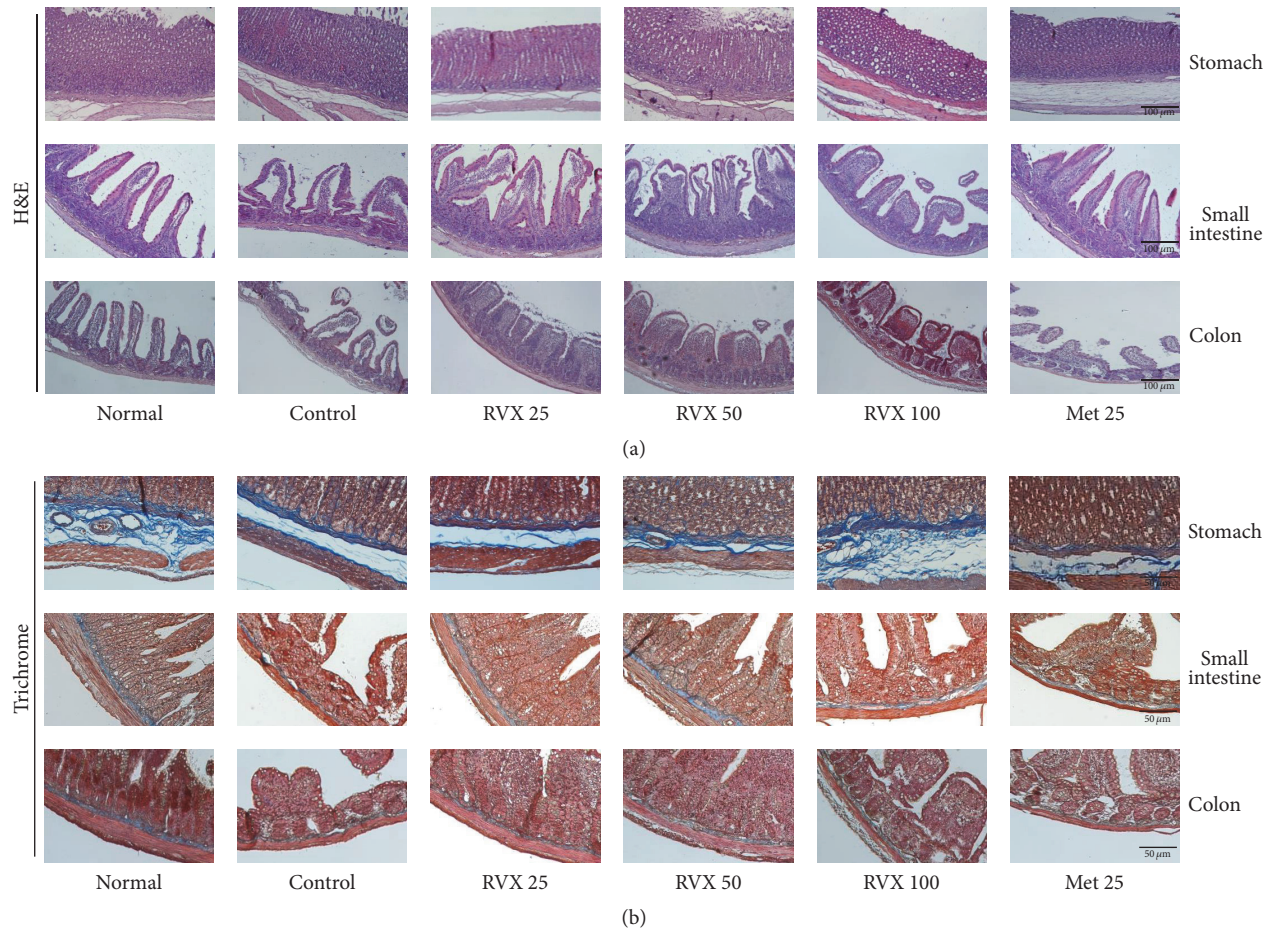


Figure 3: Histopathological findings of the gastrointestinal tract. H&E staining was conducted for the stomach, small intestine, and colon (a) and Masson's trichrome staining (b). The stained tissues were examined under a light microscope (200x magnification).

($p < 0.05$, 0.01, or 0.001) compared with the control group (Table 1). This positive finding was observed in the metoclopramide group, for thymus weight, but not spleen weight.

4. Discussion

R. verniciflua has been traditionally used to treat *"blood stasis* (瘀血)*"* which is associated with chronic diseases, including pain, stomach problem, and cancer, according to the theory of traditional Oriental medicine [14]. *R. verniciflua* was described in the herbal formulae extract part of traditional Chinese medicine literature (*Ben-cao-gang-mu*) for ameliorating gastro enteric dysfunctions [15]. Besides, *R. verniciflua* has been used to treat digestive troubles by adding it to chicken soup in traditional Korean medicine [20, 21]. Regarding the adverse effect of chemotherapy, particularly emesis, the pharmacological mechanisms of *R. verniciflua* still remain unclear. Herein, thus, we evaluated the pharmaceutical effects of *R. verniciflua* against cisplatin-induced adverse effects, especially gastrointestinal distortion. Cisplatin is an anticancer drug of a platinum-containing class used to treat various types of cancers, including sarcomas, small cell lung cancer, ovarian cancer, and lymphomas, but it presents a number of side effects [22, 23]. Low reproducibility has frequently became an issue in pharmaceutical studies using herbal materials [24]. To minimize biased results due to poor quality of samples, we conducted fingerprint analysis and gene sequence-based vivification of the plant species, *R. verniciflua*.

As expected, a single injection of cisplatin remarkably increased the kaolin diet intake (increased emesis) and reduced the normal diet intake (increased anorexia) in the control group. Approximately 90% of the patients receiving cisplatin suffer from nausea and vomiting, and anorexia is also often accompanied by emesis in these patients [25]. This study adapted a pica model in which animals responded to emetic stimuli by consuming nonnutritive substances, such as kaolin [26]. The behavioral changes in food intake have frequently been assessed in animals incapable of vomiting [27]. In general, chemotherapy-induced emesis is divided into two phases: acute (24 h) and delayed (24–120 h), respectively [28], and RVX treatment ameliorated phases of emesis and anorexia.

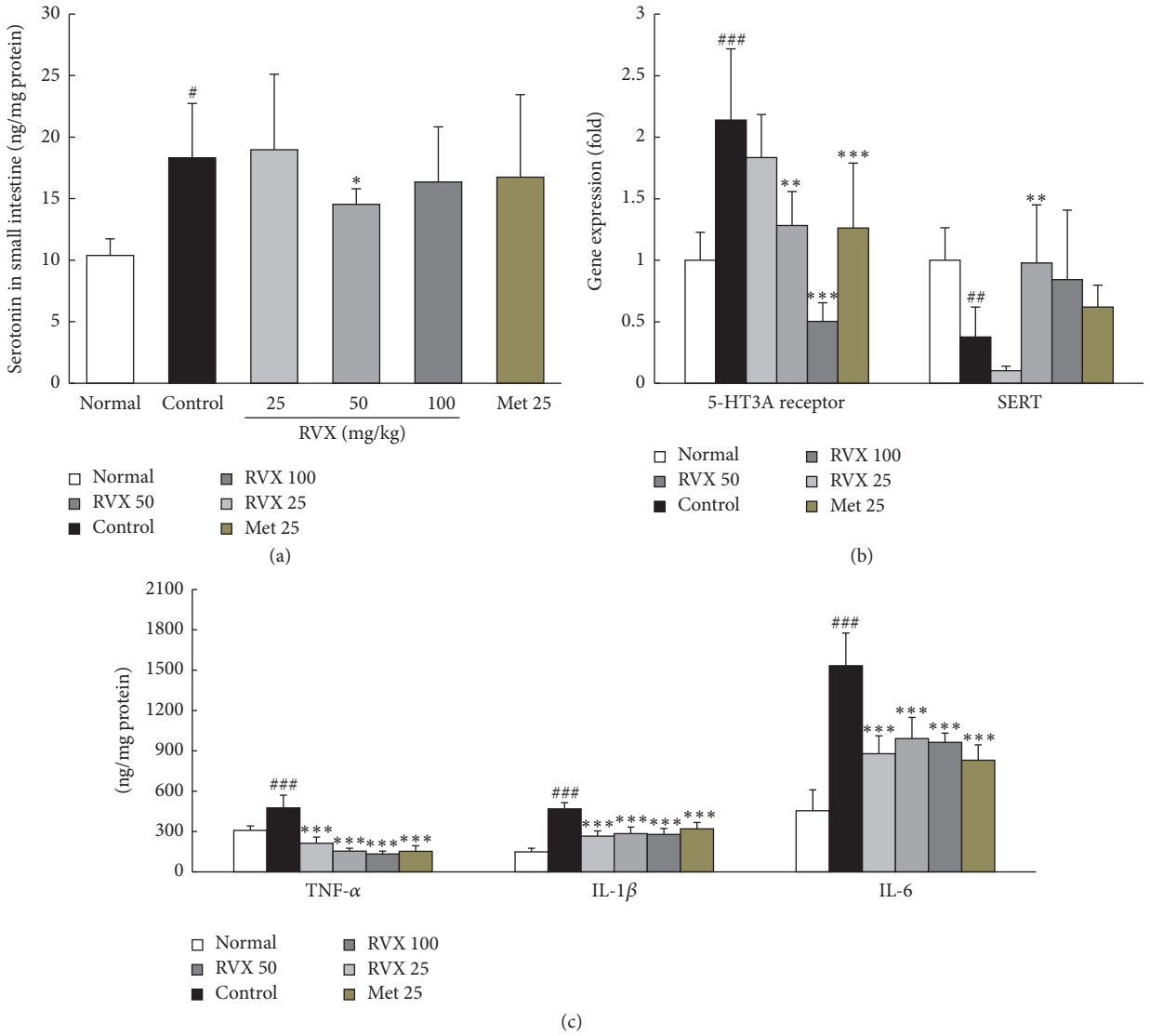


Figure 4: Serotonin and its related genes and proinflammatory cytokines. Serotonin concentrations in the small intestine were measured using ELISA (a). The gene expression levels of 5-HT3A receptor and SERT were measured using real-time PCR (b). Protein levels of TNF-α, IL-6, and IL-1β (c) were performed using ELISA method. Data are expressed as the mean ± SD ($n = 6$). $^{\#}P < 0.05$, $^{\#\#}p < 0.01$, and $^{\#\#\#}p < 0.001$ compared with the normal group; $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ compared with the control group.

The activation of 5-HT3 receptor with serotonin (5-HT) plays a key role in chemotherapy-induced-emesis [29]. Chemotherapy can induce 5-HT release from the enterochromaffin (EC) cells, consequently stimulating 5-HT3 receptor on the vagal nerve and resulting in the activation of the vomiting reflex [30]. As expected, RVX treatment significantly improved the cisplatin-induced upregulation of 5-HT3 receptor gene expression as well as the activated release of 5-HT in the intestine. Intestinal 5-HT is transported via SERT and then is metabolized in the epithelial cells of the gastrointestinal tract; therefore, damage to epithelial cells can impair the 5-HT metabolism, leading to prolonged contact time of 5-HT with 5-HT3 receptor [31]. RVX treatment normalized the downregulated gene expression of SERT in the intestine after cisplatin injection. Metoclopramide 25 mg/kg exerted antiemetic effects in only delayed phase, and it affected the gene expression of 5-HT3 receptor but not of SERT.

Histopathological findings showed severe gastrointestinal tract damage by cisplatin injection in our study, as evidenced by remarkable loss of villus cells, disruption of the epithelial architecture, and considerable reduction in collagen contents via H&E staining as well as trichrome staining. These alterations were more notable in the small intestine and colon than stomach; however, the stomach showed the intensive nuclei in a blue color, supposedly because of migration of gastric chief cells to the outer layer of the stomach lining. Gastric chief cells stain basophilic upon H&E staining owing to the large proportion of rough endoplasmic reticulum in its cytoplasm. These cells are generally located deep in the

Table 1: Hematologic parameters and organ weights.

Parameters	Normal	Control	RVX 25	RVX 50	RVX 100	Met 25
WBC (k/μL)	5.44 ± 1.19	3.12 ± 0.45$^{\#\#\#}$	3.20 ± 0.61	3.87 ± 0.63	5.59 ± 1.62***	4.14 ± 0.28
Neutrophils (k/μL)	0.52 ± 0.12	0.68 ± 0.24	0.62 ± 0.25	0.73 ± 0.18	1.01 ± 0.33	0.93 ± 0.60
Lymphocytes (k/μL)	4.73 ± 1.06	2.27 ± 0.43$^{\#\#\#}$	2.46 ± 0.48	2.97 ± 0.44*	4.33 ± 1.23***	3.06 ± 0.40*
Monocytes (k/μL)	0.03 ± 0.02	0.04 ± 0.01	0.04 ± 0.04	0.05 ± 0.02	0.13 ± 0.06***	0.08 ± 0.03
Basophil (k/μL)	0.01 ± 0.00	0.03 ± 0.02$^{\#}$	0.02 ± 0.01	0.03 ± 0.01	0.03 ± 0.01	0.03 ± 0.01
Eosinophil (k/μL)	0.05 ± 0.05	0.08 ± 0.06$^{\#}$	0.03 ± 0.02	0.09 ± 0.03	0.08 ± 0.04	0.05 ± 0.02
RBC (m/μL)	7.58 ± 0.37	7.09 ± 0.20$^{\#}$	6.89 ± 0.67	7.31 ± 0.27	7.31 ± 0.10	7.16 ± 0.23
Hemoglobin (g/dL)	14.68 ± 1.17	14.45 ± 0.71	14.40 ± 1.60	14.75 ± 0.55	15.35 ± 0.73	14.68 ± 0.56
Platelet (k/μL)	1091.50 ± 31.89	907.75 ± 173.30	661.00 ± 302.11	1191.75 ± 330.96*	1189.50 ± 46.26*	1138.75 ± 139.90
Thymus weight (g) (Relative%)	0.67 ± 0.11 (0.24 ± 0.04)	0.34 ± 0.08$^{\#\#\#}$ (0.15 ± 0.04$^{\#\#\#}$)	0.34 ± 0.05 (0.17 ± 0.02)	0.46 ± 0.06** (0.20 ± 0.02**)	0.50 ± 0.03*** (0.22 ± 0.01**)	0.48 ± 0.06** (0.21 ± 0.04**)
Spleen weight (g) (Relative%)	0.81 ± 0.10 (0.31 ± 0.04)	0.50 ± 0.03$^{\#\#\#}$ (0.24 ± 0.02$^{\#\#\#}$)	0.47 ± 0.02 (0.22 ± 0.02)	0.59 ± 0.10* (0.24 ± 0.01)	0.60 ± 0.05* (0.27 ± 0.02*)	0.58 ± 0.07 (0.25 ± 0.02)

Data are expressed as the mean SD ($n = 6$). $^{\#}p < 0.05$ and $^{\#\#\#}p < 0.001$, compared with normal group; $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$, compared with control group. WBC: white blood cell; RBC: red blood cell.

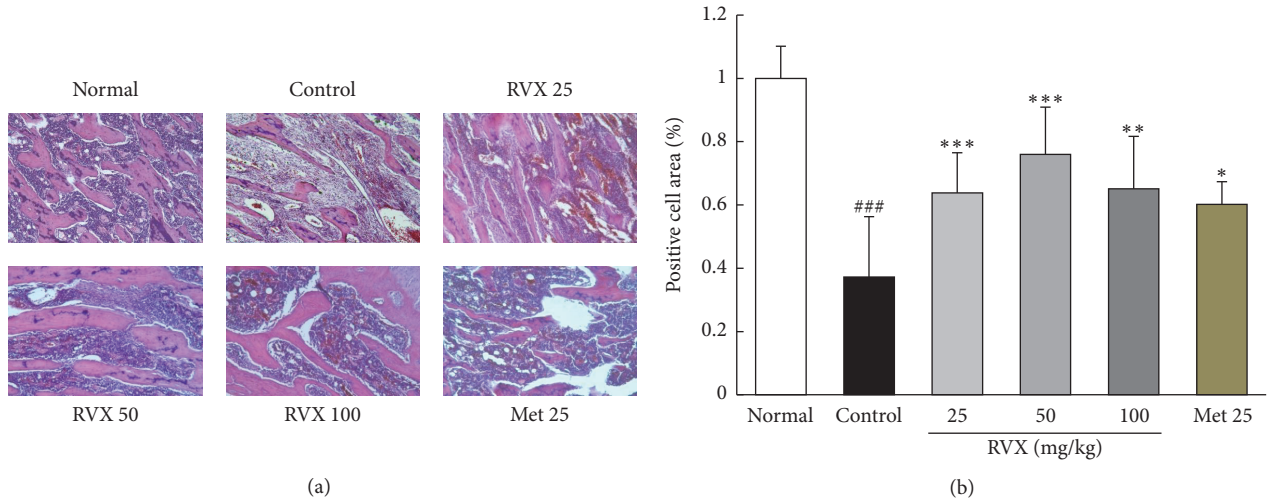


Figure 5: Histopathological finding of bone marrow. H&E staining was conducted for bone marrow, and the results were examined under a light microscope (200x magnification) (a). The positive cells area was quantitatively analyzed using Image J software, version 1.64 (b). Data are expressed as the mean ± SD ($n = 6$). $^{\#\#\#}p < 0.001$ compared with the normal group; $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ compared with control group.

mucosal layer of the stomach lining; the gastric chief cells then move into outer layer in response to inflammation [32]. This alteration was notably attenuated in groups treated with RVX, but not metoclopramide treatment. Chemotherapy treatment with cisplatin is also well known for provoking the gastritis [33]. While proinflammatory cytokines, including TNF-α, IL-6, and IL-1β, were remarkably elevated in stomach tissue, these abnormal elevations of these three cytokines were significantly improved by RVX treatment in our results.

On the other hand, bone marrow is one of the most fragile organs during chemotherapy. Myelosuppression increases the risk of infection, fatigue, and diminished quality of life (QoL) in patients; thus, it is a major dose-limiting factor for the clinical use of chemotherapy [34]. Moreover, myelosuppression, for instance, leukopenia, is primarily the reason for the delay, reduction, or cessation of chemotherapy treatment, occurring in approximately 15% of total cases of chemotherapy [35]. Our previous study reported that the most severe leukopenia was observed at 5 days after a single cisplatin injection rat model [36]. As we expected, notable bone marrow suppression and leukopenia were observed 5 days after cisplatin injection, and these pathologic conditions were considerably reserved by RVX treatment, especially at the highest dose of 100 mg/kg. In addition to the bone marrow, the spleen and thymus are representative immune organs, and losses in their weights in experimental models

of cisplatin injection have been widely reported [37], significantly attenuated by RVX treatment in our current study. These findings anticipated that RVX may have potential for use against immunosuppression caused by chemotherapy. The current results are in accordance with previous study, which RVS ameliorated significantly the cisplatin-induced adverse effects on liver and kidney functions in colon cancer cell (CT-26 cell line) injected tumor model [38].

Taken together, the current study reported for the first time comprehensive effects of *R. verniciflua* on the side effects of chemotherapy, mainly focusing on emesis and immunosuppression. In addition, modulation of 5-HT3 receptor is suggested to be an underlying mechanism of the antiemetic effects of *R. verniciflua*.

Competing Interests

The authors declare no competing financial interests.

Authors' Contributions

Hyo-Seon Kim and Hyeong-Geug Kim mainly wrote the manuscript text and conducted experiments. Sung-Bae Lee and Jin-Seok Lee performed the gene sequencing analysis of the *R. verniciflua* (Figure 1(c)). Hye-Won Lee, Sam-Keun Lee, and Chang Kyu Byun analyzed the fingerprinting analysis in Figures 1(a) and 1(b) (HPLC analysis). Won-Yong Kim prepared the *R. verniciflua* extract and reported behavioral test for kaolin diet and normal diet intake. Hwi-Jin Im supported the histopathological findings such as H&E and Masson's trichrome staining. Chang-Gue Son supervised the manuscript and directed final version of all contents. All authors reviewed and approved this manuscript. Hyo-Seon Kim and Hyeong-Geug Kim equally contributed to the current study.

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References

[1] J. Ferlay, I. Soerjomataram, R. Dikshit et al., "Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012," *International Journal of Cancer*, vol. 136, no. 5, pp. E359–E386, 2015.

[2] L. Nunney, C. C. Maley, M. Breen, M. E. Hochberg, and J. D. Schiffman, "Peto's paradox and the promise of comparative oncology," *Philosophical Transactions of the Royal Society B: Biological Sciences*, vol. 370, no. 1673, 2015.

[3] C. C. Murphy, L. C. Harlan, J. L. Lund, C. F. Lynch, and A. M. Geiger, "Patterns of colorectal cancer care in the United States: 1990–2010," *Journal of the National Cancer Institute*, vol. 107, no. 10, 2015.

[4] L. Zitvogel, L. Apetoh, F. Ghiringhelli, and G. Kroemer, "Immunological aspects of cancer chemotherapy," *Nature Reviews Immunology*, vol. 8, no. 1, pp. 59–73, 2008.

[5] M. T. Halpern and K. R. Yabroff, "Prevalence of outpatient cancer treatment in the United States: Estimates from the Medical Panel Expenditures Survey (MEPS)," *Cancer Investigation*, vol. 26, no. 6, pp. 647–651, 2008.

[6] Y. H. Yun, M. Kwak, S. M. Park et al., "Chemotherapy use and associated factors among cancer patients near the end of life," *Oncology*, vol. 72, no. 3-4, pp. 164–171, 2008.

[7] A. M. Griffin, P. N. Butow, A. S. Coates et al., "On the receiving end V: patient perceptions of the side effects of cancer chemotherapy in 1993," *Annals of Oncology*, vol. 7, no. 2, pp. 189–195, 1996.

[8] K. Jenns, "Importance of nausea," *Cancer Nursing*, vol. 17, no. 6, pp. 488–493, 1994.

[9] C. Morran, D. C. Smith, D. A. Anderson, and C. S. McArdle, "Incidence of nausea and vomiting with cytotoxic chemotherapy: a prospective randomised trial of antiemetics," *British Medical Journal*, vol. 1, no. 6174, pp. 1323–1324, 1979.

[10] K. Jordan, C. Sippel, and H.-J. Schmoll, "Guidelines for antiemetic treatment of chemotherapy-induced nausea and vomiting: past, present, and future recommendations," *Oncologist*, vol. 12, no. 9, pp. 1143–1150, 2007.

[11] R. Mahesh, R. V. Perumal, and P. V. Pandi, "Cancer chemotherapy-induced nausea and vomiting: role of mediators, development of drugs and treatment methods," *Pharmazie*, vol. 60, no. 2, pp. 83–96, 2005.

[12] S. Goodin and R. Cunningham, "5-HT_3-receptor antagonists for the treatment of nausea and vomiting: a reappraisal of their side-effect profile," *Oncologist*, vol. 7, no. 5, pp. 424–436, 2002.

[13] U. Haus, M. Späth, and L. Färber, "Spectrum of use and tolerability of 5–HT3 receptor antagonists," *Scandinavian Journal of Rheumatology*, vol. 33, supplement 117, pp. 12–18, 2009.

[14] J. H. Kim, Y. C. Shin, and S.-G. Ko, "Integrating traditional medicine into modern inflammatory diseases care: multitargeting by rhus verniciflua stokes," *Mediators of Inflammation*, vol. 2014, Article ID 154561, 17 pages, 2014.

[15] L. Shizhen, *Compendium of Materia Medica (Bencao Gangmu)*, Foreign Languages Press, Beijing, China, 2006.

[16] J. E. Moon, J.-H. Shin, O. Kwon, and J. Y. Kim, "A standardized extract of rhus verniciflua stokes protects wistar rats against lipopolysaccharide-induced acute inflammation," *Journal of Medicinal Food*, vol. 18, no. 11, pp. 1223–1230, 2015.

[17] P.-S. Oh, S.-J. Lee, and K.-T. Lim, "Hypolipidemic and antioxidative effects of the plant glycoprotein (36 kDa) from Rhus verniciflua stokes fruit in triton WR-1339-induced hyperlipidemic mice," *Bioscience, Biotechnology and Biochemistry*, vol. 70, no. 2, pp. 447–456, 2006.

[18] K.-C. Choi, W.-T. Chung, J.-K. Kwon et al., "Chemoprevention of a flavonoid fraction from Rhus verniciflua Stokes on aflatoxin B1-induced hepatic damage in mice," *Journal of Applied Toxicology*, vol. 31, no. 2, pp. 150–156, 2011.

[19] H. R. B. Raghavendran, S. Rekha, J.-W. Shin et al., "Effects of Korean ginseng root extract on cisplatin-induced emesis in a rat-pica model," *Food and Chemical Toxicology*, vol. 49, no. 1, pp. 215–221, 2011.

[20] J. H. Kim, H.-P. Kim, C.-H. Jung et al., "Inhibition of cell cycle progression via p27Kip1 upregulation and apoptosis induction by an ethanol extract of Rhus verniciflua Stokes in AGS gastric cancer cells," *International Journal of Molecular Medicine*, vol. 18, no. 1, pp. 201–208, 2006.

[21] J.-S. Shin, Y. M. Park, J.-H. Choi et al., "Sulfuretin isolated from heartwood of Rhus verniciflua inhibits LPS-induced inducible nitric oxide synthase, cyclooxygenase-2, and pro-inflammatory cytokines expression via the down-regulation of NF-κB in RAW 264.7 murine macrophage cells," *International Immunopharmacology*, vol. 10, no. 8, pp. 943–950, 2010.

[22] D. Lebwohl and R. Canetta, "Clinical development of platinum complexes in cancer therapy: an historical perspective and an update," *European Journal of Cancer*, vol. 34, no. 10, pp. 1522–1534, 1998.

[23] G. Sassi, B. Striano, and U. A. Merlo, "A reporting system for the assessment of chemotherapy toxicity," *Journal of Oncology Pharmacy Practice*, vol. 11, no. 2, pp. 63–67, 2005.

[24] J. Zhang, B. Wider, H. Shang, X. Li, and E. Ernst, "Quality of herbal medicines: challenges and solutions," *Complementary Therapies in Medicine*, vol. 20, no. 1-2, pp. 100–106, 2012.

[25] K. Nelson, D. Walsh, and F. Sheehan, "Cancer and chemotherapy-related upper gastrointestinal symptoms: the role of abnormal gastric motor function and its evaluation in cancer patients," *Supportive Care in Cancer*, vol. 10, no. 6, pp. 455–461, 2002.

[26] N. Takeda, S. Hasegawa, M. Morita, and T. Matsunaga, "Pica in rats is analogous to emesis: an animal model in emesis research," *Pharmacology, Biochemistry and Behavior*, vol. 45, no. 4, pp. 817–821, 1993.

[27] P. L. R. Andrews and C. C. Horn, "Signals for nausea and emesis: implications for models of upper gastrointestinal diseases," *Autonomic Neuroscience: Basic & Clinical*, vol. 125, no. 1-2, pp. 100–115, 2006.

[28] S. M. Grunberg, R. R. Deuson, P. Mavros et al., "Incidence of chemotherapy-induced nausea and emesis after modern antiemetics," *Cancer*, vol. 100, no. 10, pp. 2261–2268, 2004.

[29] L. X. Cubeddu, I. S. Hoffmann, N. T. Fuenmayor, and J. J. Malave, "Changes in serotonin metabolism in cancer patients: its relationship to nausea and vomiting induced by chemotherapeutic drugs," *British Journal of Cancer*, vol. 66, no. 1, pp. 198–203, 1992.

[30] L. X. Cubeddu, "Serotonin mechanisms in chemotherapy-induced emesis in cancer patients," *Oncology*, vol. 53, supplement 1, pp. 18–25, 1996.

[31] M. D. Gershon, "Review article: serotonin receptors and transporters—roles in normal and abnormal gastrointestinal motility," *Alimentary Pharmacology & Therapeutics*, vol. 20, supplement 7, pp. 3–14, 2004.

[32] A. B. Rogers and J. G. Fox, "Inflammation and Cancer. I. Rodent models of infectious gastrointestinal and liver cancer," *American Journal of Physiology—Gastrointestinal and Liver Physiology*, vol. 286, no. 3, pp. G361–G366, 2004.

[33] S. Sartori, I. Nielsen, A. Maestri, D. Beltrami, L. Trerisani, and P. Pazzi, "Acute gastroduodenal mucosal injury after cisplatin plus etoposide chemotherapy: clinical and endoscopic study," *Oncology*, vol. 48, no. 5, pp. 356–361, 1991.

[34] L. Montoya, "Managing hematologic toxicities in the oncology patient," *Journal of Infusion Nursing*, vol. 30, no. 3, pp. 168–172, 2007.

[35] M. Okera, S. Chan, U. Dernede et al., "A prospective study of chemotherapy-induced febrile neutropenia in the South West London Cancer Network. Interpretation of study results in light of NCAG/NCEPOD findings," *British Journal of Cancer*, vol. 104, no. 3, pp. 407–412, 2011.

[36] J.-Y. Son, J.-W. Shin, J.-H. Wang et al., "Chemotherapy-induced myelotoxicity and incidence of lung metastasis in an animal model," *Human and Experimental Toxicology*, vol. 30, no. 7, pp. 649–655, 2011.

[37] Y. Kouchi, Y. Maeda, A. Ohuchida, and M. Ohsawa, "Immunotoxic effect of low dose cisplatin in mice," *Journal of Toxicological Sciences*, vol. 21, no. 4, pp. 227–233, 1996.

[38] J.-H. Lee, H.-J. Lee, H.-J. Lee et al., "*Rhus verniciflua* Stokes prevents cisplatin-induced cytotoxicity and reactive oxygen species production in MDCK-I renal cells and intact mice," *Phytomedicine*, vol. 16, no. 2-3, pp. 188–197, 2009.

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Research Article

Anticancer Effects of *Salvia miltiorrhiza* Alcohol Extract on Oral Squamous Carcinoma Cells

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Researchers have reported significant effects from Danshen (*Salvia miltiorrhiza*) in terms of inhibiting tumor cell proliferation and promoting apoptosis in breast cancer, hepatocellular carcinomas, promyelocytic leukemia, and clear cell ovary carcinomas. Here we report our data indicating that Danshen extracts, especially alcohol extract, significantly inhibited the proliferation of the human oral squamous carcinoma (OSCC) cell lines HSC-3 and OC-2. We also observed that Danshen alcohol extract activated the caspase-3 apoptosis executor by impeding members of the inhibitor of apoptosis (IAP) family, but not by regulating the Bcl-2-triggered mitochondrial pathway in OSCC cells. Our data also indicate that the extract exerted promising effects in vivo, with HSC-3 tumor xenograft growth being suppressed by 40% and 69% following treatment with Danshen alcohol extract at 50 and 100 mg/kg, respectively, for 34 days. Combined, our results indicate appreciable anticancer activity and significant potential for Danshen alcohol extract as a natural antioxidant and herbal human oral cancer chemopreventive drug.

1. Introduction

Oral cancer, the sixth most common cancer worldwide [1], is ranked third in South Central Asia [2]. Over 90% of all identified oral cavity cancers are invasive oral squamous cell carcinomas (OSCCs) [3–5] whose relative five-year survival rates range from 50% to 60% [3, 6]. Primary treatments are surgery, radiation therapy, and chemotherapy, all of which have severe side effects and poor response rates [7]. Herbal medicine is one of the most frequently used alternative therapies, several of which have been used alongside conventional treatment regimens [8, 9]. The use of medicinal herbs or their extracts is currently attracting attention as a promising chemopreventive strategy. One agent receiving particularly strong interest is Danshen (*Salvia miltiorrhiza*), one of the most widely and longest-used herbal medicines for numerous maladies throughout Asia. Previous studies indicate that Danshen has anti-inflammatory [10–12], antioxidant [12, 13], and blood circulation-improvement characteristics [10, 14] and some researchers have suggested that it has therapeutic advantages for several cancer types, including breast, prostate, and lung cancer [15–18]. However, few efforts

have been made to test the effectiveness of Danshen extracts to treat oral cancer.

For the present study, we looked at the antioxidant activity of different Danshen extracts and their in vivo effects on cell proliferation and tumorigenesis in two human OSCC cell lines, HSC-3 and OC-2. Our results indicate that 95% crude Danshen alcohol extract exhibited the highest level of free radical scavenging and antitumor activity of all the extracts we examined. Further, we observed that Danshen alcohol extract induced apoptosis by regulating apoptosis protein family inhibition, with the exception of the Bcl-2-driven apoptosis pathway in OSCC cells. We therefore suggest that Danshen alcohol extract has significant potential for the treatment of oral squamous cell carcinomas.

2. Materials and Methods

2.1. Plant Extract Preparation. *Salvia miltiorrhiza* were purchased from Chuang Song-Zong Pharmaceutical Co., Ltd., in Kaohsiung City, Taiwan. Pieces of *Salvia miltiorrhiza* were isolated, washed, cut into small sections, soaked in solutions consisting of either double-distilled water (ddH_2O), 95% ethanol, or 1 : 1 water/ethanol, and extracted by heating under reflux. Extracts were concentrated and strained through 0.45 μm filters. All extraction processes were repeated twice. Extracts were powdered by freeze-drying and stored at −20°C.

2.2. 2,2-Diphenyl-1-picrylhydrazyl (DPPH) Assay. The antioxidant capacities of various Danshen extracts were determined using the DPPH radical scavenging method described in [19]. Briefly, 100 μL concentrations of the various extracts in ethanol were added to 750 μL of a 0.0025% ethanol DPPH solution. After 30 min of incubation at room temperature, absorbance was read against a blank at 517 nm. DPPH is a purple-colored stable free radical that changes to a yellowish diphenylpicrylhydrazine when reduced. The water-soluble vitamin E analogue 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) was used as a positive control. Inhibition ratios were calculated using the formula [(Ac − As)/Ac] × 100%, with Ac denoting control absorbance and As test sample absorbance. Extract concentrations providing 50% inhibition (IC_{50}) were calculated using graphs plotting inhibition percentage against extract concentration [20].

2.3. 2,2′-Azinobis(3-ethylbenzothiazoline-6-sulphonic Acid) (ABTS) Assays. The free radical scavenging capabilities of the essential oils were determined using ABTS radical cation decolorization assays as described in [20]. ABTS radical cations were produced by reacting ABTS solution with 2.45 mM potassium persulfate, and allowing the mixture to stand in darkness at room temperature for 12–16 h. Incubation mixtures (5 mL total volumes) contained 0.54 mL of ABTS radical cations, 0.5 mL of phosphate buffer, and varying concentrations of individual extract. Appropriate solvent blanks were run with each assay. Absorbance was read by a spectrophotometer at 734 nm and compared with the Trolox control [20].

2.4. Cell Viability Analyses. The effects of Danshen extracts on cell viability were assessed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assays (six replications). Cells (3×10^3) were seeded in 10% FBS-supplemented growth medium in 96-well plates for 24 h and exposed to indicated concentrations (2, 5, 10, 25, or 50 μg/mL) of Danshen extract in the same medium for 24, 48, or 72 h. Control cells were treated with DMSO or a ddH_2O vehicle at the same concentrations. Following treatment, medium was removed and replaced with 200 μL of 0.5 mg/mL MTT, after which cells were incubated in a CO_2 incubator at 37°C for 2 h. After removing supernatant from each well, reduced MTT dye was solubilized in DMSO (200 μL/well). Absorbance was determined at 595 nm using a plate reader.

2.5. Caspase-3 Activity Assays. Caspase-3 activity was determined using an FITC Active Caspase-3 Apoptosis Kit (BD Biosciences) according to manufacturer instructions. Cells were treated with DMSO or indicated concentrations of Danshen extract (10, 25, or 50 μg/mL). After 48 h, cells were collected and incubated with Cytofix/Cytoperm solution at 4°C for 20 minutes; solution was removed by centrifugation at 3000 rpm for 5 min. Cells were then incubated with FITC-conjugated monoclonal rabbit anti-active human-caspase-3 antibody for 30 minutes at room temperature. Cells were washed twice with PBS, and 500 μL of Perm/Wash buffer was added prior to flow cytometry.

2.6. Mitochondrial Membrane Potential. Mitochondrial membrane potential was quantified by flow cytometry using a MitoProbe JC-1 Assay Kit (Life Technologies). Cells were treated with Danshen extract (10, 25, or 50 μg/mL), DMSO (control), or 50 μM carbonyl cyanide 3-chlorophenylhydrazone (CCCP) as a positive control. After 48 h, cells were collected and incubated with 200 μM JC-1 dye for 30 minutes at 37°C. Cells were centrifuged at 1300 rpm for 5 minutes to remove supernatant and resuspended in 1 mL PBS prior to flow cytometry.

2.7. Protein Extraction and Western Blot Analyses. Apoptosis biomarkers were assessed by Western blotting. Treated cells were washed in PBS, resuspended in sodium dodecyl sulfate (SDS) sample buffer, sonicated for 5 sec, and boiled for 5 min. After brief centrifugation, equal amounts of total protein from each sample were fractionated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene difluoride membrane that was washed three times with Tris-buffered saline (TBS) containing 0.05% Tween 20 (TBST). After blocking with TBST containing 5% nonfat milk for 60 min, the membrane was incubated overnight with an appropriate primary antibody at 1 : 1000 dilution in TBST-5% low-fat milk at 4°C and then washed three more times with TBST. The membrane was probed with goat anti-rabbit or anti-mouse IgG-horseradish peroxidase conjugate (1 : 10000) for 1 h at room temperature and washed three more times with TBST. Hybridized immunocomplexes were detected with Renaissance Chemiluminescence Reagent Plus (NEN Life Science Products, Boston).

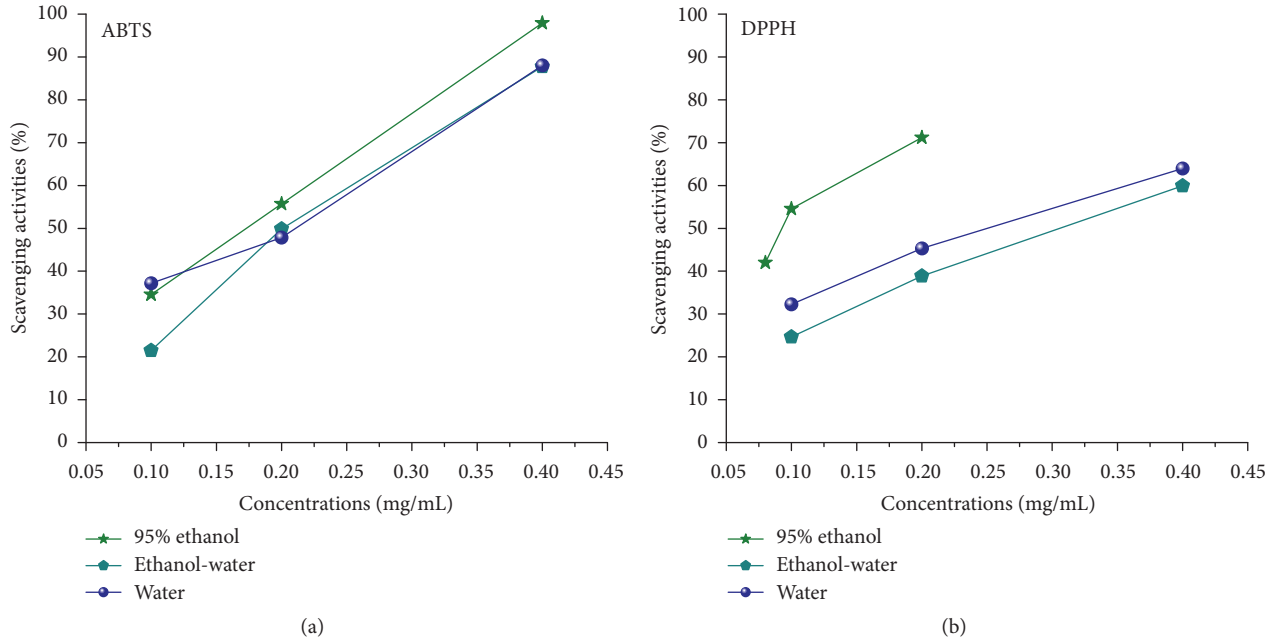

Figure 1: Radical scavenging activity for three Danshen extract types. Effect data for different concentrations of three Danshen extracts from free radical scavenging tests. (a) Assay data for scavenging of free radical ABTS with SC_{50} values of 0.197, 0.232, and 0.223 μg/mL. (b) Data for DPPH with SC_{50} values of 0.094, 0.311, and 0.26 μg/mL.

2.8. Animal Experiments. Male BALB/c NU mice (6–8 weeks old) (purchased from BioLASCO Co., Ltd., Taiwan) were subcutaneously injected with HSC-3 cells (2×10^6). As tumors became established, mice were randomly assigned to one of three groups and treated daily with either Danshen extract or DMSO (50 or 100 mg/kg) by intraperitoneal injection. Mouse weights and tumor volumes (length × width^2 × 0.5) were measured daily. Mice were sacrificed when tumor volumes reached 2,000 mm^3. Portions of each tumor were snap-frozen in liquid nitrogen and stored at −80°C until needed for Western blot analysis of relevant biomarkers. All experimental procedures were performed in accordance with protocols approved by the Institutional Laboratory Animal Care and Use Committee of Kaohsiung Medical University.

2.9. Statistical Analyses. All results are presented as mean ± SEM. Statistical analyses of control and treatment data were executed in the form of Student's t-tests, with significance defined as $p < 0.05$ in all cases.

3. Results and Discussion

3.1. Determination of Antioxidant Activity of Danshen Extracts. In mammalian systems, reactive oxygen species are produced naturally due to oxidative metabolism. In addition to contributing to a variety of physiological and biochemical lesions, free radicals can induce degenerative illnesses such as coronary artery disease and cancer [21, 22]. For this study we analyzed three types of Danshen extracts in terms of their antioxidant and radical scavenging capabilities. ABTS and DPPH assay data are presented in Figure 1. As shown, the scavenging ability of Danshen alcohol extract had significantly higher values (0.197 for ABTS and 0.094 for DPPH) compared to water/alcohol (0.232 for ABTS and 0.311 for DPPH) and water-only extracts (0.223 for ABTS and 0.26 for DPPH). In comparison, the SC50 values of Trolox (a positive control) were only 0.048 and 0.022 for ABTS and DPPH scavenging, respectively. The data clearly indicate greater antioxidant potency for Danshen alcohol extract.

3.2. Cytotoxicity of Danshen Alcohol Extract in OSCC Cells. Three different Danshen extraction methods were assessed in vitro using MTT assays to determine their antiproliferative capabilities against HSC-3 cells (Figures 2(a)–2(c)). Cells were treated with a Danshen extract (water, 95% alcohol, or a 1 : 1 mixture) at various concentrations. At 24 h after treatment, alcohol and alcohol/water extracts exhibited significantly stronger antiproliferative effects among the three types (IC_{50} values of 39.8 and 47.1 μg/mL, resp.). Next, we attempted to verify Danshen alcohol extract cytotoxicity against HSC-3 and OC-2 cells. Our results indicate appreciable dose- and time-dependent inhibitory effects in both cell lines (Figures 2(d)–2(g)). IC_{50} values of 26.67 and 30.68 μg/mL were observed 48 h after treatment for HSC-3 and OC-2 cells, respectively. We noted that these IC_{50} values exceeded that for 50 μg/mL of normal oral keratinocytes (Figure S1; see Supplementary Material available online at https://doi.org/10.1155/2017/5364010).

3.3. Apoptosis Induction in Danshen Alcohol Extract-Treated HSC-3 Cells. Cell apoptosis is triggered via intrinsic and extrinsic pathways. Intrinsic pathways are initiated by the loss of mitochondrial membrane potential ($\Delta\psi$), leading to

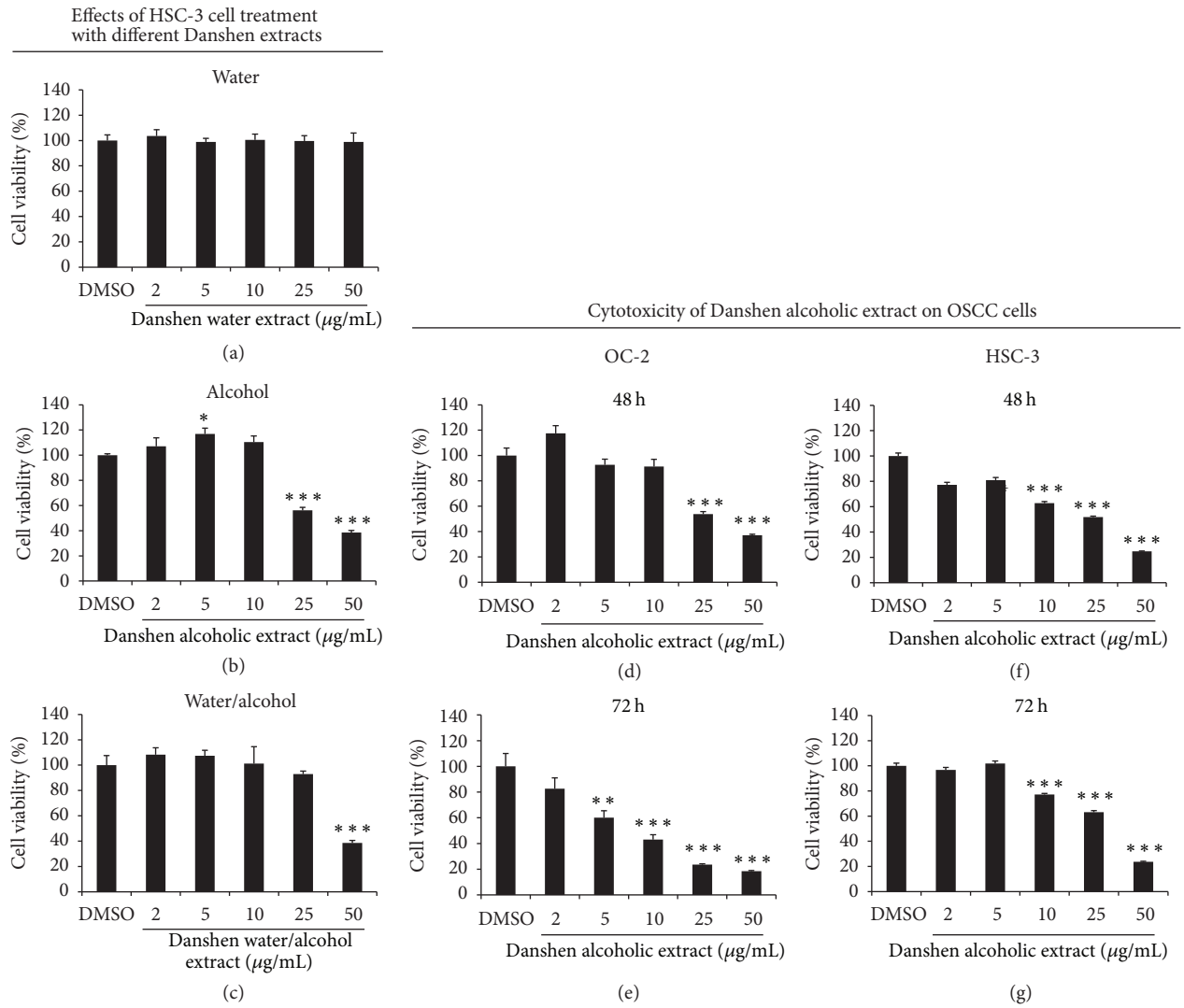

Figure 2: Cytotoxicity of different Danshen extracts on OSCC cells. (a–c) Cytotoxic effects of different Danshen extracts on HSC-3 cells at 24 h after treatment. (d, e) OC-2 and (f, g) HSC-3 cells were treated with 0, 2, 5, 10, 25, or 50 μg/mL of Danshen alcohol extract for 48 or 72 h. MTT assay data are shown as mean ± SEM ($n = 6$). $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

the release of cytochrome c from mitochondrial intermembranes and resulting in the formation of caspase activation platforms (apoptosomes) that trigger apoptotic protease cascades [23, 24]. For further characterization, we measured active caspase-3 (a marker for cells undergoing apoptosis) and found that it gradually increased 48 h following treatment with Danshen alcohol extract at 10, 25, or 50 μg/mL (Figure 3(a)). To identify the upstream trigger of caspase-3 activation, we used cytometric analysis with JC-1 staining to evaluate apoptosis-related mitochondrial changes associated with Danshen alcohol extract and surprisingly found no change in detected $\Delta\psi$ (Figure 3(b)). This finding is consistent with our data for other apoptotic markers; levels of antiapoptotic proteins Bcl-2 and Bcl-xL and the proapoptotic proteins Bax and Bad [25, 26] remained relatively unchanged following HSC-3 cell treatment with Danshen alcohol extract (Figure 4). At the same time, we observed dramatic decreases in the expression of both XIAP and survivin, two members of the inhibitor of apoptosis protein (IAP) family. Combined, the data suggest that IAP family members, but not intrinsic apoptosis regulators, triggered the Danshen alcohol extract-induced apoptosis that we observed.

3.4. In Vivo Antitumor Growth Efficacy of Danshen Alcohol Extract. To further clarify the clinical implications of Danshen alcohol extract, we examined its antitumor effects in vivo. Male BALB/c NU mice (6–8 weeks old) were given subcutaneous injections of oral squamous carcinoma HSC-3 cells, followed by daily intraperitoneal injections of Danshen alcohol extract at 50 and 100 mg/kg body weight for 34 days; control mice were treated with DMSO. Mouse body weights were recorded and tumor volumes monitored daily. As shown in Figure 5(a), no significant impacts on mouse body weights were observed over the 34-day injection period, which indicated that no overt signs of toxicity were noted in any of the treated mice. However, the mice treated with

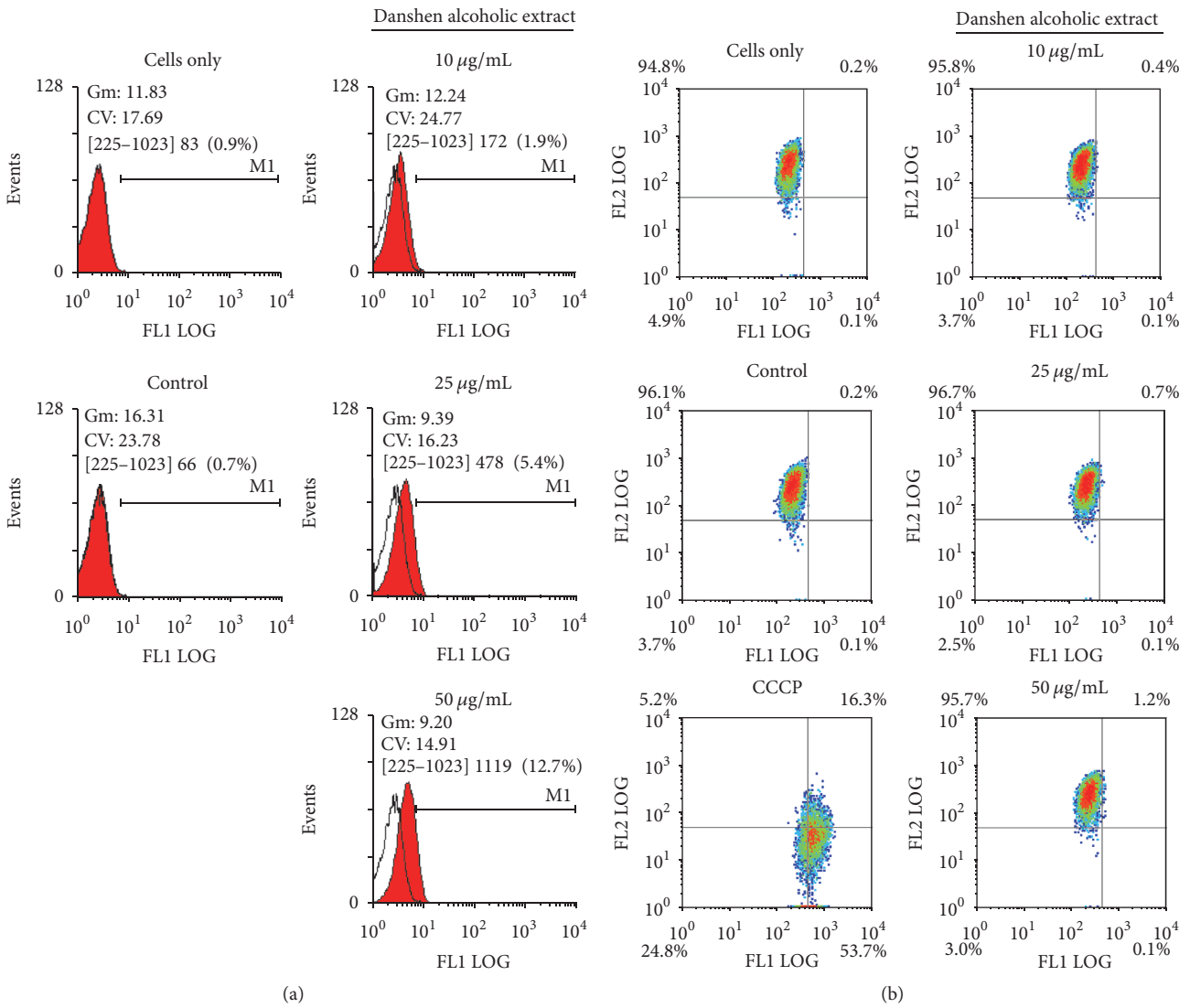

FIGURE 3: Danshen alcohol extract induces caspase-3 apoptotic pathway in HSC-3 cells. (a) Cells were treated with indicated concentrations of Danshen alcohol extract. Apoptosis was estimated using ITC Active Caspase-3 Apoptosis Assays. DMSO and camptothecin (Camp) served as a control and positive control, respectively, for caspase-3 activity. (b) Cells were incubated with different concentrations of Danshen alcohol extract. Mitochondrial membrane potential ($\Delta\Psi m$) was measured by flow cytometry with JC-1 staining. DMSO and carbonyl cyanide 3-chlorophenylhydrazone (CCCP) served as a control and positive control, respectively.

the Danshen alcohol extract had significantly smaller tumor volumes ($1056.06 \pm 66.64\,mm^2$ and $552.02 \pm 133.40\,mm^2$ for mice treated with 50 mg/kg and 100 mg/kg, resp.; $p < 0.01$) (Figure 5(b)). Compared to control group mice ($1761.11 \pm 302.86\,mm^2$), average tumor growth in mice treated with Danshen alcohol extract was reduced by 39.9% for the 50 mg/kg dosage group and 68.7% for the 100 mg/kg dosage group. To examine biological markers in vivo, we randomly selected tumor tissue taken from 2 mice in each group to examine protein expression and found that, similar to the in vitro data, treatment with Danshen alcohol extract resulted in the downregulation of XIAP and survivin but not Bcl-2 family members (Figure 5(c)).

3.5. Potential Mechanisms and Active Ingredients. Many studies of Danshen's antitumor potential have produced significant findings. Active components of Danshen include danshensu, tanshinones, and salvianolic acids, all of which have been shown to exert antioxidant, antimicrobial, anti-inflammatory, anticancer, and cardiovascular-protective effects [27–29]. These clinical effects are generally attributed to two major Danshen components: tanshinone IIA (Tan-IIA) and salvianolic acid B (Sal-B) [29–31]. According to one report, Tan-IIA is capable of inducing cell apoptosis and inhibiting cell proliferation in hepatocellular carcinomas [32], promyelocytic leukemia [33–35], erythroleukemia [34, 35], and ER-positive breast cancer cells [36]. Tan-IIA has also

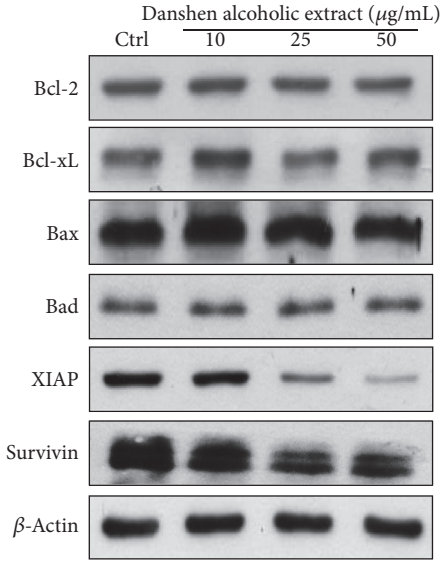

Figure 4: HSC-3 cells were analyzed by Western blotting 48 h following treatment with Danshen alcohol extract at concentrations of 10, 25, or 50 μg/mL.

been shown to prevent cells from oxidant damage [37–39] and lipid peroxidation [40]. In oral cavities, the hyperexpression of cycloxygenase-2 (COX-2) increases the risk of developing head and neck cancers, but these risks are reduced by Sal-B [41]. Sal-B anticancer mechanisms involve the attenuation of OSCC cell growth by blocking COX-2 pathways, inhibiting angiogenesis, and inducing apoptosis [28]. Results from previous studies suggest that the anticancer effects of Danshen may be due to apoptosis modulation and angiogenesis regulation, in addition to its anti-inflammatory and antioxidative properties [28]. However, the mechanisms underlying its therapeutic potential remain unclear. This served as our motivation to analyze various Danshen extracts in terms of their antiproliferative activity and antioxidant effects and to specifically analyze Danshen alcohol extract in terms of its anticancer potential against human OSCC cells. Our data reveal high levels of radical scavenging and chemopreventive activity in vitro and in vivo.

Acknowledging that different types of Danshen might contain different component ratios that affect antiproliferative and antioxidant efficacy and due to the fact that weather, soil conditions, and other factors can affect the herb's therapeutic value, we also investigated the active ingredients and antioxidant effects of Danshen extracts from different sources and extraction methods. Specifically, we examined Taiwan- and China-grown Danshen samples extracted by water, 1 : 1 water/ethanol, and 95% ethanol. All extracts were found to be rich in Tan-IIA and Sal-B and to exhibit linear correlations between the two components and antioxidant capacity, especially Taiwan-grown samples extracted with alcohol (Tables S1–S3). The higher Tan-IIA and Sal-B content was the primary reason for using Taiwan-grown Danshen in this study. It is plausible to suggest that those major components significantly influenced the antioxidant activity of the Danshen extracts used in this research, thereby directly or indirectly affecting their antitumor capacities. Our findings are consistent with those from previous studies demonstrating the anticarcinogenic capacities of Tan-IIA and Sal-B via the prevention of oxidative damage [37].

Multiple studies have shown that caspase activation and the loss of mitochondrial membrane potential initiate apoptosis. However, some researchers have reported that mitochondria are not always involved as apoptotic stimuli [42, 43]. In the present study we also observed an apoptotic effect with no change in mitochondrial membrane potential. Caspase-3 activity is known to be inhibited by a group of proteins collectively labeled "inhibitors of apoptosis proteins." We also looked for XIAP and survivin expression due to previous reports showing that they directly bind and inhibit caspase-3 [44, 45]. Survivin, a bifunctional protein that regulates cell division and suppresses apoptosis, is commonly expressed in normal tissue and overexpressed in several types of human cancers [20]. Survivin prevents cell apoptosis via the overexpression of procaspase-3 and the suppression of active caspase-3, thereby increasing tumor cell proliferation and decreasing chemotherapy response [44]. The complete removal of XIAP and survivin that we observed serves as evidence that Danshen alcohol extract induced cell death by mitigating apoptosis inhibition.

4. Conclusion

Danshen extracts, especially alcohol extract, exhibit appreciable antioxidant and antitumor activity. Our data indicate that Danshen alcohol extract induced OSCC cell apoptosis, inhibited XIAP, and activated caspase-3. We also found evidence that Danshen alcohol extract may suppress HSC-3 tumor growth in vivo without notable side effects such as body weight loss. Combined, our results indicate appreciable anticancer activity from Danshen alcohol extract and potential for it to be applied as a natural antioxidant and herbal human oral cancer chemopreventive drug.

Competing Interests

The authors declare no competing financial interests.

Authors' Contributions

All authors contributed to this study. Zong-Shiow Chen and Wan-Chi Tsai conceived and designed the experiments. Ling-Ya Chu, Chia-Ying Lee, and Yu-Chang Tyan performed the experiments and/or analyzed the data. Wen-Hung Wang, Kuo-Yu Hsuan, and Wan-Chi Tsai prepared the manuscript. All authors approved the final version of this paper. Wen-Hung Wang and Kuo-Yu Hsuan contributed equally to this work.

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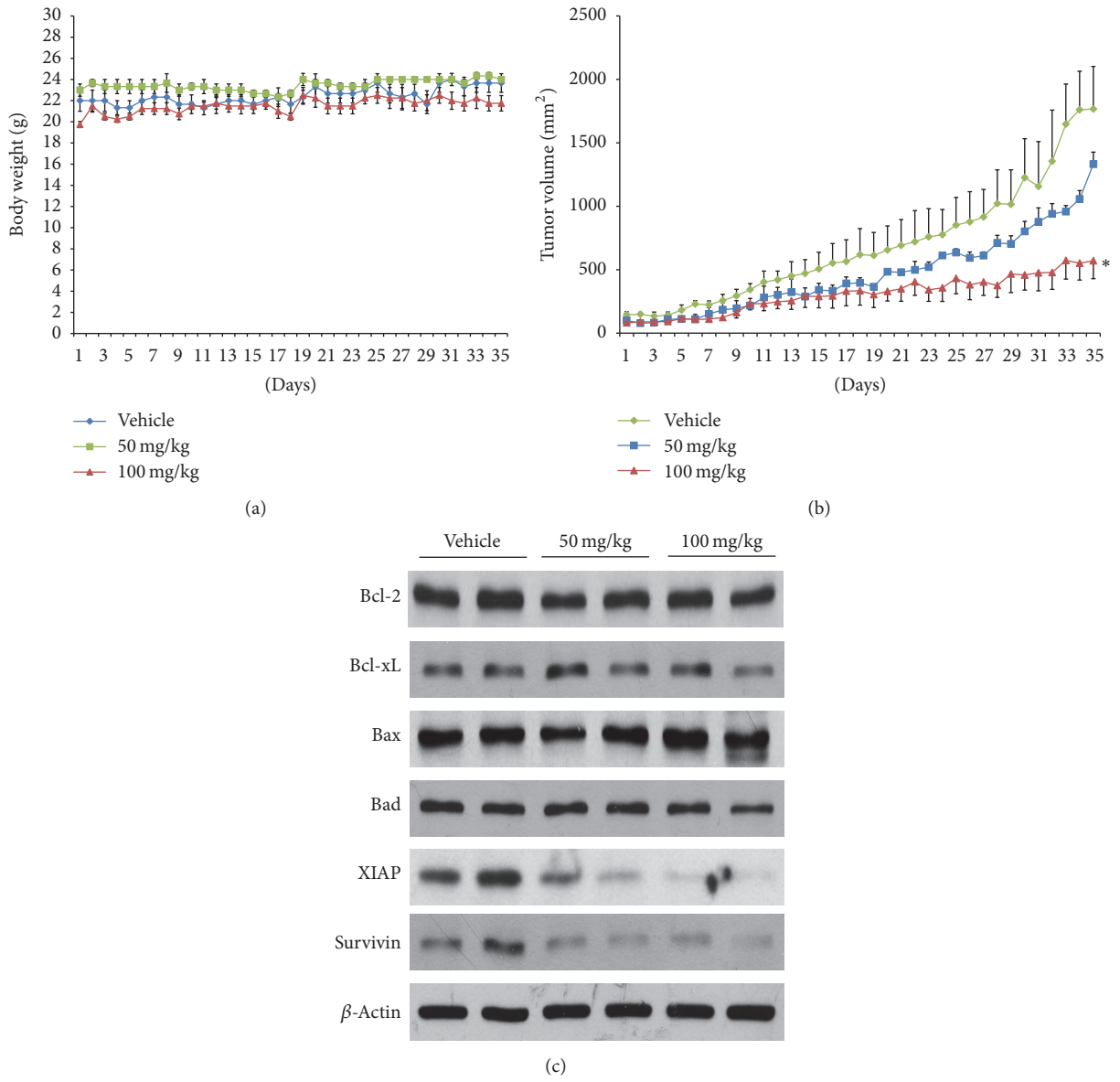

FIGURE 5: Effect of treatment with Danshen alcohol extract on tumor growth in BALB/c NU mice. (a) Body weights of mice from control, 50 mg/kg, and 100 mg/kg treatment groups. (b) Tumor volume data. Results are presented as mean ± SEM ($n = 3$). $^*P < 0.05$ compared to vehicle control at the endpoint of the experiment. (c) Western blot analysis data for protein expression of Bcl-2, Bcl-xL, Bax, Bad, XIAP, and survivin, using tumor protein extracts from 2 mice in each group. β-actin was served as loading control.

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References

[1] X. Liu, A. Kolokythas, J. Wang, H. Huang, and X. Zhou, "Gene expression signatures of lymph node metastasis in oral cancer: molecular characteristics and clinical significances," *Current Cancer Therapy Reviews*, vol. 6, no. 4, pp. 294–307, 2010.

[2] Z. Khan, J. Tönnies, and S. Müller, "Smokeless tobacco and oral cancer in South Asia: a systematic review with meta-analysis," *Journal of Cancer Epidemiology*, vol. 2014, Article ID 394696, 11 pages, 2014.

[3] P. Specenier and J. B. Vermorken, "Advances in the systemic treatment of head and neck cancers," *Current Opinion in Oncology*, vol. 22, no. 3, pp. 200–205, 2010.

[4] M. Click and M. W. Johnson, "Oral and oropharyngeal cancer: what are the next steps?" *The Journal of the American Dental Association*, vol. 142, no. 8, pp. 892–894, 2011.

[5] N. W. Johnson, S. Warnakulasuriya, P. C. Gupta et al., "Global oral health inequalities in incidence and outcomes for oral cancer: causes and solutions," *Advances in Dental Research*, vol. 23, no. 2, pp. 237–246, 2011.

[6] A. O. Lawal, B. Kolude, B. F. Adeyemi, J. O. Lawoyin, and E. E. Akang, "Serum antioxidant vitamins and the risk of oral cancer in patients seen at a tertiary institution in Nigeria," *Nigerian Journal of Clinical Practice*, vol. 15, no. 1, pp. 30–33, 2012.

[7] S. D. da Silva, M. Hier, A. Mlynarek, L. P. Kowalski, and M. A. Alaoui-Jamali, "Recurrent oral cancer: current and emerging therapeutic approaches," *Frontiers in Pharmacology*, vol. 3, article 149, 2012.

[8] J. O. Rivera, A. M. Loya, and R. Ceballos, "Use of herbal medicines and implications for conventional drug therapy medical sciences," *Alternative & Integrative Medicine*, vol. 2, no. 6, 2013.

[9] Y. Li, X. Zhu, A. Bensussan et al., "Herbal medicine for hot flushes induced by endocrine therapy in women with breast cancer: a systematic review and meta-analysis," *Evidence-Based Complementary and Alternative Medicine*, vol. 2016, Article ID 1327251, 11 pages, 2016.

[10] J.-Y. Han, J.-Y. Fan, Y. Horie et al., "Ameliorating effects of compounds derived from *Salvia miltiorrhiza* root extract on microcirculatory disturbance and target organ injury by ischemia and reperfusion," *Pharmacology & Therapeutics*, vol. 117, no. 2, pp. 280–295, 2008.

[11] J. D. Adams, R. Wang, J. Yang, and E. J. Lien, "Preclinical and clinical examinations of *Salvia miltiorrhiza* and its tanshinones in ischemic conditions," *Chinese Medicine*, vol. 1, article 3, 2006.

[12] B. Liu, Y. Du, L. Cong, X. Jia, and G. Yang, "Danshen (Salvia miltiorrhiza) compounds improve the biochemical indices of the patients with coronary heart disease," *Evidence-Based Complementary and Alternative Medicine*, vol. 2016, Article ID 9781715, 9 pages, 2016.

[13] G.-R. Zhao, Z.-J. Xiang, T.-X. Ye, Y.-J. Yuan, and Z.-X. Guo, "Antioxidant activities of *Salvia miltiorrhiza* and *Panax notoginseng*," *Food Chemistry*, vol. 99, no. 4, pp. 767–774, 2006.

[14] T. O. Cheng, "Danshen: a popular chinese cardiac herbal drug," *Journal of the American College of Cardiology*, vol. 47, no. 7, pp. 1498–1500, 2006.

[15] W. Yang, J.-H. Ju, M. J. Jeon, X. Han, and I. Shin, "Danshen (*Salvia miltiorrhiza*) extract inhibits proliferation of breast cancer cells via modulation of akt activity and p27 level," *Phytotherapy Research*, vol. 24, no. 2, pp. 198–204, 2010.

[16] Y. Gong, Y. Li, Y. Lu et al., "Bioactive tanshinones in Salvia miltiorrhiza inhibit the growth of prostate cancer cells in vitro and in mice," *International Journal of Cancer*, vol. 129, no. 5, pp. 1042–1052, 2011.

[17] Y. T. Tung, H. L. Chen, C. Y. Lee et al., "Active component of danshen (*Salvia miltiorrhiza* bunge), tanshinone I, attenuates lung tumorigenesis via inhibitions of VEGF, cyclin A, and cyclin B expressions," *Evidence-Based Complementary and Alternative Medicine*, vol. 2013, Article ID 319247, 10 pages, 2013.

[18] B. Q. Wang, "Salvia miltiorrhiza chemical and pharmacological review of a medicinal plant," *Journal of Medicinal Plants Research*, vol. 4, no. 25, pp. 2813–2820, 2010.

[19] W. Brand-Williams, M. E. Cuvelier, and C. Berset, "Use of a free radical method to evaluate antioxidant activity," *LWT—Food Science and Technology*, vol. 28, no. 1, pp. 25–30, 1995.

[20] W.-H. Wang, Y.-C. Tyan, Z.-S. Chen et al., "Evaluation of the antioxidant activity and antiproliferative effect of the jaboticaba (Myrciaria cauliflora) seed extracts in oral carcinoma cells," *BioMed Research International*, vol. 2014, Article ID 185946, 7 pages, 2014.

[21] L. A. Pham-Huy, H. He, and C. Pham-Huy, "Free radicals, antioxidants in disease and health," *International Journal of Biomedical Science*, vol. 4, no. 2, pp. 89–96, 2008.

[22] K. Rahman, "Studies on free radicals, antioxidants, and co-factors," *Clinical Interventions in Aging*, vol. 2, no. 2, pp. 219–236, 2007.

[23] V. Gogvadze, S. Orrenius, and B. Zhivotovsky, "Multiple pathways of cytochrome c release from mitochondria in apoptosis," *Biochimica et Biophysica Acta—Bioenergetics*, vol. 1757, no. 5-6, pp. 639–647, 2006.

[24] N. B. van der Walt, Z. Zakeri, and M. J. Cronjé, "The induction of apoptosis in A375 malignant melanoma cells by *Sutherlandia frutescens*," *Evidence-Based Complementary and Alternative Medicine*, vol. 2016, Article ID 4921067, 14 pages, 2016.

[25] I. Boumela, Y. Guillemin, J.-F. Guérin, and A. Aouacheria, "The Bcl-2 family pathway in gametes and preimplantation embryos," *Gynécologie Obstétrique & Fertilité*, vol. 37, no. 9, pp. 720–732, 2009.

[26] E. N. Gurzov and D. L. Eizirik, "Bcl-2 proteins in diabetes: mitochondrial pathways of β-cell death and dysfunction," *Trends in Cell Biology*, vol. 21, no. 7, pp. 424–431, 2011.

[27] Y.-S. Chen, S.-M. Lee, Y.-J. Lin, S.-H. Chiang, and C.-C. Lin, "Effects of danshensu and salvianolic acid B from salvia miltiorrhiza bunge (Lamiaceae) on cell proliferation and collagen and melanin production," *Molecules*, vol. 19, no. 2, pp. 2029–2041, 2014.

[28] Y. Zhao, Y. Guo, and X. Gu, "Salvianolic acid B, a potential chemopreventive agent, for head and neck squamous cell cancer," *Journal of Oncology*, vol. 2011, Article ID 534548, 8 pages, 2011.

[29] T.-H. Lin and C.-L. Hsieh, "Pharmacological effects of Salvia miltiorrhiza (Danshen) on cerebral infarction," *Chinese Medicine*, vol. 5, article no. 22, 2010.

[30] Y. X. Guo, L. L. Zhou, T. Li, and L. H. Wang, "Preparative separation of lithospermic acid B from Salvia miltiorrhiza by polyamide resin and preparative high-performance liquid chromatography," *Journal of Chromatography A*, vol. 1218, no. 29, pp. 4606–4611, 2011.

[31] Q. Xu, Y. Xu, C. Yang et al., "Sodium tanshinone IIA sulfonate attenuates scopolamine-induced cognitive dysfunctions via improving cholinergic system," *BioMed Research International*, vol. 2016, Article ID 9852536, 9 pages, 2016.

[32] M. A. Mosaddik, "In vitro cytotoxicity of Tanshinones isolated from *Salvia miltiorrhiza* Bunge against P388 lymphocytic leukemia cells," *Phytomedicine*, vol. 10, no. 8, pp. 682–685, 2003.

[33] S.-L. Yuan, Y.-Q. Wei, X.-J. Wang, F. Xiao, S.-F. Li, and J. Zhang, "Growth inhibition and apoptosis induction of tanshinone II-A on human hepatocellular carcinoma cells," *World Journal of Gastroenterology*, vol. 10, no. 14, pp. 2024–2028, 2004.

[34] Z.-Z. Tang, Y. Tang, and L.-B. Fu, "Effect of tanshinone IIA on the growth behavior of human hepatoma cell line BEL-7402 in vitro and its mechanism," *Academic Journal of the First Medical College of PLA*, vol. 23, no. 6, pp. 595–601, 2003.

[35] Z. Tang, Y. Tang, and L. Fu, "Growth inhibition and apoptosis induction in human hepatoma cells by tanshinone II A," *Journal of Huazhong University of Science and Technology—Medical Science*, vol. 23, no. 2, pp. 166–168, 2003.

[36] X. Wang, Y. Wei, S. Yuan et al., "Potential anticancer activity of tanshinone IIA against human breast cancer," *International Journal of Cancer*, vol. 116, no. 5, pp. 799–807, 2005.

[37] J.-R. Liu, G.-F. Chen, H.-N. Shih, and P.-C. Kuo, "Enhanced antioxidant bioactivity of Salvia miltiorrhiza (Danshen) products prepared using nanotechnology," *Phytomedicine*, vol. 15, no. 1-2, pp. 23–30, 2008.

[38] L. Yang, X.-J. Zou, X. Gao et al., "Sodium tanshinone IIA sulfonate attenuates angiotensin II-induced collagen type I expression in cardiac fibroblasts in vitro," *Experimental & Molecular Medicine*, vol. 41, no. 7, pp. 508–516, 2009.

[39] R. Yang, A. Liu, X. Ma, L. Li, D. Su, and J. Liu, "Sodium tanshinone IIA sulfonate protects cardiomyocytes against oxidative stress-mediated apoptosis through inhibiting JNK activation," *Journal of Cardiovascular Pharmacology*, vol. 51, no. 4, pp. 396–401, 2008.

[40] Q. Shang, H. Xu, and L. Huang, "Tanshinone IIA: a promising natural cardioprotective agent," *Evidence-Based Complementary and Alternative Medicine*, vol. 2012, Article ID 716459, 7 pages, 2012.

[41] Y.-L. Chen, C.-S. Hu, F.-Y. Lin et al., "Salvianolic acid B attenuates cyclooxygenase-2 expression in vitro in LPS-treated human aortic smooth muscle cells and in vivo in the apolipoprotein-E-deficient mouse aorta," *Journal of Cellular Biochemistry*, vol. 98, no. 3, pp. 618–631, 2006.

[42] P. Chen, Y.-F. Hu, L. Wang et al., "Mitochondrial apoptotic pathway is activated by H_2O_2-mediated oxidative stress in BmN-SWU1 cells from bombyx mori ovary," *PLoS ONE*, vol. 10, no. 7, Article ID e0134694, 2015.

[43] L.-F. Wu, G.-P. Li, J.-L. Feng, and Z.-J. Pu, "Molecular mechanisms of adenosine-induced apoptosis in human HepG2 cells," *Acta Pharmacologica Sinica*, vol. 27, no. 4, pp. 477–484, 2006.

[44] S. J. Riedl, M. Renatus, R. Schwarzenbacher et al., "Structural basis for the inhibition of caspase-3 by XIAP," *Cell*, vol. 104, no. 5, pp. 791–800, 2001.

[45] S. Shin, B.-J. Sung, Y.-S. Cho et al., "An anti-apoptotic protein human survivin is a direct inhibitor of caspase-3 and -7," *Biochemistry*, vol. 40, no. 4, pp. 1117–1123, 2001.

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Research Article

Phylogenetic Tree Analysis of the Cold-Hot Nature of Traditional Chinese Marine Medicine for Possible Anticancer Activity

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Traditional Chinese Marine Medicine (TCMM) represents one of the medicinal resources for research and development of novel anticancer drugs. In this study, to investigate the presence of anticancer activity (AA) displayed by cold or hot nature of TCMM, we analyzed the association relationship and the distribution regularity of TCMMs with different nature (613 TCMMs originated from 1,091 species of marine organisms) via association rules mining and phylogenetic tree analysis. The screened association rules were collected from three taxonomy groups: (1) Bacteria superkingdom, Phaeophyceae class, Fucales order, Sargassaceae family, and *Sargassum* genus; (2) Viridiplantae kingdom, Streptophyta phylum, Malpighiales class, and Rhizophoraceae family; (3) Holothuroidea class, Aspidochirotida order, and *Holothuria* genus. Our analyses showed that TCMMs with closer taxonomic relationship were more likely to possess anticancer bioactivity. We found that the cluster pattern of marine organisms with reported AA tended to cluster with cold nature TCMMs. Moreover, TCMMs with salty-cold nature demonstrated properties for softening hard mass and removing stasis to treat cancers, and species within Metazoa or Viridiplantae kingdom of cold nature were more likely to contain AA properties. We propose that TCMMs from these marine groups may enable focused bioprospecting for discovery of novel anticancer drugs derived from marine bioresources.

1. Introduction

The nature of traditional Chinese medicines (TCMs) can be classified into three categories (cold, hot, and neutral) that represent the types of body reactions after the administration of specific TCM [1, 2]. The therapeutic effect of CMs depends mainly on the nature of the drugs as well as the processes they regulate to recover the balance between Yin and Yang in the human body [3, 4]. According to TCM, the rationale for the correct remedy selection is based upon a corresponding TCM syndrome (Zheng, 证, or pattern) [5]. A patient will present with a syndrome upon disruption of Yin-Yang balance, which may be caused either by external and/or internal pathological factors. This can be regarded as clinical phenotype, such as cold or hot syndrome [5, 6]. The standard therapeutic guideline used to treat cold or hot syndrome is to "cure cold syndrome by medication with hot nature" and to "cure hot syndrome by medication with cold nature" [5]. This therapeutic practice has been validated and developed over thousands of years, and most CMs have thus been labeled with different nature types as an outcome of this repeated clinical practice [1]. Recent literature shows that different biological effects conferred by a specific TCM could serve as the basis to discriminate cold and hot nature of TCMs

[7, 8]. Thus, conversely, the cold-hot nature of a specific TCM could potentially serve as clues to its bioactivities including anticancer properties.

As a vital part of TCMs, Traditional Chinese Marine Medicine (TCMM) has been used to treat and prevent diseases for thousands of years, and it is based on a unique theoretical framework, diagnosis, and treatment [9]. TCMM appears to be effective in treating various diseases such as cancers, malaria, diabetes, cardiocerebrovascular diseases, immunodeficiency diseases, and senile dementia, and therefore has become an important medicinal resource for the research and development of new drugs [10].

Cancer poses serious threat to human health worldwide, and there have been efforts in screening for compounds possessing anticancer activity (AA) from TCMMs [17]. Marine organisms including TCMMs have evolved efficient and highly potent metabolites that exhibit strong biological activity at low concentrations to circumvent rapid dilution caused by their aqueous environment [18], and this confers a potential advantage over metabolites of terrestrial origin including TCMs originating from nonmarine sources. Moreover, TCMMs contain significant differences from TCMs of terrestrial origin including their bioactivity properties, cold and hot nature [10, 19, 20]. Marine organisms have been demonstrated to be promising source of novel antitumor compounds [18, 21] and several of the marine families of TCMMs have been explored and reportedly show anticancer potential [10, 22].

High-throughput screening for novel anticancer drugs are widely conducted; however it is costly and might yield chemical hits with low actual clinical efficacy and/or high toxicity [23]. It has been reported that distinct plant species yields potent bioactive compounds at higher rates than other plant species, and most drugs are derived from preexisting drug-productive families [24]. Clues to drug-productive species can be obtained from the species-distribution profiles of phylogenetic tree [19]. Almost 80% of the approved drugs and 67% of the clinical-trial drugs concentrated in 17 and 30 drug-prolific families, respectively, including Fabid and Malvid groups of the Rosidae subclass, the Lamiid and Campanulid groups of the Asterid subclass, and the Ranunculales order [24]. Eribulin mesylate is a structurally simplified synthetic analogue of halichondrin B used for the treatment of metastatic breast cancer, which is a natural product isolated from the marine sponge *Halichondria okadai*, which originates from a drug-productive family Halichondriidae [25–27]. Taken together, these instances provide the basis to screen for natural resources possessing AA activities through phylogenetic tree analysis.

Thus, in this study, in order to examine the phylogenetic tree and cold-hot nature of TCMM for identifying TCMMs with potential AA properties, association rules mining and phylogenetic tree construction methodologies were used to investigate the association relationship and distribution regularity of TCMMs with different nature possessing AA properties.

2. Materials and Methods

2.1. Datasets Preparation. The cold-hot nature categorization of 613 TCMMs related to 1,091 marine bioresources species were retrieved from the "Chinese Marine Materia Medica" [22]. Latin name and taxonomy data of the related bioresources were retrieved from the National Center for Biotechnology Information (NCBI) Taxonomy Database (https://www.ncbi.nlm.nih.gov/taxonomy) [28]. The 1,091 marine species were clustered into three groups: cold, hot, and neutral.

The anticancer bioactivity information of each marine bioresources species were retrieved from PubMed literature database by using the following retrieval formula.

"Latin name of each marine bioresource species" [All Fields] AND (anticancer [All Fields] OR antitumer [All Fields] OR antitumor [All Fields]).

Each species was labeled with presence or absence of anticancer bioactivity according to the retrieved results. All the results were independently checked by two researchers, F. X. and S. X.

2.2. Phylogenetic Tree Construction. The phylogenetic trees were generated by using the NCBI taxonomy-based automatic tree generator against known families in the Bacteria, Viridiplantae, and Metazoa kingdoms or superkingdoms [24, 29].

First, TAX ID of each marine bioresources species were retrieved from The NCBI Taxonomy System (https://www.ncbi.nlm.nih.gov/taxonomy) [28]. Then data of TAX ID were input to phylot web (http://phylot.biobyte.de/index.html) to construct phylogenetic tree, and the visualization of the tree was conducted in iTOL (version 3.3.2) and EvolView [19, 24, 30, 31]. Family or species names were labeled at branch ends. Reported anticancer bioactivity clusters and different nature were labeled or marked in the phylogenetic trees.

2.3. Association Rules Mining. The association relationship between reported anticancer bioactivity and taxonomy or nature types was mined by aRules package [32] based on the R platform to elucidate the association rules.

An association rule is an implication of the form $X \Rightarrow Y$, where $X \subset I$, $Y \subset I$, and $X \cap Y = \varnothing$. The rule $X \Rightarrow Y$ holds in the database D with confidence and support [33]. The support is a measure of the frequency of a rule, and the confidence is a measure of the strength of the relation between sets of items [34]. In this study, the cold-hot nature and taxonomy data of TCMMs were taken as X, while the AA of each TCMM was regarded to Y; the association rules whose confidence and support were larger than the set thresholds (50.00% for confidence and 0.5% for support) were chosen as strong association rules.

3. Results and Discussion

3.1. Cluster Pattern of Marine Organism from TCMMs with Reported AA. In this study, 613 TCMMs originated from 1,091 species of marine organisms were screened for potential AA properties (Table 1). Majority of the species ($n = 870$ of 1,091; 79.74%) were from Metazoa kingdom. Among the 1,091 species investigated, 194 species were reported to have AA with nearly half of them ($n = 92$; 47.42%) from the

Table 1: The distribution of marine species with AA in different kingdom or superkingdom.

Kingdom or Superkingdom[#]	Number of species (%)[a]	Number of species with AA (%)[b]
Metazoa	870 (79.74%)	92 (10.57%)
Viridiplantae	89 (8.16%)	45 (50.56%)
Others in Eukaryota[#]	123 (11.27%)	51 (41.46%)
Bacteria[#]	9 (0.82%)	6 (66.67%)
Total	1,091 (100%)	194 (17.78%)

[a]Percentage derived from division with the total number of species within all kingdom and superkingdom combined (n = 1,091).
[b]Percentage derived from division with the number of species within each corresponding kingdom or superkingdom.
refers to superkingdom.

Metazoa kingdom. More than half of the interrogated species within the Viridiplantae (green plants) kingdom (n = 45 of 89; 50.56%) and Bacteria superkingdom (n = 6 of 9; 66.67%) demonstrated AAs. This implies that the marine species of TCMMs from Viridiplantae kingdom and Bacteria superkingdom are more likely to possess AA.

Metazoa kingdom showed 61 AA families concentrated in 38 clusters (Figure 1; Table S1 in Supplementary Material available online at https://doi.org/10.1155/2017/4365715). These families were present in seven of eight phylums known to possess AAs (Table S2). Four phylums contained more than ten AA species including 24 in Mollusca, 23 in Echinodermata, 22 in Chordata, and 10 in Cnidaria. There were 18 AA classes from 25 known classes of Metazoa (Table S3), and three classes (Holothuroidea, Actinopteri, and Bivalvia) contained more than ten AA species. Three orders (Aspidochirotida, Veneroida, and Alcyonacea) contained more than five AA species (Table S4).

This implies that TCMMs from Metazoa are potential candidates for anticancer drug discovery. Diverse peptides with a wide range of biological activities including antimicrobial and antitumoral have been isolated from different phyla of Mollusca, Cnidaria, and Echinodermata [35]. Two novel marine anticancer compounds, kahalalide F and ES285, have been isolated from the Indopacific mollusc *Elysia rufescens* and the North Atlantic mollusc *Spisula polynyma*, respectively [36]. The phylum Cnidaria is unique such that practically all of its members are toxic and contain Cnidarian toxins which are a rich source of polypeptides with a wide variety of biological activities including pore-forming cytolysins, phospholipases, neurotoxins, and protease inhibitors [35]. These marine organisms could be an important source of structurally bioactive secondary metabolites. There have been 12 reported novel and highly potent antitumor natural products derived from seven species of cnidarians of marine origin [37].

Figure 2 presents the distribution of marine families with AA in phylogenetic tree of Viridiplantae kingdom, Eukaryota superkingdom, and Bacteria superkingdom. A total of 52 AA families were concentrated in 18 clusters (Figure 2; Table S5). These families were distributed in five AA phylums (Table S6) in which Streptophyta phylums of Viridiplantae contained 35 AA species. There were two classes from the Eukaryota superkingdom containing more than 20 AA species (Table S7), the Florideophyceae (24 AA species) and Phaeophyceae (23 AA species). One order (Fucales) contained more than ten AA species (Table S8).

Compared with Figure 1, Figure 2 showed more concentrated anticancer family clusters in Bacteria and Eukaryota superkingdom than Metazoa kingdom. Bacteria have widely contributed to some of the most useful chemotherapeutic drugs [38], while marine cyanobacteria contain antiproliferative properties, yielding several potent inhibitors of malignancies [39]. All of the six AA species of TCMMs from Bacteria superkingdom are of Cyanobacteria phylum.

Viridiplantae (green plants) are an ancient group of eukaryotes comprising of two main clades: the Chlorophyta and the Streptophyta. The former consists of a wide diversity of green algae while the latter consists of freshwater green algae and terrestrial plants [40]. There are four phyla of algae including red algae (Florideophyceae), brown algae (Phaeophyceae), green algae (Chlorophyta), and diatom (Bacillariophyceae) and two phyla of plants from coastal wetlands including Pteridophyta and Angiospermae [10]. Marine plants serve as main sources of potential anticancer agents [38].

3.2. Cold-Hot Nature Distribution of Marine Organism from TCMMs with Reported AA. Within the 1,091 marine organisms, 380 can be grouped into TCMMs with cold nature, 233 with hot nature, and 366 with neutral nature (Table 2). More than half of AA species were from the cold group (n = 51.03%), followed by the neutral (25.26%) and hot group (12.37%).

It was reported that basic pharmacological effects of herbals with cold nature are antibacterial, anti-inflammatory, antitumor, antipyretic, diuretic, lowering blood pressure, sedation, and analgesic [41]. Most frequently used TCMMs are generally of cold nature [20]. Studies have shown that salty flavor and cold nature (such as *Sargassum* and *Laminariae Thallus*) are representative of TCMMs [10, 20]. In terms of medicinal effects, the most representative efficacies of TCMMs with salty-cold flavor and nature (e.g., *Sargassum*, *Laminariae Thallus*, *Ostreae Concha*, and *Meretricis Concha*) include softening hard mass and removing stasis to treat cancers [10]. This might serve as an explanation, at least partially, as to why AA species are often from the cold group.

3.3. The Association Rules and Phylogenetic Tree of Marine Organisms. The association rules mining resulted in 12

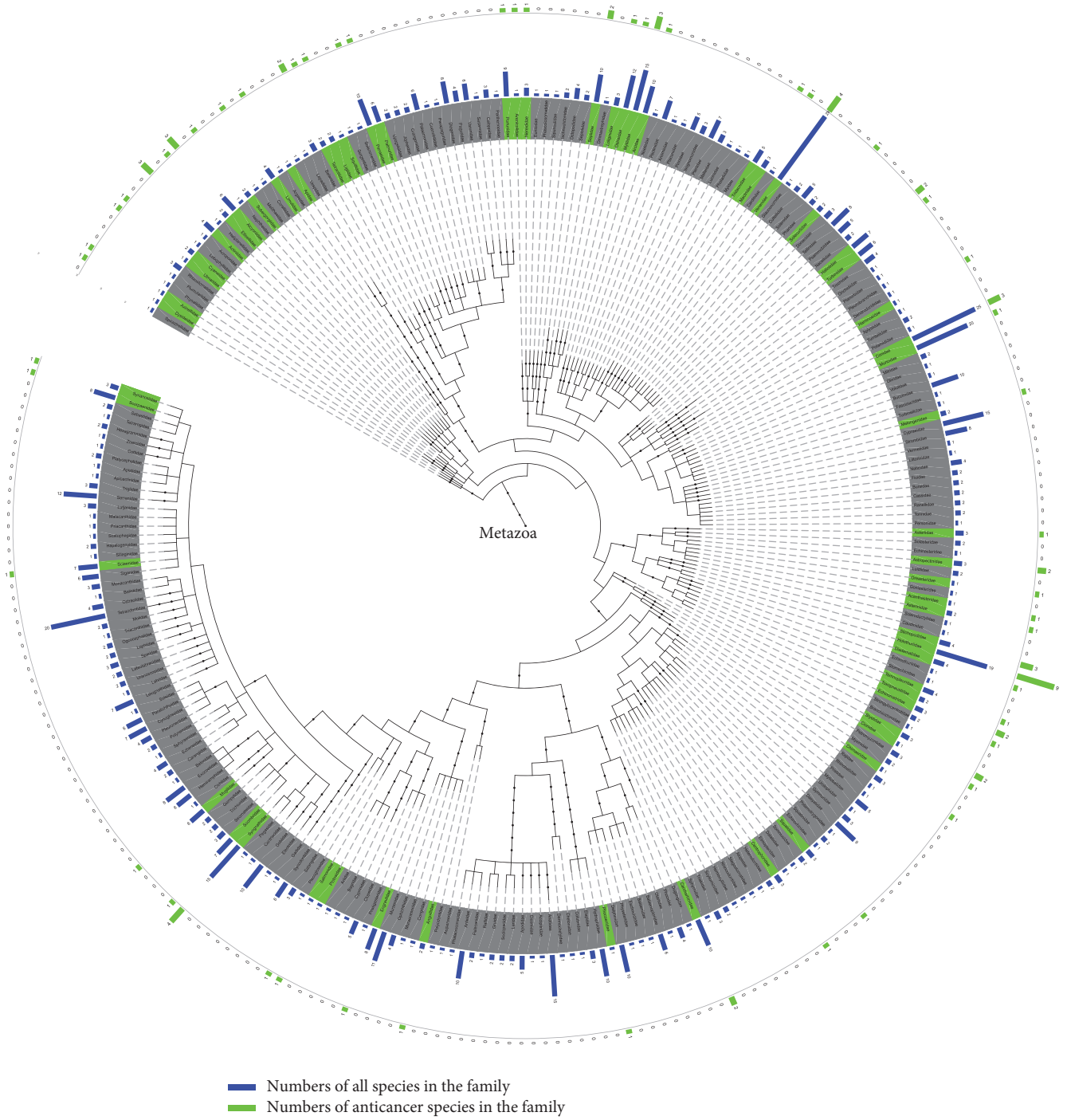


Figure 1: The distribution of marine anticancer activity families (green background color) phylogenetic tree of Metazoa kingdom. The family names are provided at branch ends, which can be viewed more clearly by enlarging the figure in the electronic version. The length of the blue and green bar outside the circle represents the number of all species and the AA species, respectively, in the family.

screened rules (Table 3). There were 11 rules with single item and one with double items. In the single item rules, one was of superkingdom (Bacteria with confidence of 66.67%) and one was associated with Streptophyta phylum, while two were related to Holothuroidea and Phaeophyceae classes. The Malpighiales order and Rhizophoraceae family showed strong association with AA with confidences of 87.50% and 85.71%, respectively. The double items of cold and Viridiplantae kingdom also showed strong association with AA, implying that the species of TCMMs with cold nature from Viridiplantae kingdom tend to have AA.

Figure 3 shows the cluster pattern of marine AA families, in the phylogenetic tree of marine organisms, tended to cluster with cold nature TCMMs. In contrast, few of

Figure 2: The distribution of marine families with AA in phylogenetic tree of Viridiplantae kingdom, Eukaryota superkingdom, and Bacteria superkingdom. Coloring and labeling schemes are as described in Figure 1.

the AAs-containing families clustered with hot and neutral nature TCMMs. The screened association rules labeled at the corresponding branch collected at three species groups (from superkingdom to genus) in the phylogenetic tree. The cluster pattern (Figure 3) contained three major groups: (1) the first group consisting of Bacteria superkingdom, Phaeophyceae class, Fucales order, Sargassaceae family, and *Sargassum* genus; (2) the second group consisting of Viridiplantae kingdom, Streptophyta phylum, Malpighiales class, and Rhizophoraceae family; (3) the third group comprising three levels of Holothuroidea class, Aspidochirotida order, and *Holothuria* genus at the same branch.

The results above suggest that certain species of TCMMs with closer taxonomic relationship are more likely to have

Table 2: The distribution of marine organisms with AA in different nature categories.

Nature	Number of species (%)[a]	Number of species with AA (%)[b]
Cold	380 (34.83)	99 (26.05)
Hot	233 (21.36)	24 (10.30)
Neutral	366 (33.55)	49 (13.39)
None	112 (10.27)	22 (19.64)
Total	1,091 (100.00)	194 (17.78)

[a]Percentage derived from division with the total number of species within all different natures combined (n = 1,091).
[b]Percentage derived from division with the number of species within each corresponding nature.

Table 3: The results of association rules mining (support > 0.5%, confidence > 50%, lift > 2).

Rule ID	Single or double items	Taxonomic rank	Rules	Support	Confidence	Lift
1	Single item	Super kingdom	{*Bacteria*} ⇒ {anticancer}	0.55%	66.67%	3.75
2	Single item	Phylum	{*Streptophyta*} ⇒ {anticancer}	3.21%	52.24%	2.94
3	Single item	Class	{*Phaeophyceae*} ⇒ {anticancer}	2.20%	63.16%	3.55
4			{*Holothuroidea*} ⇒ {anticancer}	1.28%	51.85%	2.92
5	Single item	Order	{*Malpighiales*} ⇒ {anticancer}	0.64%	87.50%	4.92
6			{*Fucales*} ⇒ {anticancer}	1.19%	65.00%	3.66
7			{*Aspidochirotida*} ⇒ {anticancer}	1.10%	52.17%	2.93
8	Single item	Family	{*Rhizophoraceae*} ⇒ {anticancer}	0.55%	85.71%	4.82
9			{*Sargassaceae*} ⇒ {anticancer}	1.19%	68.42%	3.85
10	Single item	Genus	{*Sargassum*} ⇒ {anticancer}	1.01%	68.75%	3.87
11			{*Holothuria*} ⇒ {anticancer}	0.73%	61.54%	3.46
12	Double items	Nature & kingdom	{cold, *Viridiplantae*} ⇒ {anticancer}	2.57%	56.00%	3.15

AA. The AA of marine organisms is mostly based on the secondary metabolites of each species [42, 43]. The distribution of secondary metabolites has some value for taxonomy [44]. Chemical structure of secondary metabolites forms the molecular basis for its bioactivity [45], and marine natural products are important sources of chemical scaffolds [46]. Natural products from marine species with closer taxonomic relationship contain similar scaffolds and bioactivities [47]. For instance, the marine organisms *Sargassum fusiforme*, *Sargassum hemiphyllum*, *Sargassum pallidum*, *Sargassum carpophyllum*, *Sargassum horneri*, and *Sargassum thunbergii* are from the Sargassaceae family that form the TCMM seaweed (known as "haizao" in Chinese or 海藻) with cold nature. All of the *Sargassum* seaweed possessed phytosterols compounds with the same scaffold (compounds 1–5 in Table 4) or similar structure (compounds 6 in Table 4) containing similar anticancer bioactivity.

In addition to the grouped species of TCMMs with closer taxonomic relationship, TCMM species with cold nature from Viridiplantae kingdom also showed a tendency for AA with confidence of 56%. As discussed above, marine plants contain potential anticancer agents and cold nature TCMMs render softening of hard mass and stasis removal. Hence, the species (e.g., *Ulva pertusa* [48]) with combination of Viridiplantae kingdom and cold nature are more likely to demonstrate AA. For example, *Ulva pertusa* from the Ulvaceae family of Viridiplantae kingdom was used as classic TCMM with cold nature to treat thyroid neoplasm from Tang dynasty and recorded in the herbal book of "Bencao Shiyi (Supplement to Materia Medica, 本草拾遗)" [22]. It is reported that *Ulva pertusa* showed antitumor activity against Meth-A fibrosarcoma by intraperitoneal administration of 50 mg/kg daily for seven days [49].

Nonetheless, we acknowledge limitations of the study as follows: (1) the studies included in the phylogenetic tree analysis took into account results from in vitro investigations. However, in vitro studies have remained the prerequisite before a candidate compound is tested further in in vivo or human trials settings, and excluding results from in vitro studies could significantly reduce the sensitivity of our analysis; (2) this study represented TCMMs originated from species of marine organisms for potential AA properties available currently and several more are being actively discovered.

Our previous study showed that the TCMM from the organisms in the same family may have the same nature, while marine plants such as Chlorophyta, Florideophyceae, and Phaeophyceae were associated with cold nature, and marine animals including Decapoda, Malacostraca, and Arthropoda contained close relationship with hot nature [19]. The different nature types seem to affect different biological processes based on the pluralistic character of molecular structure [50]. For distribution of secondary metabolites from marine species with closer taxonomic relationship, they contain similar scaffolds and bioactivities [47]. Marine algae associated

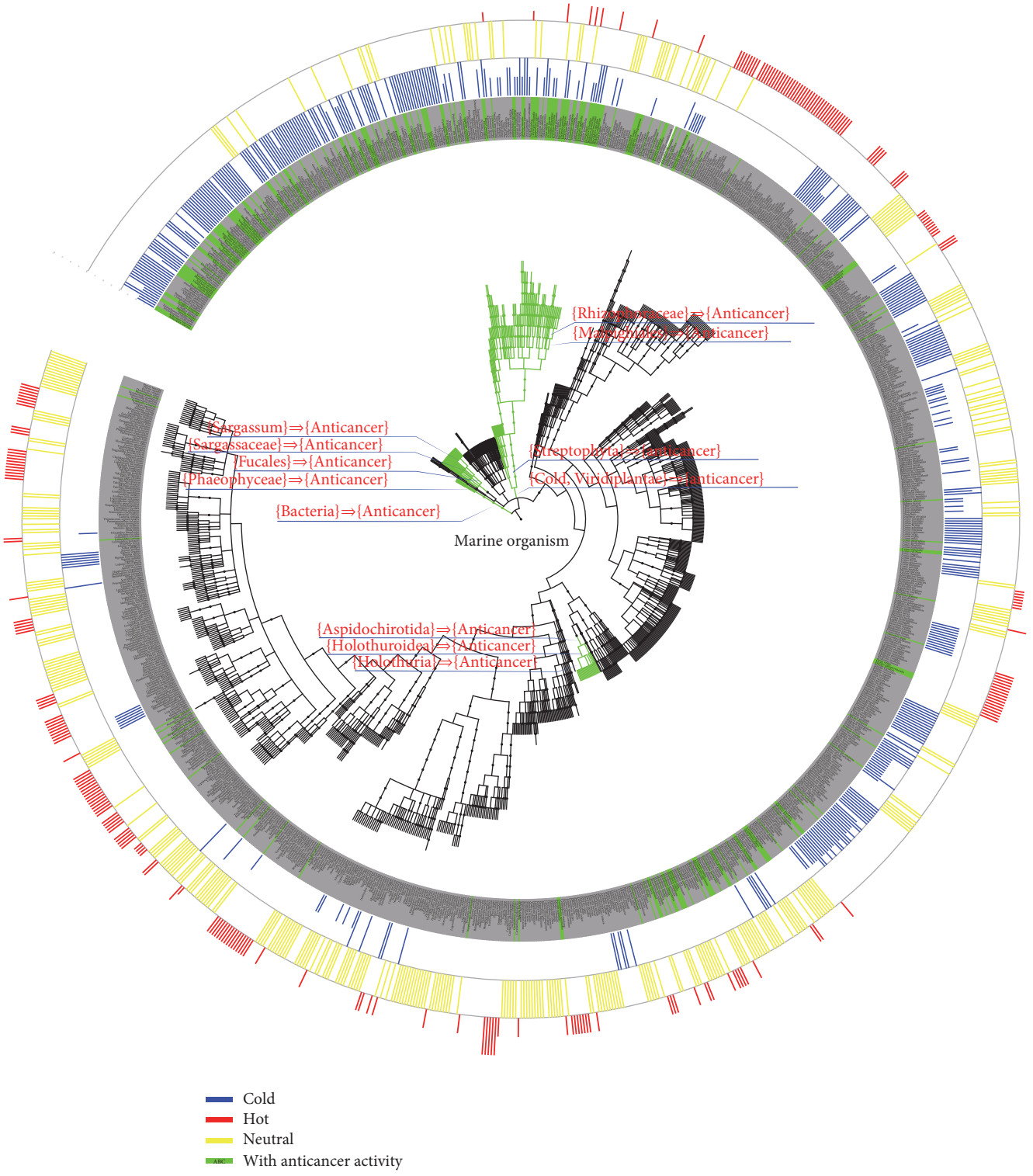
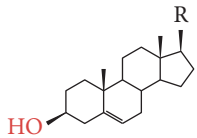
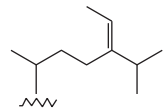
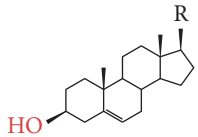
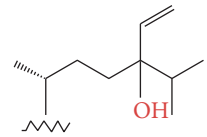
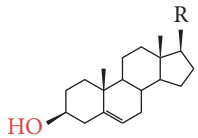
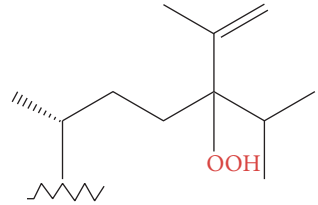
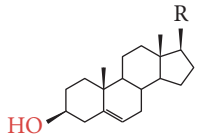
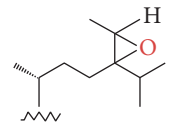
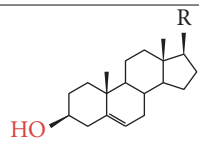
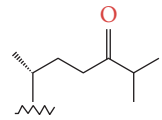
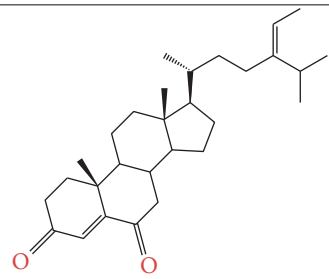


Figure 3: The distribution of marine AA species (green background) in the phylogenetic tree of marine organisms. The cold-hot nature of each species was labeled outside of the circle by different colors. The length of the bar represents the degree of nature of TCMMs. The screened association rules were labeled at the corresponding branch (the branches marked with different color). The names of species of the marine organisms of TCMMs are provided at branch ends, which can be viewed more clearly by enlarging the figure in the electronic version.

Table 4: Anticancer compounds from species of Sargassaceae family of the TCMM Seaweed (Haizao, 海藻).

ID	Name	Structure		Species	AA
		Scaffold	R		
1	Fucosterol [11–16]			(1) *Sargassum fusiforme* (2) *Sargassum hemiphyllum* (3) *Sargassum pallidum* (4) *Sargassum carpophyllum* (5) *Sargassum horneri* (6) *Sargassum thunbergii*	(1) Induce the deformation activity of rice blast fungus (2) Inhibit the growth of P-388, T47D, and HT29 cell lines.
2	Sargasterol [13, 14]			(1) *Sargassum fusiforme* (2) *Sargassum hemiphyllum* (3) *Sargassum pallidum* (4) *Sargassum carpophyllum*	Induce the deformation activity of rice blast fungus
3	24- Hydrogen peroxide based -24- vinyl cholesterol [13]			(1) *Sargassum fusiforme* (2) *Sargassum hemiphyllum* (3) *Sargassum carpophyllum*	(1) Induce the deformation activity of rice blast fungus (2) Inhibit the growth of HL-60 cell line.
4	24S, 28S- epoxy -24- ethyl cholesterol [13]			(1) *Sargassum fusiforme* (2) *Sargassum carpophyllum*	(1) Induce the deformation activity of rice blast fungus (2) Inhibit the growth of MCF-7, HCT-8, 1A9, HOS, and PC-3 cell lines.
5	Cholesterol -24- ketone [13]			*Sargassum carpophyllum*	Induce the deformation activity of rice blast fungus
6	24-Ethylcholest-4-en-3,6-dione [13]			*Sargassum carpophyllum*	(1) Induce the deformation activity of rice blast fungus (2) Inhibit the growth of P-388 cell line.

with cold nature such as Chlorophyta, Florideophyceae, and Phaeophyceae contain antitumor properties [51].

4. Conclusions

Our analysis demonstrated that potential AA derived from Metazoa or Viridiplantae species with cold nature tended to have close taxonomic relationship than distantly distributed in phylogenetic tree. The clustered patterns with mined association rules presented in this work provide information pertaining to the groups of species with anticancer properties. Moreover, we have shown that phylogenetic tree analysis can be utilized to shortlist plant or animal species that possess potential AA. Future bioprospecting studies on TCMMs are thus warranted with aims of producing novel anticancer drugs.

Competing Interests

The authors declare no competing financial interest.

Authors' Contributions

Xianjun Fu conceived the main theme on which the work is performed and ensured that the scientific aspect of the study is rationally valid and dedicated a substantial contribution to the conception and design of the project and analysis and interpretation of the data and wrote this manuscript. Xuxia Song and Jiaoyang Li contributed in data preparation. Kah Keng Wong, Xuebo Li, Fengcong Zhang, and Changyun Wang helped in the analysis and interpretation of data. Zhenguo Wang ensured that the scientific aspect of the study was rationally valid. All authors have read and approved the final manuscript.

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References

[1] X.-J. Fu, Z.-G. Wang, Y. Qu, P. Wang, Y. Zhou, and H.-Y. Yu, "Study on the networks of 'nature-family-component' of Chinese medicinal herbs based on association rules mining," *Chinese Journal of Integrative Medicine*, vol. 19, no. 9, pp. 663–667, 2013.

[2] F. Liang, L. Li, M. Wang et al., "Molecular network and chemical fragment-based characteristics of medicinal herbs with cold and hot properties from Chinese medicine," *Journal of Ethnopharmacology*, vol. 148, no. 3, pp. 770–779, 2013.

[3] C. Zhou, J. Wang, X. Zhang et al., "Investigation of the differences between the 'COLD' and 'HOT' nature of Coptis chinensis Franch and its processed materials based on animal's temperature tropism," *Science in China Series C: Life Sciences*, vol. 52, no. 11, pp. 1073–1080, 2009.

[4] P. P. Li, "Toward an integrative framework of indigenous research: the geocentric implications of Yin-Yang Balance," *Asia Pacific Journal of Management*, vol. 29, no. 4, pp. 849–872, 2012.

[5] T. Ma, C. Tan, H. Zhang, M. Wang, W. Ding, and S. Li, "Bridging the gap between traditional Chinese medicine and systems biology: the connection of cold syndrome and NEI network," *Molecular BioSystems*, vol. 6, no. 4, pp. 613–619, 2010.

[6] F. Mohd Fauzi, A. Koutsoukas, R. Lowe et al., "Chemogenomics approaches to rationalizing the mode-of-action of traditional chinese and ayurvedic medicines," *Journal of Chemical Information and Modeling*, vol. 53, no. 3, pp. 661–673, 2013.

[7] L.-P. Huang, M. F. Zhu, R. Y. Yu, J. Q. Du, and H. N. Liu, "Study on discrimination mode of cold and hot properties of traditional chinese medicines based on biological effects," *Zhongguo Zhongyao Zazhi*, vol. 39, no. 17, pp. 3353–3358, 2014.

[8] H. Zhao, Y. Zhao, J. Wang et al., "Expression of the difference between the Cold (Han) and Hot (Re) natures of traditional Chinese medicines (Strobal and Rhubarb) based on the cold/hot plate differentiating assay," *Science in China, Series C: Life Sciences*, vol. 52, no. 12, pp. 1192–1197, 2009.

[9] X. Fu, H. Guan, W. Qiangming et al., "Elementary exploration of the orion and development of marine Chlnelle materla medica," *Chinese Journal of Medical History*, vol. 39, no. 3, pp. 168–172, 2009.

[10] X. Fu, M. Zhang, C. Shao et al., "Chinese marine materia medica resources: status and potential," *Marine Drugs*, vol. 14, no. 3, p. 46, 2016.

[11] M. Terasaki, A. Hirose, B. Narayan et al., "Evaluation of recoverable functional lipid components of several brown seaweeds (phaeophyta) from japan with special reference to fucoxanthin and fucosterol contents," *Journal of Phycology*, vol. 45, no. 4, pp. 974–980, 2009.

[12] S.-E. N. Ayyad, S. T. Ezmirly, S. A. Basaif, W. M. Alarif, A. F. Badria, and F. A. Badria, "Antioxidant, cytotoxic, antitumor, and protective DNA damage metabolites from the red sea brown alga *Sargassum* sp," *Pharmacognosy Research*, vol. 3, no. 3, pp. 160–165, 2011.

[13] H.-F. Tang, Y.-H. Yi, X.-S. Yao, Q.-Z. Xu, S.-Y. Zhang, and H.-W. Lin, "Bioactive steroids from the brown alga Sargassum carpophyllum," *Journal of Asian Natural Products Research*, vol. 4, no. 2, pp. 95–101, 2002.

[14] Y. M. Ham, K. N. Kim, W. J. Lee, N. H. Lee, and C. G. Hyun, "Chemical constituents from *Sargassum micracanthum* and antioxidant activity," *International Journal of Pharmacology*, vol. 6, no. 2, pp. 147–151, 2010.

[15] M. Khanavi, R. Gheidarloo, N. Sadati et al., "Cytotoxicity of fucosterol containing fraction of marine algae against breast and colon carcinoma cell line," *Pharmacognosy Magazine*, vol. 8, no. 29, pp. 60–64, 2012.

[16] M. Kobayashi, A. Hasegawa, and H. Mitsuhashi, "Marine sterols. XV. Isolation of 24-vinyloxycholesta-5,23-dien-3β-ol from the brown alga Sargassum thumbergii," *Chemical and Pharmaceutical Bulletin*, vol. 33, no. 9, pp. 4012–4013, 1985.

[17] H. Yang, J. Li, Y. Zheng et al., "Drug activity screening based on microsomes-hydrogel system in predicting metabolism induced antitumor effect of oroxylin A," *Scientific Reports*, vol. 6, article no. 21604, 2016.

[18] M. Sugumaran and W. E. Robinson, "Bioactive dehydrotyrosyl and dehydrodopyl compounds of marine origin," *Marine Drugs*, vol. 8, no. 12, pp. 2906–2935, 2010.

[19] X. Fu, Z. Wang, C. Wang et al., "The distribution and association relationships of marine Chinese medicine with different nature in the phylogenetic tree of marine organisms," *Modernization of Traditional Chinese Medicine and Materia Medica. World Science and Technology*, vol. 17, no. 11, pp. 2189–2196, 2015 (Chinese).

[20] X. Fu, C. Pan, S. Lin et al., "Study on nature distribution of marine Chinese medicine based on literature mining," *China Journal of Traditional Chinese Medicine and Pharmacy*, vol. 31, no. 1, pp. 96–100, 2016.

[21] P. A. Harnedy and R. J. FitzGerald, "Bioactive peptides from marine processing waste and shellfish: a review," *Journal of Functional Foods*, vol. 4, no. 1, pp. 6–24, 2012.

[22] S. G. Guan and H. S. Wang, *Chinese Marine Materia Medica*, Shanghai Scientific and Technical Publishers, Shanghai, China, 2009.

[23] S. Singh, A. E. Carpenter, and A. Genovesio, "Increasing the content of high-content screening: an overview," *Journal of Biomolecular Screening*, vol. 19, no. 5, pp. 640–650, 2014.

[24] F. Zhu, C. Qin, L. Tao et al., "Clustered patterns of species origins of nature-derived drugs and clues for future bioprospecting," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 108, no. 31, pp. 12943–12948, 2011.

[25] A. Mcbride and S. K. Butler, "Eribulin mesylate: a novel halichondrin B analogue for the treatment of metastatic breast

cancer," *American Journal of Health-System Pharmacy*, vol. 69, no. 9, pp. 745–755, 2012.

[26] F. Zhu, X. H. Ma, C. Qin et al., "Drug discovery prospect from untapped species: indications from approved natural product drugs," *PLoS ONE*, vol. 7, no. 7, Article ID e39782, 2012.

[27] E. Pean, S. Klaar, E. G. Berglund et al., "The European Medicines Agency review of eribulin for the treatment of patients with locally advanced or metastatic breast cancer: summary of the scientific assessment of the committee for medicinal products for human use," *Clinical Cancer Research*, vol. 18, no. 17, pp. 4491–4497, 2012.

[28] D. L. Wheeler, T. Barrett, D. A. Benson et al., "Database resources of the national center for biotechnology information," *Nucleic Acids Research*, vol. 35, supplement 1, pp. D5–D12, 2007.

[29] F. D. Ciccarelli, T. Doerks, C. Von Mering, C. J. Creevey, B. Snel, and P. Bork, "Toward automatic reconstruction of a highly resolved tree of life," *Science*, vol. 311, no. 5765, pp. 1283–1287, 2006.

[30] H. Zhang, S. Gao, M. J. Lercher, S. Hu, and W.-H. Chen, "EvolView, an online tool for visualizing, annotating and managing phylogenetic trees," *Nucleic Acids Research*, vol. 40, no. 1, pp. W569–W572, 2012.

[31] I. Letunic and P. Bork, "Interactive Tree Of Life (iTOL): an online tool for phylogenetic tree display and annotation," *Bioinformatics*, vol. 23, no. 1, pp. 127–128, 2007.

[32] M. Hahsler, B. Grun, and K. Hornik, "The arules package: mining association rules and frequent itemsets, version 0.6-6," 2008.

[33] J. Fürnkranz, *Association Rule*, Encyclopedia of Systems Biology, Springer, New York, NY, USA, 2013.

[34] E. Dasseni, V. S. Verykios, A. K. Elmagarmid, and E. Bertino, "Hiding association rules by using confidence and support," in *Information Hiding*, vol. 2137 of *Lecture Notes in Computer Science*, pp. 369–383, Springer, Berlin, Germany, 2001.

[35] F. Lazcano-Pérez, S. A. Román-González, N. Sánchez-Puig, and R. Arreguín-Espinosa, "Bioactive peptides from marine organisms: a short overview," *Protein and Peptide Letters*, vol. 19, no. 7, pp. 700–707, 2012.

[36] G. Faircloth and M. del Carmen Cuevas Marchante, "Kahalalide F and ES285: potent anticancer agents from marine molluscs," in *Molluscs: From Chemo-Ecological Study to Biotechnological Application*, vol. 43 of *Progress in Molecular and Subcellular Biology*, pp. 363–379, Springer, Berlin, Germany, 2006.

[37] B. Pejin, M. Mojovic, and A. G. Savic, "Novel and highly potent antitumour natural products from cnidarians of marine origin," *Natural Product Research*, vol. 28, no. 24, pp. 2237–2244, 2014.

[38] N. G. M. Gomes, F. Lefranc, A. Kijjoa, and R. Kiss, "Can some marine-derived fungal metabolites become actual anticancer agents?" *Marine Drugs*, vol. 13, no. 6, pp. 3950–3991, 2015.

[39] L. A. Salvador-Reyes, N. Engene, V. J. Paul, and H. Luesch, "Targeted natural products discovery from marine cyanobacteria using combined phylogenetic and mass spectrometric evaluation," *Journal of Natural Products*, vol. 78, no. 3, pp. 486–492, 2015.

[40] F. Leliaert, A. Tronholm, C. Lemieux et al., "Chloroplast phylogenomic analyses reveal the deepest-branching lineage of the Chlorophyta, Palmophyllophyceae class. nov.," *Scientific Reports*, vol. 6, Article ID 25367, 2016.

[41] X. Kai and M. Mingsan, "The characteristics and the modern research of potency cold," *Chinese Journal of Chinese Medicine*, vol. 28, no. 182, pp. 1006–1008, 2013.

[42] T. L. Simmons, E. Andrianasolo, K. McPhail, P. Flatt, and W. H. Gerwick, "Marine natural products as anticancer drugs," *Molecular Cancer Therapeutics*, vol. 4, no. 2, pp. 333–342, 2005.

[43] E. A. Gontang, S. P. Gaudêncio, W. Fenical, and P. R. Jensen, "Sequence-based analysis of secondary-metabolite biosynthesis in marine actinobacteria," *Applied and Environmental Microbiology*, vol. 76, no. 8, pp. 2487–2499, 2010.

[44] M. Wink and G. I. A. Mohamed, "Evolution of chemical defense traits in the Leguminosae: mapping of distribution patterns of secondary metabolites on a molecular phylogeny inferred from nucleotide sequences of the *rbc*L gene," *Biochemical Systematics and Ecology*, vol. 31, no. 8, pp. 897–917, 2003.

[45] C. W. Levy, A. Roujeinikova, S. Sedelnikova et al., "Molecular basis of triclosan activity," *Nature*, vol. 398, no. 6726, pp. 383–384, 1999.

[46] D.-X. Kong, Y.-Y. Jiang, and H.-Y. Zhang, "Marine natural products as sources of novel scaffolds: achievement and concern," *Drug Discovery Today*, vol. 15, no. 21-22, pp. 884–886, 2010.

[47] V. J. Paul and W. Fenical, "Natural products chemistry and chemical defense in tropical marine algae of the phylum Chlorophyta," in *Bioorganic Marine Chemistry*, vol. 1 of *Bioorganic Marine Chemistry*, pp. 1–29, Springer, Berlin, Germany, 1987.

[48] Y. Pengzhan, Z. Quanbin, L. Ning, X. Zuhong, W. Yanmei, and L. Zhi'en, "Polysaccharides from *Ulva pertusa* (Chlorophyta) and preliminary studies on their antihyperlipidemia activity," *Journal of Applied Phycology*, vol. 15, no. 1, pp. 21–27, 2003.

[49] H. Noda, H. Amano, K. Arashima, and K. Nisizawa, "Antitumor activity of marine algae," *Hydrobiologia*, vol. 204-205, no. 1, pp. 577–584, 1990.

[50] F. Xianjun, W. Peng, and W. Zhenguo, "Hypothesis on building of chemical constituent element system of cold-heat nature based on study of 'nature-structure relationship' of traditional Chinese medicine," *Modernization of Traditional Chinese Medicine and Materia Medica-World Science and Technology*, vol. 13, no. 5, pp. 919–924, 2011.

[51] C. Xiao, D. Li, and H. Wang, "Medicinal composition of main economic algae in our China," *Northwest Pharmaceutical Journal*, vol. 23, no. 6, pp. 408–410, 2008.

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Research Article

Lavandula angustifolia Mill. Oil and Its Active Constituent Linalyl Acetate Alleviate Pain and Urinary Residual Sense after Colorectal Cancer Surgery: A Randomised Controlled Trial

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Pain and urinary symptoms following colorectal cancer (CRC) surgery are frequent and carry a poor recovery. This study tested the effects of inhalation of *Lavandula angustifolia* Mill. (lavender) oil or linalyl acetate on pain relief and lower urinary tract symptoms (LUTS) following the removal of indwelling urinary catheters from patients after CRC surgery. This randomised control study recruited 66 subjects with indwelling urinary catheters after undergoing CRC surgery who later underwent catheter removal. Patients inhaled 1% lavender, 1% linalyl acetate, or vehicle (control group) for 20 minutes. Systolic and diastolic blood pressure (BP), heart rate, LUTS, and visual analog scales of pain magnitude and quality of life (QoL) regarding urinary symptoms were measured before and after inhalation. Systolic BP, diastolic BP, heart rate, LUTS, and QoL satisfaction with urinary symptoms were similar in the three groups. Significant differences in pain magnitude and urinary residual sense of indwelling catheters were observed among the three groups, with inhalation of linalyl acetate being significantly more effective than inhalation of lavender or vehicle. Inhalation of linalyl acetate is an effective nursing intervention to relieve pain and urinary residual sense of indwelling urinary catheters following their removal from patients who underwent CRC surgery.

1. Introduction

The incidence of colorectal cancer (CRC) in Korea has increased markedly due to a transition to Western dietary patterns [1]. At present, CRC is the third most common cancer in both men and women, with 27,618 patients newly diagnosed with CRC in 2013, accounting for 12.3% of the 225,343 patients newly diagnosed with cancer during that year [2]. Surgical resection is the treatment of choice for CRC, but it entails various side effects and complications. The most frequent postoperative complications include micturition disorders, ileus, changes in bowel habit, postoperative pain, enterostomy related problems, surgical site infection and bleeding, anastomotic leakage, and pulmonary complications, all of which can lead to physical pain and distress [3]. Post-CRC pain is both sharp and acute, adding to overall malaise and fatigue. Micturition disorders resulting from CRC surgery have been attributed to injuries to the iliohypogastric and/or pelvic splanchnic nervous systems [4]. The intrapelvic autonomic nervous system involved in the neural control of micturition includes the pelvic splanchnic and iliohypogastric nerves and the pelvic nervous plexus. The hypogastric nerves, which arise from the preaortic sympathetic plexus, divide to the left and right within the pelvis, forming the pelvic autonomic nerve plexus upon meeting the parasympathetic sacral splanchnic nerves that originate from the second, third, and fourth sacral foramina along the pelvic wall [5]. Therefore, any injury to the pelvic splanchnic or iliohypogastric nervous system causes a dysfunction in micturition. Injury to the parasympathetic nervous circuitry causes the sensory function of the bladder to deteriorate, increases voiding difficulty, and weakens detrusor contraction, whereas injury to the sympathetic nervous circuitry causes urinary urgency, tonic urinary incontinence, and increased urinary frequency [6]. The main neurogenic causes of micturition disorders resulting from CRC surgery may

be intraoperative injuries to the ureter or pelvic autonomic nervous system. Moreover, it has been estimated that 10~15% of patients who underwent CRC surgery experience micturition disorders [7].

Efficient and immediate pain control following CRC surgery can facilitate earlier hospital discharge by reducing the length of hospital stay, thereby minimizing complications in the respiratory system, such as atelectasis and pneumonia, as well as gastrointestinal complications, including intestinal obstruction and abdominal inflation. Typical types of pain relief management after CRC surgery include patient-controlled anesthesia (PCA), narcotic (opioid) analgesics, nonsteroidal anti-inflammatory drugs, and epidural anesthesia [3]. Despite pharmacotherapy, however, many patients complain of severe pain in the area of surgery. Acute micturition disorders after CRC surgery impede return to the activities of daily life. Moreover, lower urinary tract symptoms arising from voiding difficulty degrade patient quality of life (QoL) and aggravate patient discomfort until the recovery of urinary function. To prevent this, CRC surgery, consisting of total mesorectal excision, is designed to preserve pelvic autonomic function; indeed, it was found that such autonomic nerve-preserving total mesorectal excision prevents local recurrence as well as preserving urinary functions [4].

Aromatherapy, defined as inhalation of herb oil essences, is a type of complementary and alternative therapy. Analyses of hexane extracts of *Lavandula angustifolia* Mill. (lavender) oil have shown that lavender essential oil has a diuretic effect [8]. Although lavender essential oil was less effective than benzodiazepines in mouse models of anxiety, lavender oil had an antianxiety effect [9]. Inhaled lavender oil has been shown to have relaxant effects on tracheal and ileal smooth muscles of guinea pigs and urinary bladder muscles of white mice, with their anticonvulsant activities involving cyclic AMP-dependent pathways [10]. Furthermore, an urodynamic investigation targeting female patients with urinary incontinence found that inhalation of lavender scent did not mitigate stresses induced by diuretic activity [11]. Studies with clary sage and lavender oils, the main constituent of which was linalyl acetate, showed that inhalation of clary sage oil provided pain relief to patients with periodontitis and efficiently controlled systolic blood pressure and heart rate [12]. Moreover, inhalation of clary sage oil had an antidepressive effect in mouse models of depressive behavior, via a mechanism by which clary sage oil controls dopamine activity [13].

Although lavender oil and its core constituent linalyl acetate relieve pain and anxiety reduce blood pressure and heart rate and have diuretic activity, no study to date has assessed the effect of aromatherapy with lavender and linalyl acetate on the degree of pain relief and urination satisfaction after the removal of indwelling urinary catheters. This study therefore analyzed the effects of lavender and linalyl acetate on the relief of pain in the surgery area and the level of urination satisfaction. These results may indicate an effective nursing intervention for patients following the removal of indwelling urinary catheters.

2. Methods

2.1. Study Design and Sample Size. This randomised pre- and posttest controlled trial assessed the effects of inhalation of 1% (v/v) lavender and 1% (v/v) linalyl acetate in almond oil on pain relief and lower urinary tract symptoms in patients who have undergone removal of indwelling urinary catheters after CRC surgery. Using a G-power program, it was calculated that the minimum number of subjects necessary for intergroup comparisons to achieve a significance level of 0.05, an effect size of 0.40, and test power of 0.80 was >21 subjects per group. Twenty-two (22) subjects were assigned to each group and randomised using table of random numbers to inhalation of 1% lavender oil, 1% linalyl acetate, or almond oil (used as solvent, control group).

2.2. Participants. The study protocol was approved by the institutional bioethics committee of Korea University Anam Hospital (ED15045). All subjects were informed about the objectives and procedures of this study, and all provided written informed consent. All patients had been diagnosed with and underwent robotic or laparoscopic surgery for CRC at Korea University Medical Center and were given postoperative fentanyl as a painkiller and ketorolac tromethamine as an anti-inflammatory agent, with all attaining a specific stable drug dosage at the time of testing. None of the included patients had any complications, inflammatory diseases, loss of consciousness, communication disorders, or disorientation after surgery. None had olfactory impairments or allergy to any of the essential oils; and none had been treated with drugs, hormones, or aromatherapy for a psychiatric disorder.

2.3. Procedures. Prior to testing, the selected subjects were informed about the study objectives and asked to fill out a questionnaire regarding general characteristics, VAS pain, and urination. After a 10-minute rest, blood pressure and heart rate were measured in the supine position; all measurements were taken by a single trained researcher to minimize the effects of emotional stimuli on cardiovascular responses. All subjects were not informed about the types, concentrations, and efficacy of aroma oils.

Following the removal of the indwelling urinary catheter implanted after CRC surgery, curtains were drawn to exclude the effects of experimental cues. After completing the above survey, the subjects were allowed to rest for 10 minutes and placed in the supine position. In taking three deep breaths through a 4×2 cm sized gauze suspended in above the philtrum of the subject, 1 ml 1% lavender oil, 1% LA, or almond oil was dropped onto the gauze, and the subject were allowed to inhale the aroma for about 20 minutes. To ensure the objectivity of this study, the researcher was not involved in formulating the inhaled oils, and testing was performed in a double blind fashion.

After each test, subjects were asked to complete a posttest survey, using the same method as that employed for the pretest survey. Subjects were allowed to rest for about 10 minutes, and blood pressure and heart rate were measured in the supine position, as above.

The main components of the lavender oil (Aromarant Co., Ltd., Rottingen, Germany), as determined by gas chromatography, were [11] linalyl acetate (38.5%), linalool (33.3%), caryophyllene (3.9%), myrcene (3.9%), trans-ocimene (2.4%), lavandulyl acetate (2.2%), and terpinen-4-ol (2.1%).

2.4. Pain Scale. Pain was measured by a visual analog scale (VAS) score [14]. This tool is the most common pain scale for quantification of endometriosis related pain, as it allows each respondent to directly specify his/her level of agreement using a horizontal line, usually 10 cm in length, with scores of 0 and 10 indicating no pain and the worst pain imaginable, respectively.

2.5. Blood Pressure and Heart Rate. Blood pressure (BP) and heart rate (HR) are parameters revealing autonomic nervous system reactions in a state of pain and were used to measure physiological reactions. BP was measured after a rest of 10 minutes or longer using an electronic manometer (3BMI-3, Microlife, Switzerland), placed on the brachial artery with the subject comfortably seated in a supine position before and after essential oil inhalation. HR was measured in the radial artery for 1 minute before and after essential oil inhalation.

2.6. Questionnaire regarding Lower Urinary Tract Function. Micturition function was assessed using a self-administered questionnaire that measures lower urinary tract symptoms (LUTS) based on seven items (urinary residual sense, urinary frequency, urinary intermittency, urinary urgency, urinary weak stream and urinary hesitancy, and nocturia). Each response was scored on a 5-point scale, assessing the frequency of events during the past five urination episodes, with scores of 0–5 points indicating never, 1 in 5 times, 1 in 3 times, 1 in 2 times, 2 in 3 times, and always, respectively. Scores of each of the seven items were summed to yield the total symptom score. Current urinary satisfaction as a QoL measure was scored on a 6-point scale, ranging from very happy (0 point) to terrible (6 points).

2.7. Statistical Analysis. Data were analyzed using SPSS 20.0 software. To preliminarily determine homogeneity across the three patient groups, categorical variables were assessed using Chi-squared or Fisher's exact tests. Continuous variables were analyzed using Kolmogorov-Smirnov tests to determine regularity, with normally distributed variables analyzed by ANOVA (analysis of variance) and nonnormally distributed variables analyzed by Kruskal-Wallis tests. Differences in dependent variables across the three groups before and after testing were analyzed by Kruskal-Wallis tests, whereas within group differences in dependent variables from before to after testing were analyzed by Wilcoxon's rank sum tests.

3. Results

3.1. General Characteristics of Subjects and Verification of Homogeneity. The mean age of the 66 subjects who participated in this study was 60.9 years. Of these subjects, 42 (63.6%) were male and 29 (43.9%) were smokers, with a mean smoking period of 11.5 years and a mean 7.2 cigarettes per day. Of these 66 subjects, 36 (54.5%) exercised every day, 33 (50%) consumed alcohol, 23 (34.8%) took antihypertensive drugs, 11 (16.7%) took hypoglycemic agents, 66 (100%) consumed at least one cup of coffee per day, 23 (34.6%) underwent robotic surgery, and 21 (31.8%) had metastatic disease. None of these factors differed significantly among the three groups (Table 1).

Prior to essential oil inhalation, the mean VAS pain score after removing indwelling urinary catheters was 4.25, mean systolic BP was 126.69 mmHg, mean diastolic BP was 73.87 mmHg, mean HR was 74.54 beats/min, mean total LUTS score was 5.44 points, and mean QoL score was 2.65 points. None of these scores differed significantly among the three groups of subjects (Table 2).

3.2. Effects on Pain. Pain magnitude score after essential oil inhalation differed significantly in the linalyl acetate and control groups (22 in each group, $P = 0.035$, Figure 1(a)). Relative to pretreatment levels, pain magnitude pain magnitude scores after the intervention were reduced $8.22 \pm 4.77\%$ (0.37 ± 0.21 points) in the control group, $14.39 \pm 4.14\%$ (0.56 ± 0.16 points) in the lavender group, and $19.65 \pm 4.44\%$ (0.86 ± 0.19 points) in the linalyl acetate group, with the differences between the lavender ($P = 0.002$) and linalyl acetate ($P < 0.001$) groups being significantly more reduced than in the control group (Figure 1(a)).

Pain magnitude in the three groups was not significantly affected by administration of an antihypertensive drug after the intervention (Figure 1(b)). Pain magnitude was not significantly reduced in either control subgroup (nonhypertensives, $n = 15$; hypertensives, $n = 7$) but was reduced significantly in both the lavender (nonhypertensives, $n = 14$; hypertensives, $n = 8$) and linalyl acetate subgroups (nonhypertensives, $n = 14$; hypertensives, $n = 8$).

Patients in the linalyl acetate ($n = 7$, $P = 0.001$) and lavender ($n = 9$, $P = 0.027$) groups who underwent robotic surgery showed significantly greater reductions in pain score compared with control patients who underwent robotic surgery (Figure 1(c)). Pain magnitude after the intervention increased in control patients who underwent robotic surgery ($n = 7$) but decreased in control patients who underwent laparoscopic surgery ($n = 15$). Pain magnitude in both the lavender and linalyl acetate groups decreased after both robotic (9 in the lavender group; 7 in the linalyl acetate group) and laparoscopic surgery (13 in the lavender group; 15 in the linalyl acetate group).

These findings indicate that inhalation of lavender and linalyl acetate relieved postoperative pain in patients who underwent CRC surgery. Lavender was effective in subjects who did not receive antihypertensive drugs, whereas linalyl acetate was effective in patients who did and did not receive antihypertensive drugs. Furthermore, pain relief was greater in linalyl acetate than in lavender and control patients who underwent robotic surgery.

3.3. Effects on BP and HR. Aroma inhalation reduced systolic BP, diastolic BP, and HR slightly, but similarly, in the three

Table 1: General characteristics of the subjects ($N = 66$).

Characteristics	Control n (%)	Lavender n (%)	Linalyl acetate n (%)	P value
Age (years)	60.64 (13.83)	61.18 (10.06)	61 (12.52)	0.989*
Gender				0.822
Male	14 (63.64)	15 (68.18)	13 (59.09)	
Female	8 (36.36)	7 (31.82)	9 (40.91)	
Smoking				0.782
Yes	9 (40.91)	11 (50)	9 (40.91)	
No	13 (59.09)	11 (50)	13 (59.09)	
Smoking period (years)	10.95 (15.49)	15.14 (18.07)	8.86 (12.04)	0.570‡
Smoking amount (cigs/day)	7.36 (10.80)	8.18 (9.07)	6.05 (8.40)	0.730‡
Exercise frequency (times/wk)				0.177†
1-2	2 (9.09)	0 (0)	2 (9.09)	
3-4	2 (9.09)	5 (22.73)	3 (13.64)	
≥5	4 (18.18)	6 (27.27)	12 (54.55)	
Alcohol drinking				0.475†
Yes	10 (45.45)	14 (63.64)	9 (40.91)	
No	12 (54.55)	8 (36.36)	13 (59.09)	
Drinking amount (glasses/wk)				0.217†
1-2	5 (22.73)	1 (4.55)	2 (9.09)	
3-4	0 (0)	3 (13.64)	1 (4.55)	
5-6	1 (4.55)	1 (4.55)	2 (9.09)	
7–9	0 (0)	4 (18.18)	1 (4.55)	
≥10	4 (18.18)	5 (22.73)	3 (13.64)	
Caffeine intake (glasses/day)				0.248†
0-1	14 (63.64)	12 (54.55)	11 (50)	
2–4	7 (31.82)	9 (40.91)	11 (50)	
5-6	0 (0)	1 (4.55)	0 (0)	
≥7	1 (4.55)	0 (0)	0 (0)	
Antihypertensives				
Yes	7 (31.82)	8 (36.36)	8 (36.36)	0.935
No	15 (68.18)	14 (63.64)	14 (63.64)	
Surgery				
Robotic	7 (32.82)	9 (40.91)	7 (32.82)	0.774
Laparoscopic	15 (68.18)	13 (59.09)	15 (68.18)	
Oral antidiabetics				
Yes	4 (18.18)	4 (18.18)	3 (13.64)	>0.999
No	18 (81.82)	18 (81.82)	19 (86.36)	
Metastasis				
Yes	6 (27.27)	8 (36.36)	7 (31.82)	0.811
No	16 (72.73)	14 (63.64)	15 (68.18)	

Data reported as mean (standard deviation) or n (%).
Chi-square test.
*Analysis of variance (ANOVA).
‡Kruskal-Wallis test.
†Fisher's exact test.

groups, with no statistically significant differences across the three groups (data unshown).

3.4. Effects on Urinary Symptoms and Satisfaction with Urination. Inhalation altered symptom scores slightly, but not significantly, in each of the three groups. Comparisons among the three groups showed that inhalation increased mean LUTS score by 0.63 ± 6.39 in the control group but decreased mean LUTS score by 2.83 ± 4.69 in the lavender group and 0.48 ± 7.15 in the linalyl acetate group, with no significant difference among these three groups ($P = 0.234$; Table 3). Subanalysis showed that urinary residual sense decreased

Table 2: Measured variables prior to essential oil inhalation in the three groups.

Variables	Group (n)	Mean (SD)	P value
VAS Pain	Control (22)	4.48 (1.42)	0.413
	Lavender (22)	3.92 (1.51)	
	Linalyl acetate (22)	4.37 (1.53)	
sBP (mmHg)	Control (22)	125.05 (18.40)	0.798†
	Lavender (22)	128.18 (10.48)	
	Linalyl acetate (22)	126.86 (16.52)	
dBP (mmHg)	Control (22)	74.50 (11.45)	0.453†
	Lavender (22)	75.27 (6.76)	
	Linalyl acetate (22)	71.86 (9.26)	
HR (beats/min)	Control (22)	77.73 (18.40)	0.524
	Lavender (22)	73.14 (11.67)	
	Linalyl acetate (22)	72.77 (15.88)	
Urinary residual sense score	Control (19)	0.79 (1.55)	0.907
	Lavender (18)	1.28 (2.08)	
	Linalyl acetate (21)	1.19 (1.89)	
Total LUTS score	Control (19)	3.74 (4.49)	0.148
	Lavender (18)	6.78 (5.84)	
	Linalyl acetate (21)	5.81 (5.44)	
Quality of life score	Control (19)	2.32 (0.67)	0.075
	Lavender (18)	2.83 (0.86)	
	Linalyl acetate (21)	2.81 (0.87)	

Data reported as mean (standard deviation)
VAS, visual analog scale; sBP, systolic blood pressure; dBP, diastolic blood pressure; HR, heart rate; LUTS, lower urinary tract symptoms.
Kruskal-Wallis test.
†ANOVA.

Table 3: Difference in total urinary symptoms and satisfaction with urination among the three groups.

Variables	Group (n)	Pretest	Posttest	P value	Difference	P value
Urinary residual sense score	Control (19)	0.79 (1.55)	0.16 (0.69)	0.135	−0.63 (1.67)	0.540
	Lavender (18)	1.28 (2.08)	0.00 (0.00)	0.004	−1.28 (2.08)	
	Linalyl acetate (21)	1.19 (1.89)	0.38 (1.12)	0.046	−0.81 (1.69)	
Total LUTS score	Control (19)	3.74 (4.49)	4.37 (4.86)	0.660	0.63 (6.39)	0.234
	Lavender (18)	6.78 (5.84)	3.94 (4.01)	0.059	−2.83 (4.69)	
	Linalyl acetate (21)	5.81 (5.44)	5.33 (7.23)	0.727	−0.48 (7.15)	
Quality of life score	Control (19)	2.32 (0.67)	2.32 (0.75)	>0.999	0.00 (0.82)	0.488
	Lavender (18)	2.83 (0.86)	2.50 (0.79)	0.111	−0.33 (0.77)	
	Linalyl acetate (21)	2.81 (0.87)	2.57 (0.87)	0.216	−0.24 (1.00)	

Data reported as mean (standard deviation).
LUTS: lower urinary tract symptoms.
Kruskal-Wallis test.

more in the lavender (1.28 ± 2.08; $P = 0.004$) and linalyl acetate (0.81 ± 1.69; $P = 0.046$) groups than in the control (0.63 ± 1.67) group but that the postintervention scores were similar in the three groups ($P = 0.540$). Change in satisfaction with urination from before to after inhalation was similar in the three groups ($P = 0.488$).

4. Discussion

This study assessed the effects of inhalation of linalyl acetate and lavender oil on pain relief and urinary symptoms following the removal of indwelling urinary catheters from patients who underwent CRC surgery. Inhalation of linalyl acetate resulted in a significantly greater reduction in pain magnitude compared with inhalation of almond oil. Moreover, pain magnitude was significantly lower after than before inhalation of lavender and linalyl acetate.

Although antihypertensive drugs did not significantly affect pain magnitude in any of these groups, patients in the lavender and linalyl acetate groups who did and did not take antihypertensive agents showed significant reductions in pain magnitude. Patients in the linalyl acetate and lavender groups who underwent robotic surgery showed significantly greater reductions in pain score compared with control patients who

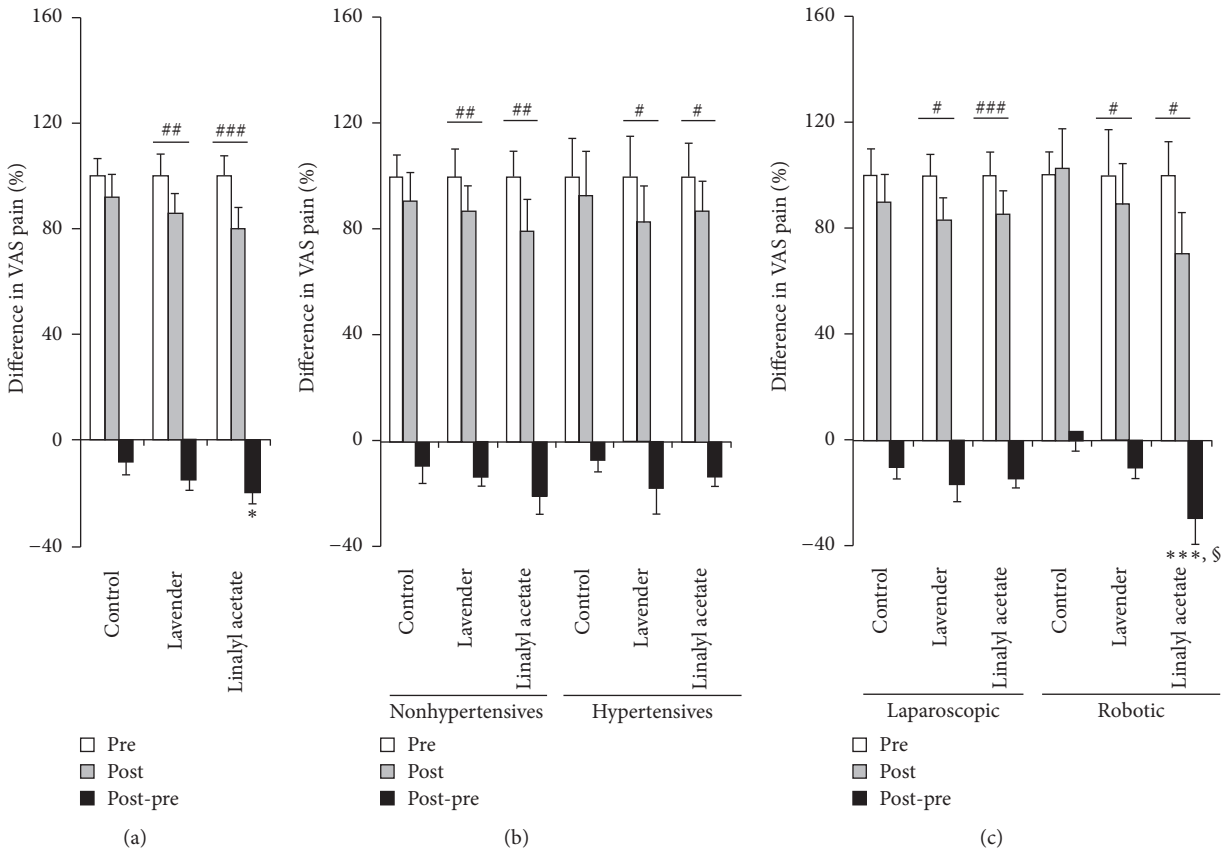

Figure 1: Effects of lavender or linalyl acetate inhalation on VAS pain scores in (a) all patients, (b) patients who were not treated with antihypertensive drugs (nonhypertensives) and were treated with antihypertensive drugs (hypertensives), and (c) patients who underwent laparoscopic and robotic operations. Results are presented as mean ± standard error of the mean, with differences compared by the Kruskal-Wallis test or Wilcoxon's rank sum test. $^{*}P < 0.05$ versus control, $^{***}P < 0.001$ versus control undergoing robotic surgery, $^{\S}P < 0.05$ versus lavender undergoing robotic surgery, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, and $^{\#\#\#}P < 0.001$. VAS: visual analog scale.

underwent robotic surgery. Pain magnitude after the intervention increased in control patients who underwent robotic surgery but decreased in control patients who underwent laparoscopic surgery. Pain magnitude in both the lavender and linalyl acetate groups decreased after both robotic and laparoscopic surgery. Taken together, these findings indicate that linalyl acetate was more effective at reducing pain than lavender, consistent with reports showing the analgesic effect of linalyl acetate aromatherapy. For example, inhalation of lavender oil containing 35.35% linalyl acetate as its main component relieved migraine headaches [15]; inhalation of neroli oil containing 19.5% linalyl acetate [16] and inhalation of *Lavandula hybrida* Reverchon cv. Grosso oil containing 36.2% linalyl acetate [17] reduced peripheral and acentric pain in a mouse model of pain induced by acetic acid and a hot plate; and inhalation of bergamot essential oil containing 70.26% linalyl acetate reduced capsaicin-induced peripheral pain in mice [18].

The main aim of this study was to assess the effects of inhalation of lavender or linalyl acetate on pain relief and lower urinary tract symptoms. This implied a limitation in a small sample size which might explain the effects of lavender or linalyl acetate on lower urinary tract symptoms. Further studies with more patients may be necessary to adequately assess the effects of lavender or linalyl acetate. However, both systolic and diastolic BP were reduced in all three patient groups but tended to be reduced more in the lavender and linalyl acetate groups than in the control group, suggesting that inhalation of lavender essential oil has vasorelaxation effects. This finding is in agreement with results showing that inhalation of (R)-(−) linalool and lavender essential oils by healthy individuals reduced HR [19]. Both total symptom (LUTS) score and satisfaction at urination tended to improve more in the lavender and linalyl acetate groups than in the control group. Intragroup differences in LUTS scores, particularly in urinary residual sense, in the lavender and linalyl acetate groups suggested that inhalation affected diuretic activity, consistent with a prior study assessing the effects on BP of the diuretic action of lavender oil, as shown by urodynamic testing for urinary incontinence in women [11]. Additionally, the sample size of the subgroups of this research was relatively small. Further research undertaken for a longer duration of the study is needed. These findings suggest that inhalation of lavender oil or its main component

linalyl acetate following the removal of an indwelling urinary catheter from patients with micturition disorders can reduce LUTS, especially urinary residual sense.

5. Conclusions

The results described in this study indicate that inhalation of essential oils containing linalyl acetate can relieve pain and urinary residual sense following the removal of indwelling urinary catheters from patients who have undergone CRC surgery. Future research should determine the concentration dependence of these effects, especially with regard to satisfaction with urination. Inhalation of linalyl acetate by these patients may be an effective nursing intervention to enhance comfort and control pain and micturition disorders.

Disclosure

This manuscript is a condensed form of the first author's master's thesis from Korea University.

Competing Interests

The authors declare that they have no conflict of interests.

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References

[1] S. Jun, K. Ha, S. Chung, and H. Joung, "Meat and milk intake in the rice-based Korean diet: impact on cancer and metabolic syndrome," *Proceedings of the Nutrition Society*, vol. 75, no. 3, pp. 374–384, 2016.

[2] National Cancer Information Center, http://www.cancer.go.kr/mbs/cancer/.

[3] P. Kirchhoff, P.-A. Clavien, and D. Hahnloser, "Complications in colorectal surgery: risk factors and preventive strategies," *Patient Safety in Surgery*, vol. 4, no. 1, article no. 5, 2010.

[4] K. Sugihara, Y. Moriya, T. Akasu, and S. Fujita, "Pelvic autonomic nerve preservation for patients with rectal carcinoma: oncologic and functional outcome," *Cancer*, vol. 78, no. 9, pp. 1871–1880, 1996.

[5] K. Havenga, M. C. De Ruiter, W. E. Enker, and K. Welvaart, "Anatomical basis of autonomic nerve-preserving total mesorectal excision for rectal cancer," *British Journal of Surgery*, vol. 83, no. 3, pp. 384–388, 1996.

[6] N. K. Kim, T. W. Aahn, J. K. Park et al., "Assessment of sexual and voiding function after total mesorectal excision with pelvic autonomic nerve preservation in males with rectal cancer," *Diseases of the Colon and Rectum*, vol. 45, no. 9, pp. 1178–1185, 2002.

[7] D. W. Lee, S. J. Baek, J. W. Shin, J. S. Cho, J. Kim, and S. H. Kim, "Robotic-assisted resection of primary rectal cancer: an analysis of consecutive 185 cases," *Journal of the Korean Society of Endoscopic & Laparoscopic Surgeons*, vol. 14, no. 2, pp. 61–67, 2011.

[8] M. Elhajili, K. Baddouri, S. Elkabbaj, F. Meiouat, and A. Settaf, "Diuretic activity of the infusion of flowers from *Lavandula officinalis*," *Reproduction Nutrition Development*, vol. 41, no. 5, pp. 393–399, 2001.

[9] D. Shaw, K. Norwood, and J. C. Leslie, "Chlordiazepoxide and lavender oil alter unconditioned anxiety-induced c-fos expression in the rat brain," *Behavioural Brain Research*, vol. 224, no. 1, pp. 1–7, 2011.

[10] M. Lis-Balchin and S. Hart, "Studies on the mode of action of the essential oil of lavender (Lavandula angustifolia P. Miller)," *Phytotherapy Research*, vol. 13, no. 6, pp. 540–542, 1999.

[11] G. H. Seol, Y. H. Lee, P. Kang, J. H. You, M. Park, and S. S. Min, "Randomized controlled trial for salvia sclarea or lavandula angustifolia: differential effects on blood pressure in female patients with urinary incontinence undergoing urodynamic examination," *Journal of Alternative and Complementary Medicine*, vol. 19, no. 7, pp. 664–670, 2013.

[12] J. Yu, M. Kim, H. Moon, S. Min, and G. H. Seol, "Effect of clary sage-inhalation on pain and stress during the treatment of periodontitis," *Journal of Korean Academy of Oral Health*, vol. 35, no. 1, pp. 32–40, 2011.

[13] G. H. Seol, H. S. Shim, P.-J. Kim et al., "Antidepressant-like effect of Salvia sclarea is explained by modulation of dopamine activities in rats," *Journal of Ethnopharmacology*, vol. 130, no. 1, pp. 187–190, 2010.

[14] M. E. Cline, J. Herman, E. R. Shaw, and R. Donald Morton, "Standardization of the visual analogue scale," *Nursing Research*, vol. 41, no. 6, pp. 378–380, 1992.

[15] P. Sasannejad, M. Saeedi, A. Shoeibi, A. Gorji, M. Abbasi, and M. Foroughipour, "Lavender essential oil in the treatment of migraine headache: a placebo-controlled clinical trial," *European Neurology*, vol. 67, no. 5, pp. 288–291, 2012.

[16] P. Khodabakhsh, H. Shafaroodi, and J. Asgarpanah, "Analgesic and anti-inflammatory activities of Citrus aurantium L. blossoms essential oil (neroli): involvement of the nitric oxide/cyclic-guanosine monophosphate pathway," *Journal of Natural Medicines*, vol. 69, no. 3, pp. 324–331, 2015.

[17] E. Barocelli, F. Calcina, M. Chiavarini et al., "Antinociceptive and gastroprotective effects of inhaled and orally administered *Lavandula hybrida Reverchon 'grosso'* essential oil," *Life Sciences*, vol. 76, no. 2, pp. 213–223, 2004.

[18] T. Sakurada, H. Mizoguchi, H. Kuwahata et al., "Intraplantar injection of bergamot essential oil induces peripheral antinociception mediated by opioid mechanism," *Pharmacology Biochemistry and Behavior*, vol. 97, no. 3, pp. 436–443, 2011.

[19] K. Kuroda, N. Inoue, Y. Ito et al., "Sedative effects of the jasmine tea odor and (R)-(-)-linalool, one of its major odor components, on autonomic nerve activity and mood states," *European Journal of Applied Physiology*, vol. 95, no. 2-3, pp. 107–114, 2005.

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Research Article

Protective Effect of Green Tea (*Camellia sinensis* (L.) Kuntze) against Prostate Cancer: From In Vitro Data to Algerian Patients

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Green tea (GT) has been studied for its effects as antioxidant and cancer-preventive agent. Epidemiological studies showed that GT consumption decreases the risk for prostate cancer (PC). To investigate whether erythrocyte oxidative stress (OS) is associated with PC and whether daily consumption of GT improves the oxidative phenotype, we performed a study in a group of Algerian PC patients, preceded by an in vitro study to characterize composition and antioxidant/antiproliferative activities of the GT used. This contained a high content of phenolic and flavonoid compounds, demonstrating in vitro antioxidant activity and significant antiproliferative effect on human prostate cancer PC-3 cell line. Seventy PC patients and 120 age-matched healthy subjects participated in the study, with glutathione (GSH), malondialdehyde (MDA), and catalase activity evaluated before and after GT consumption. The results showed a reduced GSH and catalase activity and a high level of MDA in erythrocytes from PC patients. The consumption of 2-3 cups per day of GT during 6 months significantly increased GSH concentration and catalase activity and decreased MDA concentration. In conclusion, GT significantly decreased OS in Algerian PC patients. Regular consumption of GT for a long period may prevent men from developing PC or at least delay its progression.

1. Introduction

Prostate cancer (PC) is the second most common malignancy diagnosed in men and the fifth leading cause of mortality in the world. In fact, PC was one of the most frequently diagnosed male neoplasias and the sixth leading cause of death in Algeria in 2012 [1]. As in most cancers, the etiological factors of PC still remain poorly understood although many studies suggested that aging [2, 3], diet [4], and inflammation [5] are involved in PC development and progression, with oxidative stress possibly being a common link. In fact, several studies in humans showed significant alterations in oxidant-antioxidant balance in PC patients when compared to controls. Significant high levels of malondialdehyde, ceruloplasmin, and lower levels of reduced glutathione and glutathione peroxidase, catalase, and superoxide dismutase activities were observed in PC patient blood [6–8].

Green tea, a beverage prepared from the dry leaves of *Camellia sinensis* (L.) Kuntze, has been extensively studied for its effect as a potent antioxidant [9, 10] and on cancer prevention [11–13]. Epidemiological studies found that green tea consumption may decrease the risk for PC [14, 15]. Using PC cell lines, it was demonstrated that green tea polyphenols, especially catechins, the major species, inhibit carcinogenesis through different mechanisms of action including induction of cell cycle arrest [16], apoptosis [17], inhibition of the insulin-like growth factor receptor [18] and androgen receptor downregulation by interaction with its ligand-binding

domain [19]. In many preclinical trials, the administration of an oral infusion of green tea catechins in TRAMP (transgenic adenocarcinoma of mouse prostate) mice confirmed the efficacy of green tea in decreasing PC progression [20, 21]. However, few studies were performed in human [22–24] with only a few finding encouraging results [22, 24].

The current study aimed to investigate whether PC was associated with increased oxidative stress in erythrocytes in a set of Algerian patients and whether green tea intake inversely correlated with oxidative stress, a possible factor involved in PC development and progression. We selected a popular type of commercial Chinese green tea in Algeria and measured its phenol and flavonoid content, as well as antioxidant and antiproliferative activities in vitro. In the follow-up translational step, we evaluated oxidative stress markers in peripheral blood in Algerian PC patients before and after green tea consumption.

2. Materials and Methods

2.1. Materials. Dulbecco's modified Eagle's medium (DMEM, D5648), Roswell Park Memorial Institute medium (RPMI, 1640), penicillin, streptomycin, fetal bovine serum (FBS), and 0.25% Trypsin-EDTA were purchased from Gibco-Invitrogen (Grand Island, NY). Sodium chloride (NaCl), sulforhodamine B sodium salt (SRB), Tris, and trypan-blue solution were obtained from Sigma-Aldrich Chemical Co. (Saint Louis, MO, USA). Ellman's Reagent (DTNB, D8130), thiobarbituric acid (TBA, T5500), L-glutathione reduced (GSH, G4251) were purchased from Sigma-Aldrich (St. Louis, MO, USA). All other reagents and chemical compounds used were of the greatest degree of purity commercially available. In the preparation of every solution, including buffers, ultrapure distilled water (conductivity < 18 $\mu S \cdot cm^{-1}$), filtered by the Milli-Q from a Millipore (Billerica, MA) system, was always used in order to minimize as much as possible contamination with metal ions.

2.2. Experimental Approach: In Vitro

2.2.1. Green Tea Extraction. Two thousand grams of commercial Chinese green tea leaves of *Camellia sinensis* (L.) Kuntze plant was macerated with EtOH/H_2O (7 : 3 v/v) for 48 h three successive times. The combined filtrate was dried by evaporation and the ethanolic extract obtained was solubilized in 800 mL H_2O. The aqueous filtrate was successively extracted with chloroform ($CHCl_3$), ethyl acetate (EtOAc), and *n*-butanol (*n*-BuOH). The organic phases were concentrated in vacuum to obtain the following extracts: ($CHCl_3$) (4.7 g), EtOAc (27.66 g), and *n*-BuOH (46.31 g).

2.2.2. Determination of Total Phenolic and Flavonoid Content. The total phenolic content in green tea extracts ($CHCl_3$, EtOAc, and *n*-BuOH) was measured using the method of Singleton et al. [25]. To 20 μl of each green tea extract prepared in methanol (1 mg/ml), 100 μl of Folin-Ciocalteu and 1580 μl of distilled water were added successively, flowed three min later by 300 μl of sodium carbonate (20%). Samples were stirred for 2 h at room temperature, and the absorbance was then evaluated at 765 nm. The concentration of total phenolic compounds was determined as μg of gallic acid equivalent (GAE) per mg of extract using a standard curve which was prepared using gallic acid solutions (0 to 500 mg/ml) solubilized in MeOH/H_2O (1 : 9 v/v).

The total flavonoid content in green tea extracts was determined according to the method of Wang et al. [26]. 0.5 ml of 2% $AlCl_3$ was mixed with 0.5 ml of sample. After 1 h incubation at room temperature, the absorbance was measured at 420 nm. The concentration of flavonoids was determined as μg of quercetin equivalent (QE) per mg of extract using standard quercetin calibration curve.

2.2.3. HPLC-TOF/MS Analysis of Green Tea Extracts. To analyze the phenolic content of the different green tea extracts, high-performance liquid chromatography/time-of-flight mass spectrometry (HPLC-TOF/MS) analysis was used. Agilent Technologies 1260 Infinity HPLC System was coupled to a 6210 time-of-flight (TOF) LC/MS detector and ZORBAX SB-C18 (4,6 × 100 mm, 3.5 μm) column. The mobile phases A and B were ultrapure water solution with 0.1% formic acid and acetonitrile, respectively. Flow rate was 0.6 ml/min and column temperature was 35°C. The green tea extracts (200 ppm) and stock solutions of 23 standard phenolic compounds (2.5 ppm) were prepared in methanol at room temperature. The samples were filtered passing through a PTFE (0.45 μm) filter by an injector to remove particulates. The injection volume was 10 ml and the solvent program was as follows: 0 min 10% B; 0-1 min 10% B; 1–20 min 50% B; 20–23 min 80% B; 23–25 min 10% B; 25–30 min 10% B. The ionization mode of MS-TOF instrument was ES negative with gas temperature of 325°C, gas flow of 10.0 l/min, and nebulizer of 40 (psi). The phenolic content of green tea extracts was determined by comparing retention times and *m/z* values of green tea extracts and standard phenolic compounds.

2.2.4. Evaluation of the Antioxidant Activity

(1) DPPH Radical-Scavenging Activity Assay. The ability of green tea extracts to quench DPPH (2,2-diphenyl-1-picrylhydrazyl) radical was evaluated by the method of Braca et al. [27]. To increasing concentrations (1, 2.5, 5, 10, 20, and 25 μg/ml) of methanol-dissolved extracts, 3 ml of methanol DPPH solution (0.004%) was added. Test tubes were incubated at room temperature for 30 min, and absorbance was then measured at 517 nm. Tests were carried out in triplicate and ascorbic acid was used as positive control. The percentage of DPPH scavenging activity (I%) was calculated using the following equation (1):

$$I\% = \left[\frac{(A_0 - A_1)}{A_0}\right] \times 100, \quad (1)$$

where A_0 is the absorbance of DPPH solution alone and A_1 is the absorbance of DPPH solution + extract or vitamin C. The half inhibition concentration (IC_{50}) of green tea extracts was calculated from the plot of percentage of inhibition against concentration of green tea extracts.

(2) Inhibition of Lipid Peroxidation. The lipid peroxidation assay was performed according to the modified protocol of Cao and Ikeda using egg *vitellose* [28]. To evaluate the capacity of green tea extracts to inhibit lipid peroxidation, 0.5 ml of 10% egg *vitellose* homogenate as lipid-rich media was mixed with 50 μl of $FeSO_4$ (0.07 M) and then incubated with increasing concentrations of green tea extracts or vitamin C at 37°C for 30 min. After incubation, 1 ml TCA 20% (trichloroacetic acid) and 1.5 ml TBA 1% (thiobarbituric acid) were successively added. The samples were mixed and then heated for 15 min at 95°C. After centrifugation (400g for 20 min), the resulting thiobarbituric reacting substances (TBARS) were measured in the supernatant at 532 nm. The lipid peroxidation inhibition was calculated as percentage (I%) according to (1), where A_0 is the absorbance of the control (without extract or vitamin C) and A_1 is the absorbance of sample + extract or vitamin C.

2.2.5. Evaluation of the Cytotoxicity Effect of Green Tea Extracts

(1) Cell Culture and Treatments. The human metastatic prostate cancer cell line PC-3 [29] and human foreskin BJ fibroblasts [30], purchased from America Tissue Type Collection (Manassas, VA), were cultured in RPMI (1640) and DMEM (D5648) media, purchased from Gibco-Invitrogen (Grand Island, NY), supplemented with 1.5 g/l sodium bicarbonate, 10% fetal bovine serum, 100 U/ml of penicillin, and 100 μg/ml of streptomycin in tissue-culture dishes at 37°C in a humidified atmosphere of 5% CO_2. All cells were passaged by trypsinization when reaching 70–80% confluence and all experiments were performed in log-phase growth cultures. Green tea extracts ($ChCl_3$, EtOAc, and n-BuOH) were prepared in dimethyl sulfoxide (DMSO) and stored at 4°C in the dark. The total volume of DMSO was always smaller than 0.1%, which had negligible effects in all experiments. Green tea extracts were directly added to the culture medium at the described concentrations. Vehicle controls received an equivalent amount of DMSO only.

(2) Cell Proliferation Measurements. The sulforhodamine B (SRB) assay was used to measure cell protein, which is dependent on the amount of cells in each well [31]. In the present study, we used both human foreskin BJ fibroblasts, which have a long lifespan and are commonly used as a nontumor control cell line [32, 33] and the human PC-3 cell line, commonly used as an in vitro model for PC studies [29]. Both cell lines were seeded in 48-well plates with a final volume of 500 μl per well at a density of 10,000 and 20,000 cells per ml, respectively. The two cell lines were treated with increasing concentrations of green tea extracts (5, 10, 25, and 50 μg/ml). Twenty-four hours after drug addition, the incubation medium was removed, wells rinsed with 1% PBS, and cells were fixed in 1% acetic acid in ice-cold methanol for at least one day. Cells were then incubated with 0.05% (w/v) SRB reagent dissolved in 1% acetic acid for 1 h at 37°C. Unbound dye was removed with 1% acetic acid. Dye bound to cell proteins was extracted with 10 mM Tris-base solution, pH 10. After SRB labeling, absorbance was measured at 540 nm in a plate reader and the amount of dye released which is proportional to the number of cells present in the dish was measured [31]. The results were expressed as a percentage of control.

2.3. Human Studies

2.3.1. Study Subjects. Ninety patients diagnosed with histologically confirmed PC at the Clinic of Urology-Nephrology and Kidney Transplant Daksi, Constantine, Algeria, were interviewed and invited to participate in the study. Patients received oral and written information about the study and gave their written consent. Three patients refused to participate. Eighty-seven volunteers received the same brand of green tea after the evaluation of its antioxidant and cytotoxic effect (in vitro approach). The offered quantity of green tea was weighed and divided into doses of 2 g for each. Subjects were asked to drink 5 cups of green tea infusion per day for 6 months. Green tea was prepared every day at the same temperature (70–80°C), time of infusion (5 minutes), and concentration (2 g of tea leaves in 100 ml of water for each cup) [34]. The information about total Gleason score, serum PSA level at diagnosis, and primary treatment taken for patients were obtained from the medical record. No smoking or alcohol drinking age-matched 120 healthy subjects were selected carefully in the Clinic of Urology-Nephrology and Kidney Transplant Daksi, Constantine, Algeria, and in the blood sample collection room of the Laboratory of Biochemistry of Establishment Public Hospital of Chelghoum Laid City, Algeria. Controls were divided into two groups: green tea drinkers (n = 35) and nontea drinkers (n = 85). The Ethics Committee of the EHS Daksi certified that the data collection was performed at the Department of Urology and Renal Transplant without any risk for patients.

2.3.2. Samples Collection and Preparation of Hemolysates. Heparinized venous blood samples were collected after an overnight fast. Patients sample collection was at the beginning (T = 0), after green tea consumption (T = 3 months) (T = 6 months). Blood samples were immediately centrifuged at 300g for 15 min and then serum aliquots were removed and stored at −80°C until assayed. Erythrocytes were washed three times by centrifugation (300g, 15 min) in an ice-cold isotonic sodium chloride solution (1 : 10, v/v). The supernatant and buffy coat of white cells was carefully removed after each wash. A portion of the erythrocytes obtained were used for GSH determination, while the remaining was resuspended in the washing solution to give a 50% suspension. Hemolysis of the washed cell suspension was achieved by mixing 1 volume of cells with 9 volumes of cold distilled water. After removing the cell debris by centrifugation (300g, 15 min), the hemolysate obtained was used for determination of enzymatic activity of CAT and TBARS measurement. The hemoglobin content in the red blood cell lysate was measured according to the cyanmethemoglobin method using Drabkin's reagent [35].

2.3.3. Biochemical Assays. The GSH concentration was measured using the method described by Beutler et al. [36].

Briefly, 0.2 ml fresh erythrocytes pellet was added to 1.8 ml distilled water followed by 3 ml precipitating solution (1.65 g metaphosphoric acid, 0.2 g EDTA, and 30 g NaCl in 100 ml distilled water). The mixture was allowed to stand 5 min and centrifuged next (300*g*, 15 min). Eight ml of 0.3 mM disodium phosphate solution and 1 ml of DTNB were added to 2 ml of supernatant. A blank was prepared replacing erythrocytes for 0.2 ml of distilled water. GSH standard was prepared with 0.2 ml the glutathione solution, 8 ml of 0.3 mM disodium phosphate solution, and 1 ml of DTNB. The optical density was measured at 412 nm in a spectrophotometer.

Erythrocytes catalase (EC 1.11.1.6) activity was estimated in the hemolysate following the method of Greenwald (1985) [37] based on the scavenging of hydrogen peroxide (0.019 M) in the presence of phosphate buffer (0.1 M, pH 7.4) by catalase present in the sample. Catalase activity was expressed as international unit per g hemoglobin (UI/gHb).

Lipid peroxidation in erythrocytes was evaluated by measuring malondialdehyde (MDA) in the hemolysate according to the double heating method (Draper and Hadley (1990)) [38]. This method is based on the reaction of lipid peroxides (MDA) with thiobarbituric acid (TBA, 0.67%) in acidic environment (TCA, 10%) at 90–100°C to form a pink pigment with absorption maximum at 532 nm. The concentration of MDA was calculated by the absorbance coefficient of MDA-TBA complex $1.56 \times 10^5\,\mathrm{cm}^{-1}\,\mathrm{M}^{-1}$ and was expressed in μmol/gHb.

3. Statistical Analysis

Sulforhodamine B results were expressed as mean ± SEM and multiple comparisons were performed using one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparisons test. Data from human studies were expressed as mean ± SD and differences between the different groups were using a Student's t test. Significance was accepted with $p < 0.05$.

4. Results

4.1. In Vitro Characterization of Green Tea Composition and Activity

4.1.1. Total Phenolic and Flavonoid Content of Green Tea Extracts. Total phenolic and flavonoid content in the different green tea extracts obtained showed a high concentration of phenolic and flavonoid compounds especially in the EtOAc and *n*-BuOH extracts. The latter contained 548.33 ± 54.62 and 394.66 ± 22.67 μg of gallic acid equivalent/mg extract of phenolic compounds and 12.16 ± 0.01 and 31.20 ± 0.1 μg of quercetin equivalent/mg extract of flavonoid compounds, respectively. The $CHCl_3$ extract contained minor amounts of phenolic and flavonoid compounds, namely, 82.33±5.04 μg of gallic acid equivalent/mg extract of phenolic compounds and 5.04 ± 0.59 μg of quercetin equivalent/mg extract of flavonoid compounds (Figure 1).

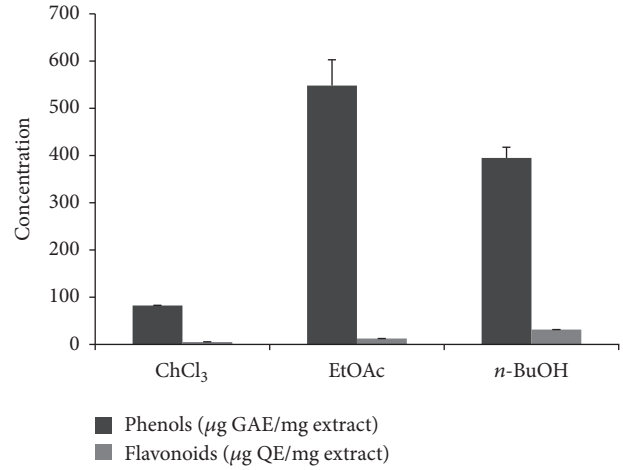

Figure 1: Total phenolic and flavonoid content of green tea extracts, measured as described in Materials and Methods. Concentrations of total phenolic compounds are expressed as μg of gallic acid equivalent (GAE) per mg of extract and total flavonoids are expressed as μg of quercetin equivalent (QE) per mg of extract. Values are means ± SD ($n = 3$).

4.1.2. Evaluation of Phenolic Content of Green Tea Extracts Using HPLC-TOF/MS. Analysis by HPLC-TOF/MS revealed the presence of different phenols in the different green tea extracts. Using 23 standard phenolic compounds, seventeen different phenols were detected in the EtOAc and *n*-BuOH extracts. A high amount of gallic acid and catechins and relevant amounts of vanillic acid, salicylic acid, rutin, and *p*-coumaric acid were found in the EtOAc extract. The *n*-BuOH extract contains also a high amount of gallic acid followed by rutin, gentisic acid, salicylic acid, and chlorogenic acid. Fifteen phenols were detected in the $CHCl_3$ extract although all present in small amounts (Figure 2, Table 1).

4.1.3. Antioxidant Activity of Green Tea Extracts. No significant antioxidant activity was observed for the $CHCl_3$ extract but significant effects were found with EtOAc and *n*-BuOH extracts. Compared to vitamin C (IC_{50} = 5 ± 0.1 μg/ml), EtOAc and *n*-BuOH extracts also significantly quenched the DPPH radical (IC_{50} = 2.98 ± 0.32 and 7.58 ± 0.74, resp.) and their effects were dose-dependent. The highest percentages of DPPH inhibition were, respectively, 93.4% and 93.2%, which were similar to vitamin C in the same concentration (20 μg/mL) (Figure 3(a), Table 2).

A dose-dependent decrease in lipid peroxidation was also observed with EtOAc (IC_{50} = 201.01 ± 2.55 μg/ml) and *n*-BuOH (IC_{50} = 302.18 ± 28.31 μg/ml) extracts, although the effects were not comparable to those of vitamin C (IC_{50} = 20 ± 1.06 μg/ml) (Figure 3(b), Table 2).

4.1.4. Cytotoxicity of Green Tea Extracts on Human Prostate Cancer PC-3 Cell Line. The results regarding cytotoxicity activity using the SRB assay indicated that green tea extracts showed significant antiproliferative activity against the human prostate cancer PC-3 cell line. Dose-dependent toxicity on that cell line was observed after 24 h treatment with EtOAc and *n*-BuOH extracts, with IC_{50} values of 36.37 and 37.74 μg/ml, respectively. However, no effects were

Table 1: The different phenols revealed in the green tea extracts and their levels (expressed as mg per kg of dry leaves of green tea).

Phenols	Phenolic content of green tea extracts (mg phenolic/kg plant)		
	$CHCl_3$ extract	EtOAc extract	*n*-BuOH extract
Gallic acid	0.25	777.93	1344.96
Gentisic acid	0.15	ND	102.14
Catechins	0.04	174.95	ND
Chlorogenic acid	ND	2.37	21.18
4-Hydroxybenzoic acid	0.31	ND	1.8
Protocatechuic acid	0.03	2.49	3.51
Caffeic acid	0.04	7.43	5.66
Vanillic acid	5.00	46.31	20.04
4-Hydroxybenzaldehyde	0.3	0	ND
Rutin	0.11	22.2	646.29
p-Coumaric acid	0.70	15.00	4.18
Ellagic acid	0.51	0.85	1.35
Chicoric acid	ND	0.2	0.67
Ferulic acid	ND	ND	1.92
Hesperidin	ND	0.65	2.5
Apigenin 7-glucoside	ND	0	1.96
Rosmarinic acid	0.03	0.237	ND
Protocatechuic acid ethyl ester	ND	0.05	0
Salicylic acid	6.5	34.69	21.23
Resveratrol	ND	ND	0.08
Quercetin	0.71	ND	ND
Naringenin	0.08	0.35	ND
Kaempferol	ND	ND	0.43

ND: not detected.

Table 2: IC_{50} values of antioxidant activities of green tea extracts and vitamin C in DPPH and LPO assays, obtained from Figure 2 data. Values are mean ± SD ($n = 3$).

Extracts and standards	IC_{50} (μg/ml)	
	DPPH	LPO
$CHCl_3$	207.94 ± 3.12	—
EtOAc	2.98 ± 0.32	201.01 ± 2.55
n-BuOH	7.58 ± 0.74	302.18 ± 28.31
Vitamin C	5 ± 0.1	20 ± 1.06

observed on the nontumor, control fibroblast BJ cell line with both extracts. No cytotoxicity effects were observed in any of the cell lines tested after 24 h treatment by increasing concentrations of $CHCl_3$ extract (Figure 4).

4.2. Human Studies

4.2.1. Study Subjects. At the beginning of the study, 87 PC patients accepted to participate and regularly received green tea. After 6 months, 17 patients were excluded from the study (6 patients died during the study, 3 refused to continue the study after 3 months, and 8 did not drink tea or drank it only a few times during the entire period of the analysis). The cohort investigated consisted then in 70 PC patients with median age of 70.64 ± 6.5 years and median serum PSA level of 62.73 ± 33.97 ng/ml. The Gleason score at diagnosis was between 5 and 7 in 40% of cases and between 8 and 10 in 60% of cases. Eleven percent of cases underwent prostatectomy followed by hormone therapy, 11% underwent radiotherapy associated with hormone therapy, 49% were under hormone therapy, and 29% were under chemotherapy associated with hormone therapy. Controls were 85 age-matched healthy subjects which never or rarely drank green tea (median age 68.5 ± 6.56 years, $p = 0.1169$) and 35 age-matched healthy subjects who normally drank 1 to 3 cups of green tea per day for at least 1 year (median age 68.81 ± 6.42 years, $p = 0.2537$) (Table 3).

4.2.2. Level of Lipid Peroxidation and Antioxidants Status. To assess oxidative stress in erythrocytes from PC patients, lipid peroxidation and antioxidants were measured in erythrocytes before starting the green tea drinking protocol ($T0$). Significant alterations in reduced glutathione (GSH) level and in catalase activity were observed and a high level of MDA was found in PC patient's erythrocytes as compared to controls that never or rarely drank tea. GSH (mg/dl) was 20.79 ± 4.32 versus 40.51 ± 4.87 ($p < 0.0001$), CAT (UI/gHb) was 15.29 ± 1.75 versus 23.84 ± 2.03 ($p < 0.0001$), and MDA (nmol/gHb) was 99.52 ± 12.49 versus 33.83 ± 5.14 ($p < 0.0001$) (Figure 5). No significant difference was observed in GSH level between control individuals who regularly drank 1 to 3 cups of green tea per day and those that never or rarely drank tea (42.02 ± 4.23 mg/dl versus 40.51 ± 4.87 mg/dl), but catalase activity was higher (25.94 ± 1.81 UI/gHb versus 23.84 ± 2.03 UI/gHb, $p < 0.005$) and MDA level was lower (29.98 ± 4.73 nmol/gHb versus 33.83 ± 5.14 nmol/gHb, $p < 0.05$) in those individuals drinking green tea (Figure 5).

After three months of green tea consumption (2 to 3 cups per day), the levels of lipid peroxidation and antioxidants in erythrocytes of PC patients were evaluated again. Significant increases in GSH level (to 31.58 ± 2.57 mg/dl, $p < 0.0001$) and in catalase activity (to 17.23 ± 1.51 UI/gHb, $p < 0.05$) were observed and a significant decrease in MDA level was found (to 85.84 ± 12.05 nmol/gHb, $p < 0.01$) (Figure 5).

After another three months of green tea drinking, reduced GSH significantly increased to 34.36 ± 3.64 mg/dl, $p < 0.0001$ and catalase activity to 22.19 ± 1.78 UI/gHb, $p < 0.0001$. Furthermore, MDA level further decreased to 45.16 ± 7.45 nmol/gHb, $p < 0.0001$ (Figure 5).

5. Discussion

The excessive production of ROS, unbalance in antioxidant defense systems, or both combined may cause oxidative stress in different cell compartments, triggering cell signaling processes associated with initiation and development of many diseases including PC. Evidence suggests that PC progress is associated with increased oxidative stress [39]. Several possible mechanisms may contribute to increased ROS

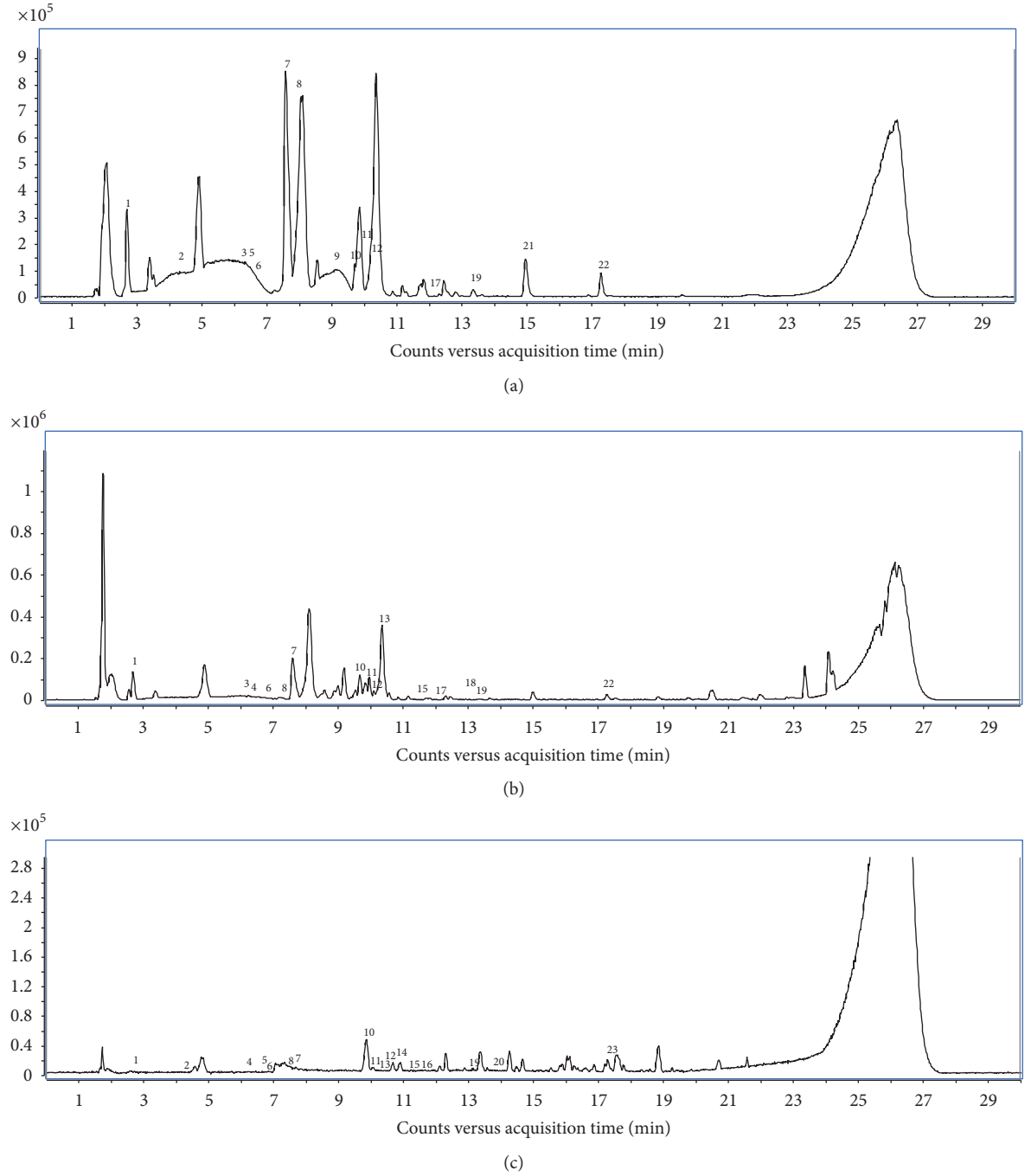

Figure 2: Chromatograms of green tea extracts. (a) Chromatogram of chloroformic ($CHCl_3$) green tea extract. (b) Chromatogram of ethyl acetate (EtOAc) green tea extract. (c) Chromatogram of *n*-butanol (*n*-BuOH) green tea extract. The chromatographic conditions were described in Materials and Methods. The phenols detected by HPLC-TOF/MS analysis are expressed as numbers from 1 to 23.

generation associated with PC development and progression including inflammation, diet, aging, NADPH oxidase, steroid hormones, and mitochondrial and nuclear DNA mutations [39]. A number of studies have investigated the effect of antioxidant supplements in preventing or reducing the risk of PC, although the results are still conflicting [40]. In the current study, we tested the capacity of green tea, described to have a general antioxidant property [10] to reduce oxidative stress in PC patients. A group of Algerian patients with PC were tested with a commercial Chinese green tea. For the safety of patients and to confirm the antioxidant/anti-proliferative activity of the Chinese green tea used, we first investigated its composition and its effects in vitro. The commercial green tea used was first extracted using different solvents ($ChCl_3$, EtOAc, and *n*-BuOH), followed by evaluating phenolic and flavonoid content, as well as each extract

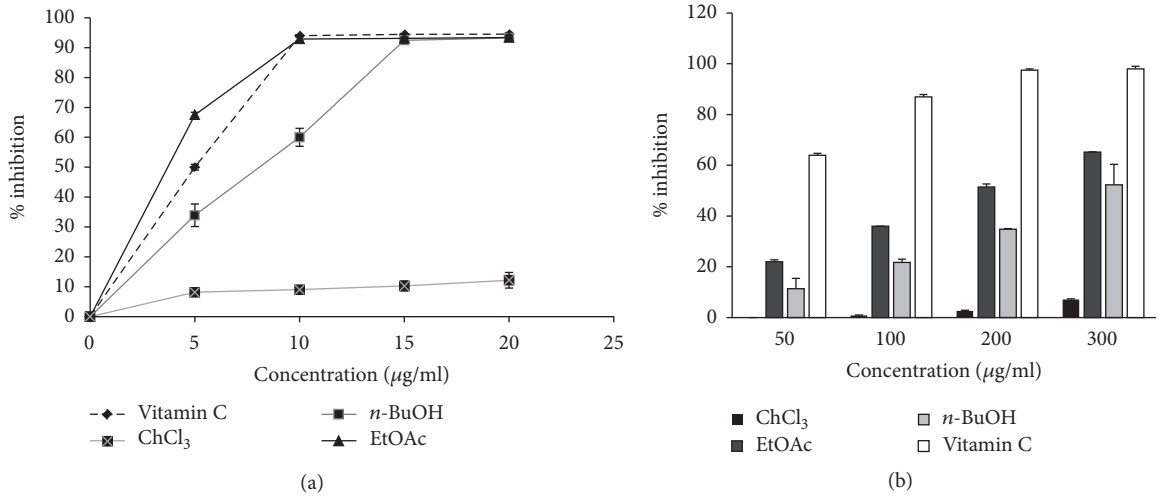

Figure 3: Antioxidant activity of green tea extracts. (a) DPPH scavenging activities of green tea extracts and vitamin C, measured as described in Materials and Methods. (b) Effect of green tea extracts and vitamin C on inhibition of $FeSO_4$-induced lipid peroxidation of egg *vitellose*, measured as previously described in Materials and Methods. Values are mean ± SD ($n = 3$).

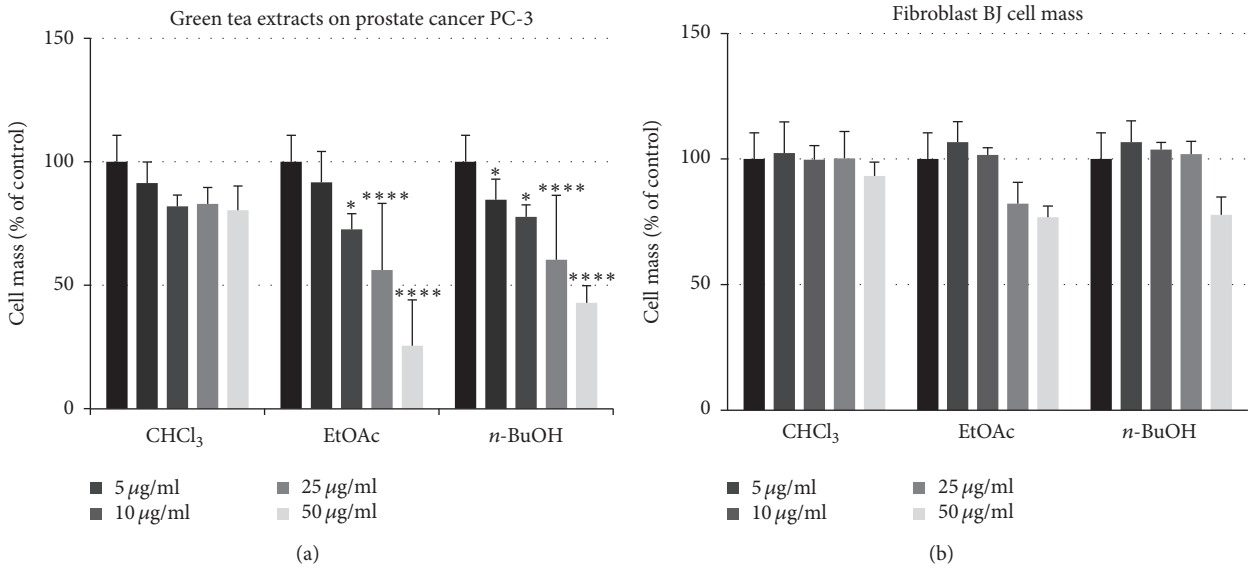

Figure 4: Effects of green tea extracts on prostate cancer PC-3 and fibroblast BJ cell mass. The control value (vehicle only) was determined as 100% to account for the differential proliferation of cell lines. Data are expressed as the means ± SEM of four different experiments, $^{*}p < 0.05$, $^{****}p < 0.0001$ versus control, nontreated cells.

antioxidant and antiproliferative activity. We confirmed here that the green tea used, namely, the extracts obtained, had significant antioxidant activity. Unlike the $ChCl_3$ extract which contained very few amounts of phenols and flavonoids, the EtOAc and *n*-BuOH extracts significantly quenched the DPPH radical and inhibited lipid peroxidation. In addition, these two latter extracts showed a significant antiproliferative effect against the human PC-3 prostate cancer cell line. The simultaneous antioxidant and antiproliferative effects observed may be ascribed to their rich content of phenols and flavonoids [10, 41]. In fact, gallic acid [42, 43] and rutin [44] in both EtOAc and *n*-BuOH extracts and catechins [17, 45] in the EtOAc extract are present within significant amounts and are known for their antioxidant and antiproliferative activity. In fact, the literature demonstrates that often natural compounds can act as both antioxidants and anticancer agents, taking advantage of the particular redox environment and signaling pathways in cancer cells [46, 47]. Interestingly, all green tea extracts used did not show any cytotoxicity effect in the normal BJ fibroblasts, which was an indirect confirmation that the use of the green tea chosen was safe generally for patients.

Seventy PC patients and 120 age-matched healthy subjects participated in the current study. The evaluation of lipid peroxidation and the antioxidant markers in the subset of Algerian PC patient's erythrocytes at the beginning of the

Table 3: Patients and respective control data.

	Patients, *n* (%)	Controls-tea[1], *n* (%)	Controls + green tea[2], *n* (%)
No. of subjects	70	85	35
Mean age ± SD	70.64 ± 6.5	68.5 ± 6.56	68.81 ± 6.42
p value[a]		0.1169[b]	0.2537[c]
Mean PSA ± SD (ng/ml)	62.73 ± 33.97	1.43 ± 0.92	1.27 ± 0.97
p value[c]		<0.0001[b]	<0.0001[c]
Gleason score			
5–7	28 (40)	—	—
8–10	42 (60)	—	—
Treatment			
Prostatectomy + hormone therapy	8 (11)	—	—
Radiotherapy + hormone therapy	8 (11)	—	—
Hormone therapy	34 (49)	—	—
Chemotherapy + hormone therapy	20 (29)	—	—

[1]Control individuals who never or rarely drank tea; [2]control individuals who usually drank 1 to 3 cups of green tea per day; [a]based on unpaired *t* test; [b]comparing PC patients to control individuals who never or rarely drank tea; [c]comparing PC patients to controls who usually drank 1 to 3 cups of green tea per day; PSA = prostate-specific antigen.

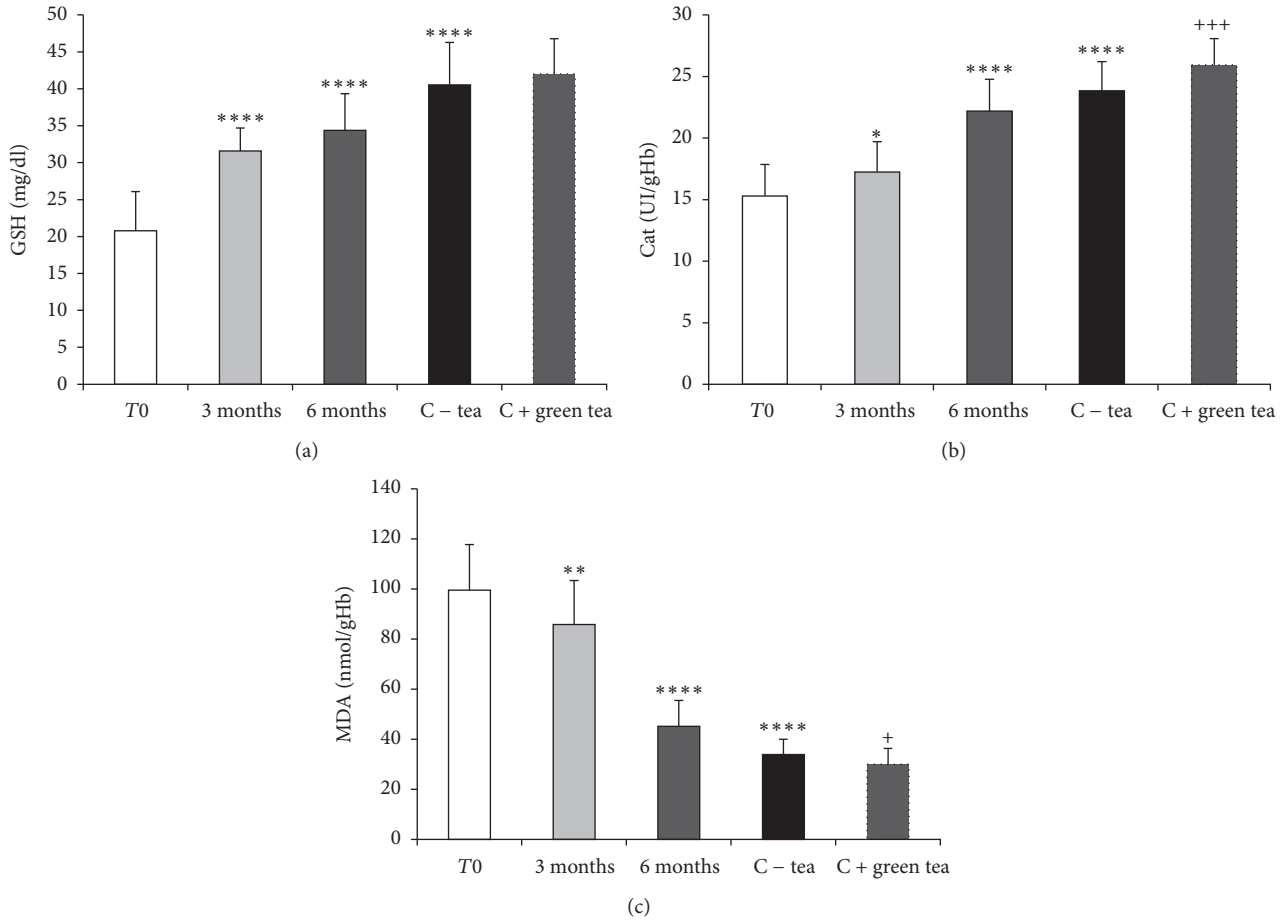

Figure 5: Effect of green tea consumption on lipid peroxidation and antioxidants status in erythrocytes from controls individuals and PC patients. (a) Effect of green tea consumption in erythrocytes GSH level in controls and PC patients. (b) Effect of green tea consumption in erythrocyte catalase activity in controls and PC patients. (c) Effect of green tea consumption in erythrocyte MDA level in controls and PC patients. *T*0: PC patients before green tea consumption; 3 months: PC patients after 3 months of green tea consumption; 6 months: PC patients after 6 months of green tea consumption; C – tea: control individuals who rarely or never drank tea; C + green tea: control individuals who usually drank 1 to 3 cups of green tea per day. Data are expressed as the means ± SD of three to four different experiments; $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{****}p < 0.0001$, compared to *T*0. $^{+++}p < 0.005$, $^{+}p < 0.05$, compared to C – tea.

study showed the presence of increased oxidative stress, including increased MDA concentration and low levels of reduced glutathione (GSH) and catalase activity were measured when compared to the respective healthy controls. These results agree with many others studies [6, 8, 48] and confirmed that this oxidant-antioxidant imbalance may be one of the major factors responsible for PC development and progression in humans. A recent review with twenty three case-control studies focusing on the role of oxidative stress in PC patients also demonstrated increased oxidative stress profiles and impairment of antioxidant defense systems in PC patients, concluding that oxidative biomarkers MDA and 8OH-dg as well as antioxidant parameters SOD, CAT, GSH enzyme family, and vitamins C and E may be potentially predictive biomarkers of PC [49].

Some epidemiological studies found that green tea consumption decreases significantly the risk of PC in different populations. Consumption of 3 daily cups of green tea in southeast China and 5 or more cups in Japan significantly decreased the risk of PC in these two populations [14, 15]. However, these results are conflicting with other studies which did not show any association between green tea consumption and PC [48]. In the present study, we found that the consumption of 2 to 3 cups per day of green tea during 3 months can significantly increase the level of GSH and catalase activity and decreased the level of MDA in PC patient's erythrocytes. After 6 months of green tea consumption, results from PC patients became close to that of controls who never or rarely drank tea. Our data confirmed that the commercial green tea used, which showed a potent effect in our in vitro assays, decreased oxidative stress markers and improved the antioxidative status in erythrocytes from the PC population studied. Hypothesizing that erythrocytes may be an adequate proxy for the prostate tissue, it is likely that green tea antioxidant effects may prevent PC progression. These results are in line with the few studies performed so far in humans [22, 24]. The study of Bettuzzi et al. [22] was the first study showing that green tea catechins were safe and very effective for treating premalignant lesions before PC development. No significant change in PSA was observed but 33% of PC inhibition was observed in a group of volunteers with high grade prostate intraepithelial neoplasia (HGPIN) after 1 year of daily treatment with green tea catechins. The work by McLarty et al. supported a potential role of tea polyphenols in the treatment or prevention of PC. A significant decrease in serum level of prostate-specific antigen (PSA), hepatocyte growth factor (HGF), vascular endothelial growth factor (VEGF), insulin-like growth factor (IGF-1), and IGF binding protein-3 (IGFBP-3) was observed with no elevation of liver enzymes in men with PC after a short-term supplementation with daily doses of polyphenon E (a total of 1.3 g of tea polyphenols) [24]. Our findings offer complementary information about the efficiency of green tea for PC management in human through its ability to regulate oxidative stress observed in PC patients in our and in many other studies [6, 8, 48]. Improving the antioxidant status in PC patients may reduce exaggerated ROS production and consequently reduce PC progression. It was previously reported that ROS activate different signaling pathways including mitogen-activated protein kinase (MAPKs) and phosphoinositide-3-kinase (PI3K)/Akt [50]. These two signaling pathways were found to be overactivated in PC and are suggested to be involved in PC development and progression [51, 52]. The study of Kumar et al. indicated that ROS generation is directly proportional to aggressive phenotype of PC and that antioxidant therapy decreased Akt expression and modulated MAPKs activities, delaying the proliferation of PC cell lines [53].

In the present study, we also reenforced the positive effect of green tea consumption in oxidative stress in humans, by comparing lipid peroxidation and antioxidants in erythrocytes from controls that never or rarely drank tea to a group of healthy men that drink usually 1 to 3 cups of green tea per day for a long period of time. The results obtained showed that green tea consumption may also improve overall the antioxidative status in healthy men. This finding suggests that regular consumption of green tea may reduce the oxidative stress produced during life and theoretically decrease its negative effects.

These benefits of the green tea may be very interesting in the case of PC characterized by a long latency period. It is typically diagnosed in 50-year-old men or older [54], but in autopsy studies both PC and high grade intraepithelial neoplasia (HGPIN) are detected in the 3rd decade, showing a steady increase with age [55].

One limitation of the present study is that the effects of green tea in PC gland tissue were not directly measured. Whether the antioxidant/antiproliferative effects of green tea extracts observed in vitro also occur in the prostate tissue or whether the oxidative stress measured in erythrocytes is also observed in the prostate gland tissue from PC patients is not known. Still, several other studies used this same approach [56–58]. This was not possible due to ethical concerns and refusal of PC patients to suffer repeated biopsies. Further studies in PC patients are needed to determine the effect of the green tea consumption in the oxidative stress in PC gland.

6. Conclusion

The current study identified increased oxidative stress in erythrocytes from Algerian PC patients. More importantly, we demonstrated here a commercial green tea investigated rich in polyphenols and flavonoids and presenting potent antioxidant and anticancer activities in vitro significantly decreased oxidative stress markers in PC patients. Regular consumption of green tea for a long period may protect individuals from the negative consequences of oxidative stress produced during life.

Additional Points

Chemical Compounds Studied in This Article. They are gallic acid (PubChem CID: 370); aluminum trichloride (PubChem CID: 24012); quercetin (PubChem CID: 5280343); 1,1-diphenyl-2-picrylhydrazyl (PubChem CID: 2735032); ascorbic acid (PubChem CID: 54670067); thiobarbituric acid (PubChem CID: 272368); penicillin (PubChem CID: 2349); streptomycin (PubChem CID: 19649); sulforhodamine B

(PubChem CID: 9916275); 5,5′-dithiobis(2-nitrobenzoic acid) (PubChem CID: 6254).

Disclosure

The funding agencies had no role in manuscript content or submission.

Competing Interests

The authors declare that they have no competing interests.

Authors' Contributions

Somia Lassed performed most of the experiments, analyzed results, generated figures, and wrote manuscript. Cláudia M. Deus and Radja Djebbari performed some experiments and analyzed the data. Paulo J. Oliveira designed some experiments and wrote part of the manuscript. Djamila Zama, Abderrezak Dahdouh, Fadila Benayache, and Samir Benayache conceived and supervised the study and designed experiments. All authors revised the final form of the manuscript.

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References

[1] J. Ferlay, I. Soerjomataram, R. Dikshit et al., "Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012," *International Journal of Cancer*, vol. 136, no. 5, pp. E359–E386, 2015.

[2] B. Kwabi-Addo, W. Chung, L. Shen et al., "Age-related DNA methylation changes in normal human prostate tissues," *Clinical Cancer Research*, vol. 13, no. 13, pp. 3796–3802, 2007.

[3] A. Minelli, I. Bellezza, C. Conte, and Z. Culig, "Oxidative stress-related aging: a role for prostate cancer?" *Biochimica et Biophysica Acta*, vol. 1795, no. 2, pp. 83–91, 2009.

[4] Y. Homma, Y. Kondo, M. Kaneko et al., "Promotion of carcinogenesis and oxidative stress by dietary cholesterol in rat prostate," *Carcinogenesis*, vol. 25, no. 6, pp. 1011–1014, 2004.

[5] W. G. Nelson, T. L. DeWeese, and A. M. DeMarzo, "The diet, prostate inflammation, and the development of prostate cancer," *Cancer and Metastasis Reviews*, vol. 21, no. 1, pp. 3–16, 2002.

[6] N. Kotrikadze, M. Alibegashvili, M. Zibribadze et al., "Activity and content of antioxidant enzymes in prostate tumors," *Experimental Oncology*, vol. 30, no. 3, pp. 244–247, 2008.

[7] B. Sandhya, S. Manoharan, G. Sirisha Lavanya, and C. Ratna Manmohan, "Lipid peroxidation and antioxidant status in prostate cancer patients," *Indian Journal of Science and Technology*, vol. 3, no. 1, pp. 83–86, 2010.

[8] D. S. L. Srivastava and R. D. Mittal, "Free radical injury and antioxidant status in patients with benign prostate hyperplasia and prostate cancer," *Indian Journal of Clinical Biochemistry*, vol. 20, no. 2, pp. 162–165, 2005.

[9] W. Al-Awaida, M. Akash, Z. Aburubaiha, W. H. Talib, and H. Shehadeh, "Chinese green tea consumption reduces oxidative stress, inflammation and tissues damage in smoke exposed rats," *Iranian Journal of Basic Medical Sciences*, vol. 17, no. 10, pp. 740–746, 2014.

[10] A. K. Dutta, M. A. Siddiquee, S. Hossain, and Y. Kabir, "Finlay green tea possesses the highest in vitro antioxidant activity among the 20 commercially available tea brands of Bangladesh," *Malaysian Journal of Pharmaceutical Sciences*, vol. 11, pp. 11–20, 2013.

[11] D. S. Albrecht, E. A. Clubbs, M. Ferruzzi, and J. A. Bomser, "Epigallocatechin-3-gallate (EGCG) inhibits PC-3 prostate cancer cell proliferation via MEK-independent ERK1/2 activation," *Chemico-Biological Interactions*, vol. 171, no. 1, pp. 89–95, 2008.

[12] T. Otsuka, T. Ogo, T. Eto, Y. Asano, M. Suganuma, and Y. Niho, "Growth inhibition of leukemic cells by (-)-epigallocatechin gallate, the main constituent of green tea," *Life Sciences*, vol. 63, no. 16, pp. 1397–1403, 1998.

[13] D. Picard, "The biochemistry of green tea polyphenols and their potential application in human skin cancer," *Alternative Medicine Review*, vol. 1, no. 1, pp. 31–42, 1996.

[14] L. Jian, L. P. Xie, A. H. Lee, and C. W. Binns, "Protective effect of green tea against prostate cancer: a case-control study in southeast China," *International Journal of Cancer*, vol. 108, no. 1, pp. 130–135, 2004.

[15] N. Kurahashi, S. Sasazuki, M. Iwasaki, M. Inoue, S. Tsugane, and J. S. Group, "Green tea consumption and prostate cancer risk in Japanese men: a prospective study," *American Journal of Epidemiology*, vol. 167, no. 1, pp. 71–77, 2008.

[16] S. Gupta, N. Ahmad, A.-L. Nieminen, and H. Mukhtar, "Growth inhibition, cell-cycle dysregulation, and induction of apoptosis by green tea constituent (-)-epigallocatechin-3-gallate in androgen-sensitive and androgen-insensitive human prostate carcinoma cells," *Toxicology and Applied Pharmacology*, vol. 164, no. 1, pp. 82–90, 2000.

[17] K. Hastak, S. Gupta, N. Ahmad, M. K. Agarwal, M. L. Agarwal, and H. Mukhtar, "Role of p53 and NF-κB in epigallocatechin-3-gallate-induced apoptosis of LNCaP cells," *Oncogene*, vol. 22, no. 31, pp. 4851–4859, 2003.

[18] M. Li, Z. He, S. Ermakova et al., "Direct inhibition of insulin-like growth factor-I receptor kinase activity by (-)-epigallocatechin-3-gallate regulates cell transformation," *Cancer Epidemiology Biomarkers & Prevention*, vol. 16, no. 3, pp. 598–605, 2007.

[19] I. A. Siddiqui, M. Asim, B. B. Hafeez, V. M. Adhami, R. S. Tarapore, and H. Mukhtar, "Green tea polyphenol EGCG blunts androgen receptor function in prostate cancer," *FASEB Journal*, vol. 25, no. 4, pp. 1198–1207, 2011.

[20] S. Gupta, K. Hastak, N. Ahmad, J. S. Lewin, and H. Mukhtar, "Inhibition of prostate carcinogenesis in TRAMP mice by oral infusion of green tea polyphenols," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 98, no. 18, pp. 10350–10355, 2001.

[21] C. E. Harper, B. B. Patel, J. Wang, I. A. Eltoum, and C. A. Lamartiniere, "Epigallocatechin-3-gallate suppresses early stage, but not late stage prostate cancer in TRAMP mice: mechanisms of action," *Prostate*, vol. 67, no. 14, pp. 1576–1589, 2007.

[22] S. Bettuzzi, M. Brausi, F. Rizzi, G. Castagnetti, G. Peracchia, and A. Corti, "Chemoprevention of human prostate cancer by oral administration of green tea catechins in volunteers with high-grade prostate intraepithelial neoplasia: a preliminary report from a one-year proof-of-principle study," *Cancer Research*, vol. 66, no. 2, pp. 1234–1240, 2006.

[23] A. Jatoi, N. Ellison, P. A. Burch et al., "A phase II trial of green tea in the treatment of patients with androgen independent metastatic prostate carcinoma," *Cancer*, vol. 97, no. 6, pp. 1442–1446, 2003.

[24] J. McLarty, R. L. H. Bigelow, M. Smith, D. Elmajian, M. Ankem, and J. A. Cardelli, "Tea polyphenols decrease serum levels of prostate-specific antigen, hepatocyte growth factor, and vascular endothelial growth factor in prostate cancer patients and inhibit production of hepatocyte growth factor and vascular endothelial growth factor in vitro," *Cancer Prevention Research*, vol. 2, no. 7, pp. 673–682, 2009.

[25] V. L. Singleton, R. Orthofer, and R. M. Lamuela-Raventos, *Methods in Enzymol: Oxidant and Antioxidants (Part A)*, Edited by M. I. S. John, N. Abelson, S. Helmut, Academic Press, 1999.

[26] H. Wang, X. D. Gao, G. C. Zhou, L. Cai, and W. B. Yao, "In vitro and in vivo antioxidant activity of aqueous extract from Choerospondias axillaris fruit," *Food Chemistry*, vol. 106, no. 3, pp. 888–895, 2008.

[27] A. Braca, N. De Tommasi, L. Di Bari, C. Pizza, M. Politi, and I. Morelli, "Antioxidant principles from *Bauhinia tarapotensis*," *Journal of Natural Products*, vol. 64, no. 7, pp. 892–895, 2001.

[28] Y. Cao and I. Ikeda, "Antioxidant activity and antitumor activity (in vitro) of xyloglucan selenious ester and surfated xyloglucan," *International Journal of Biological Macromolecules*, vol. 45, no. 3, pp. 231–235, 2009.

[29] M. E. Kaighn, K. S. Narayan, Y. Ohnuki, J. F. Lechner, and L. W. Jones, "Establishment and characterization of a human prostatic carcinoma cell line (PC-3)," *Investigative Urology*, vol. 17, no. 1, pp. 16–23, 1979.

[30] M. Lorenz, G. Saretzki, N. Sitte, S. Metzkow, and T. Von Zglinicki, "BJ fibroblasts display high antioxidant capacity and slow telomere shortening independent of hTERT transfection," *Free Radical Biology and Medicine*, vol. 31, no. 6, pp. 824–831, 2001.

[31] Z. X. Lin, J. R. S. Hoult, and A. Raman, "Sulphorhodamine B assay for measuring proliferation of a pigmented melanocyte cell line and its application to the evaluation of crude drugs used in the treatment of vitiligo," *Journal of Ethnopharmacology*, vol. 66, no. 2, pp. 141–150, 1999.

[32] S. Ali Azouaou, F. Emhemmed, N. Idris-Khodja et al., "Selective ROS-dependent p53-associated anticancer effects of the hypoxoside derivative rooperol on human teratocarcinomal cancer stem-like cells," *Investigational New Drugs*, vol. 33, no. 1, pp. 64–74, 2015.

[33] A. Tripathi, W. Fang, D. T. Leong, and L. T. Tan, "Biochemical studies of the lagunamides, potent cytotoxic cyclic depsipeptides from the marine cyanobacterium Lyngbya majuscula," *Marine Drugs*, vol. 10, no. 5, pp. 1126–1137, 2012.

[34] C. Astill, M. R. Birch, C. Dacombe, P. G. Humphrey, and P. T. Martin, "Factors affecting the caffeine and polyphenol contents of black and green tea infusions," *Journal of Agricultural and Food Chemistry*, vol. 49, no. 11, pp. 5340–5347, 2001.

[35] D. L. Drabkin and J. H. Austin, "Spectrophotometric Studies: I. Spectrophotometric constants for common hemoglobin derivatives in human, dog, and rabbit blood," *Journal of Biological Chemistry*, vol. 98, no. 2, pp. 719–733, 1932.

[36] E. Beutler, O. Duron, and B. M. Kelly, "Improved method for the determination of blood glutathione," *The Journal of Laboratory and Clinical Medicine*, vol. 61, pp. 882–888, 1963.

[37] R. A. Greenwald, *CRC Handbook of Methods for Oxygen Radical Research*, CRC Press, 1985.

[38] H. H. Draper and M. Hadley, "Malondialdehyde determination as index of lipid peroxidation," *Methods in Enzymology*, vol. 186, pp. 421–431, 1990.

[39] L. Khandrika, B. Kumar, S. Koul, P. Maroni, and H. K. Koul, "Oxidative stress in prostate cancer," *Cancer Letters*, vol. 282, no. 2, pp. 125–136, 2009.

[40] T. M. Vance, J. Su, E. T. H. Fontham, S. I. Koo, and O. K. Chun, "Dietary antioxidants and prostate cancer: a review," *Nutrition and Cancer*, vol. 65, no. 6, pp. 793–801, 2013.

[41] Y.-L. Lin, I.-M. Juan, Y.-L. Chen, Y.-C. Liang, and J.-K. Lin, "Composition of polyphenols in fresh tea leaves and associations of their oxygen-radical-absorbing capacity with antiproliferative actions in fibroblast cells," *Journal of Agricultural and Food Chemistry*, vol. 44, no. 6, pp. 1387–1394, 1996.

[42] K.-C. Liu, A.-C. Huang, P.-P. Wu et al., "Gallic acid suppresses the migration and invasion of PC-3 human prostate cancer cells via inhibition of matrix metalloproteinase-2 and -9 signaling pathways," *Oncology Reports*, vol. 26, no. 1, pp. 177–184, 2011.

[43] G.-C. Yen, P.-D. Duh, and H.-L. Tsai, "Antioxidant and pro-oxidant properties of ascorbic acid and gallic acid," *Food Chemistry*, vol. 79, no. 3, pp. 307–313, 2002.

[44] J. Yang, J. Guo, and J. Yuan, "In vitro antioxidant properties of rutin," *LWT—Food Science and Technology*, vol. 41, no. 6, pp. 1060–1066, 2008.

[45] S. Valcic, J. A. Burr, B. N. Timmermann, and D. C. Liebler, "Antioxidant chemistry of green tea catechins. New oxidation products of (−)-epigallocatechin gallate and (−)-epigallocatechin from their reactions with peroxyl radicals," *Chemical Research in Toxicology*, vol. 13, no. 9, pp. 801–810, 2000.

[46] K.-A. Hwang and K.-C. Choi, "Anticarcinogenic effects of dietary phytoestrogens and their chemopreventive mechanisms," *Nutrition and Cancer*, vol. 67, no. 5, pp. 796–803, 2015.

[47] H. Y. Khan, H. Zubair, M. F. Ullah, A. Ahmad, and S. M. Hadi, "A prooxidant mechanism for the anticancer and chemopreventive properties of plant polyphenols," *Current Drug Targets*, vol. 13, no. 14, pp. 1738–1749, 2012.

[48] N. Kikuchi, K. Ohmori, T. Shimazu et al., "No association between green tea and prostate cancer risk in Japanese men: the Ohsaki Cohort Study," *British Journal of Cancer*, vol. 95, no. 3, pp. 371–373, 2006.

[49] B. Oh, G. Figtree, D. Costa et al., "Oxidative stress in prostate cancer patients: a systematic review of case control studies," *Prostate International*, vol. 4, no. 3, pp. 71–87, 2016.

[50] G.-Y. Liou and P. Storz, "Reactive oxygen species in cancer," *Free Radical Research*, vol. 44, no. 5, pp. 479–496, 2010.

[51] V. Jendrossek, M. Henkel, J. Hennenlotter et al., "Analysis of complex protein kinase B signalling pathways in human prostate cancer samples," *BJU International*, vol. 102, no. 3, pp. 371–382, 2008.

[52] M. Royuela, M. I. Arenas, F. R. Bethencourt, B. Fraile, and R. Paniagua, "Regulation of proliferation/apoptosis equilibrium by mitogen-activated protein kinases in normal, hyperplastic, and carcinomatous human prostate," *Human Pathology*, vol. 33, no. 3, pp. 299–306, 2002.

[53] B. Kumar, S. Koul, L. Khandrika, R. B. Meacham, and H. K. Koul, "Oxidative stress is inherent in prostate cancer cells and is required for aggressive phenotype," *Cancer Research*, vol. 68, no. 6, pp. 1777–1785, 2008.

[54] J. P. Richie, W. J. Catalona, F. R. Ahmann et al., "Effect of patient age on early detection of prostate cancer with serum prostate-specific antigen and digital rectal examination," *Urology*, vol. 42, no. 4, pp. 365–374, 1993.

[55] P. T. Scardino, R. Weaver, and M. A. Hudson, "Early detection of prostate cancer," *Human Pathology*, vol. 23, no. 3, pp. 211–222, 1992.

[56] A. Woźniak, R. Masiak, M. Szpinda et al., "Oxidative stress markers in prostate cancer patients after HDR brachytherapy combined with external beam radiation," *Oxidative Medicine and Cellular Longevity*, vol. 2012, Article ID 789870, 5 pages, 2012.

[57] V. Battisti, L. D. K. Maders, M. D. Bagatini et al., "Oxidative stress and antioxidant status in prostate cancer patients: relation to Gleason score, treatment and bone metastasis," *Biomedicine and Pharmacotherapy*, vol. 65, no. 7, pp. 516–524, 2011.

[58] Z. Arsova-Sarafinovska, A. Eken, N. Matevska et al., "Increased oxidative/nitrosative stress and decreased antioxidant enzyme activities in prostate cancer," *Clinical Biochemistry*, vol. 42, no. 12, pp. 1228–1235, 2009.

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Research Article

Anticancer Effects of the Marine Sponge *Lipastrotethya* sp. Extract on Wild-Type and p53 Knockout HCT116 Cells

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Interest in marine bioresources is increasing in the drug development sector. In particular, marine sponges produce a wide range of unique metabolites that enable them to survive in challenging environments, which makes them attractive sources of candidate pharmaceuticals. In previous study, we investigated over 40 marine specimens collected in Micronesia and provided by the Korean Institute of Ocean Science and Technology, for their antiproliferative effects on various cancer cell lines, and *Lipastrotethya* sp. extract (LSSE) was found to have a marked antiproliferative effect. In the present study, we investigated the mechanism responsible for its anticancer effect on wild-type p53 (WT) or p53 knockout (KO) HCT116 cells. LSSE inhibited cell viability and induced apoptotic cell death more so in HCT116 p53 KO cells than the WT. HCT116 WT cells treated with LSSE underwent apoptosis associated with the induction of p53 and its target genes. On the other hand, in HCT116 p53 KO cells, LSSE reduced mTOR and Bcl-2 and increased Beclin-1 and LC3-II protein levels, suggesting autophagy induction. These results indicate that the mechanisms responsible for the anticancer effect of LSSE depend on p53 status.

1. Introduction

Primitive marine animals produce a diverse range of metabolites that enable them to flourish in the marine environment, and in particular, the wealth of unique metabolites produced by marine sponges has attracted the attention of those trying to develop new drugs, including anticancer drugs. Several articles have been published on the biological activities of marine sponges [1, 2], but the mechanisms responsible have yet to be elucidated.

Generally, agents that induce apoptosis or autophagy are of interest as potential cancer therapies [3, 4]. Apoptosis is called programmed cell death [5], is a critical developmental process, and is essential for tissue remodeling. Cells undergoing apoptosis exhibit characteristic features, such as DNA fragmentation and cytoplasm shrinkage, and because cancer cell apoptosis causes tumor regression, it is utilized for chemotherapy [6].

Autophagy controls cellular homeostasis and survival [7]. Initially, autophagy was regarded a tumor-suppressive mechanism, but it is also related to tumor progression [7, 8]. These conflicting effects of autophagy depend on cancer type and the microenvironment [9]. Thus, the cell death induced by autophagy could conceivably be utilized for anticancer therapy. On the other hand, p53 had an important anticancer effect and is functionally associated with apoptosis, autophagy, genome stability, and the cell cycle [10–12]. In a previous study, we found that the marine sponge *Lipastrotethya* sp. (Figure 1) inhibited the proliferation of cancer cells. In the present study, we investigated the mechanism responsible for its anticancer effects in HCT116 WT and HCT116 p53 KO cells.

2. Materials and Methods

2.1. Specimen Preparation. *Lipastrotethya* sp. extract was kindly provided by H.-S. Lee (Korea Institute of Ocean Science and Technology). The sample was collected by scuba diving in Chuuk state, Federated States of Micronesia, washed

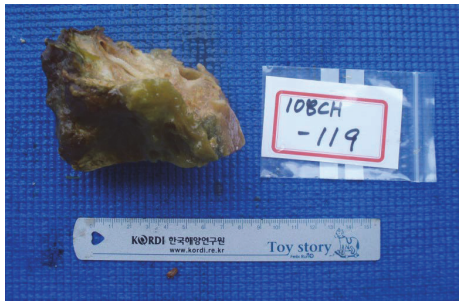


Figure 1: Morphology of *Lipastrotethya* sp.

with sterilized artificial sea water three times, immediately frozen, and stored at −20°C until required. The lyophilized specimen was extracted with methanol and dichloromethane; then it was dissolved in sterile distilled water. Aliquots of the sample were stored at −20°C until required [13, 14].

2.2. Cell Culture. Human colorectal carcinoma HCT116 (expressed wild-type p53) and HCT116p53KO cells were cultured in Dulbecco's modified Eagle's medium (DMEM, GenDEPOT) supplemented with 10% fetal bovine serum (GenDEPOT) and 1% penicillin/streptomycin (GenDEPOT) in a humidified 5% CO_2 incubator. Cells used for assays were in the exponential growth phase.

2.3. Cytotoxicity. Cell cytotoxicities were determined using the Cell Counting Kit-8 (CCK-8, DOJINDO, Japan) as previously described [14]. Briefly, cells were seeded in 96-well plates at 3×10^3 cells/well, incubated for 24 h, and treated with LSSE for 48 h. CCK-8 reagent (10 μL) was added to each well and incubated for 3 h at 37°C. Absorbance at 450 nm was determined using a microplate reader (Infinite M200 PRO, TECAN, Austria).

2.4. Western Blot. Cells were seeded in a 6-well plate at $4\sim6 \times 10^4$ cells/well. Samples were treated, added to each well, and incubated for 24 h. Cells were harvested and lysed in RIPA buffer (GenDEPOT) containing protease inhibitors (Xpert protease inhibitor cocktail solution, GenDEPOT) and phosphatase inhibitors (Xpert phosphatase inhibitor cocktail solution, GenDEPOT). Cell lysates were boiled in 5x sample buffer and separated by 10% SDS-PAGE. Proteins were transferred to PVDF membranes (Millipore) using a semidry electro blotter (Peqlab, Germany). Membranes were blocked with 5% skim milk in TBST (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.1% Tween 20) and incubated sequentially with primary antibodies at 4°C, overnight. Membranes were then incubated for 1 h at room temperature and probed with secondary antibody. Immunoreactive proteins were visualized using ECL reagents and detected using Chemi-Doc. Antibodies and the used were p53 (1 : 2000, Upstate), Hdm2/MDM2 (1 : 1000, Bioss), p14/Arf (1 : 1000, Cell Signaling), p21 (1 : 2000, Millipore), β-actin (1 : 5000, Sigma-Aldrich), Bax (1 : 1000, Cell Signaling), caspase-9 (1 : 1000, Cell Signaling), caspase-3 (1 : 1000, Cell Signaling), cleavage caspase-3 (1 : 1000, Cell Signaling), mTOR (1 : 10,000, Abcam), PUMA (1 : 1000), NOXA (1 : 1000), Bcl-2 (1 : 1000), Beclin-1 (1 : 1000, Cell Signaling), LC3 (1 : 2000, Abcam), anti-mouse IgG (H + L) horseradish peroxidase conjugate, and anti-rabbit IgG (H + L) horseradish peroxidase conjugate (1 : 3000, Bio-Rad).

2.5. Apoptosis Assay. HCT116 and HCT116 p53KO cells were seeded in six-well plates and treated with LSSE for 24 h. Incubated cells were stained using the Annexin V-FLUOS staining kit (Roche, Mannheim, Germany). Apoptotic cells were counted under an optical microscope (×20, DMi8, Leica, Germany).

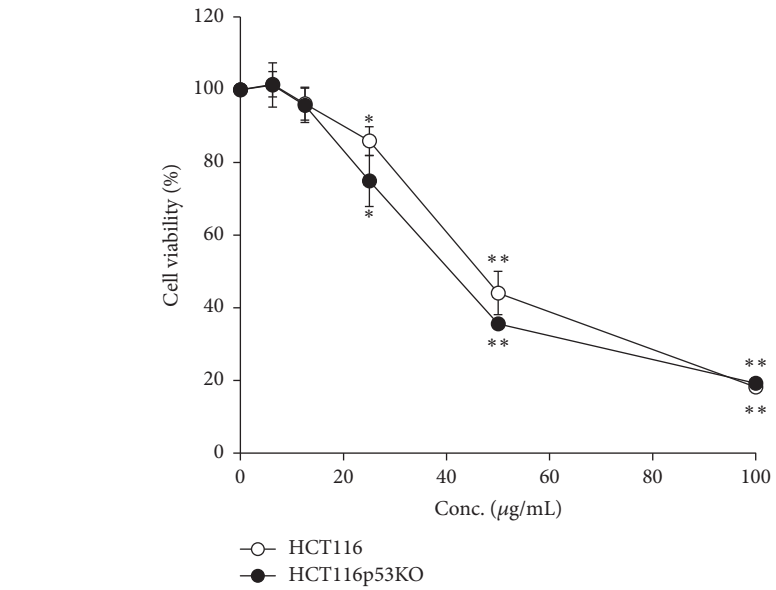
2.6. Statistical Analysis. Results are presented as means ± standard deviations. Student's t-test was used to determine the significance of intergroup comparisons, and statistical significance was accepted for p value < 0.05.

3. Results

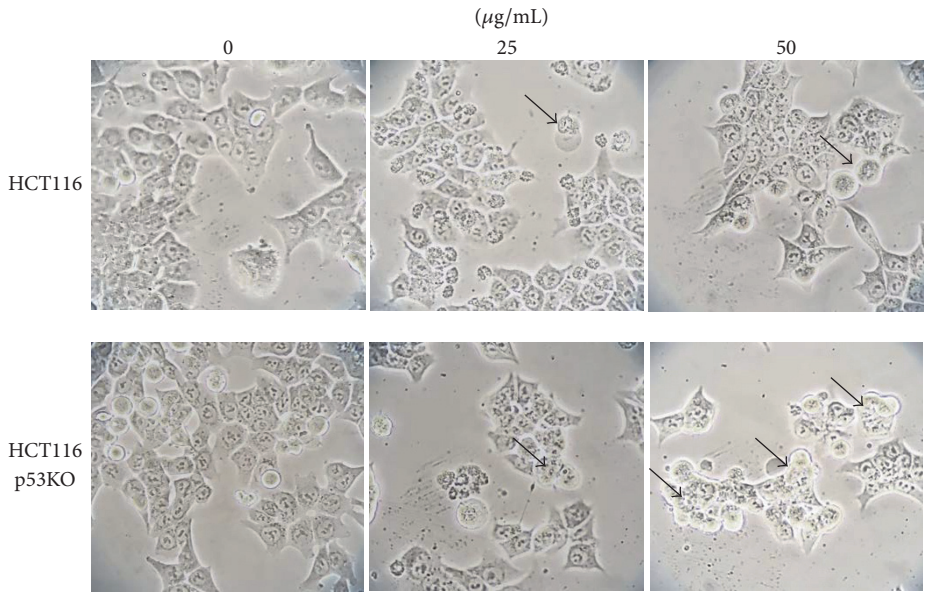
3.1. Inhibition of Cell Viability by Lipastrotethya sp. Extract. HCT116 and HCT116 p53KO cells were treated with *Lipastrotethya* sp. extract (LSSE) for 48 h and cell viability was determined using Cell Counting Kit-8. LSSE dose-dependently reduced cell viability in both cell lines (Figure 2(a)). The IC_{50} of LSSE was 44.8 and 38 μg/mL in HCT116 and HCT116 p53KO cells, respectively. LSSE inhibited the viability of HCT116 p53KO cells more so than that of HCT116 cells. Furthermore, HCT116 and HCT116 p53KO cell numbers were decreased and their morphologies were changed after the treatment with LSSE for 24 h (Figure 2(b)). In particular, HCT116 p53KO cells treated with LSSE (50 μg/mL) were spherical and floated (black arrows) on culture medium. These results suggested that HCT116 p53KO cells were more sensitive to LSSE than HCT116 cells.

3.2. Lipastrotethya sp. Extract-Induced Apoptosis. To elucidate the mechanism underlying the cell death induced by LSSE, we measured apoptotic cell numbers using an Annexin V-FITC assay. As shown in Figure 3(a), Annexin V-positive (apoptotic) cells were dose-dependently increased by LSSE in both cell lines. Percentages of apoptotic cells to total cells showed LSSE (50 μg/mL) significantly induced early apoptosis in HCT116 cells (17.2%) and HCT116 p53KO cells (30.8%) (Figure 3(b)). These results indicate LSSE induced apoptosis in both cell lines (Figure 2). Furthermore, LSSE induced more apoptosis in HCT116 p53KO cells.

3.3. Different Anticancer Mechanism of Lipastrotethya sp. Extract on HCT116 and HCT116p53KO Cells. P53 is key player in apoptotic cell death. Above all, p53 expression was confirmed in two cell lines (Figure 4(a)). As was expected in HCT116 WT cells, LSSE dose-dependently increased p53 levels and increased the levels of the p53 targeted genes p21 (Figure 4(a)) as well as NOXA, PUMA, and Bax (Figure 4(b)). Accordingly, we investigated the expressions of caspase 9 and caspase 3. Cleaved caspase 9 and cleaved caspase 3 were



(a)



(b)

Figure 2: Inhibition of cell viability by *Lipastrotethya* sp. extract. (a) Cells treated with *Lipastrotethya* sp. extract were incubated for 48 h. Cell viability was determined by Cell Counting Kit-8 as described in Materials and Methods. Data showed mean ± standard deviation ($n = 8$). $^{**}t$-test ($p < 0.01$). (b) Morphology of cells treated with *Lipastrotethya* sp. extract. Cells were incubated for 24 h and observed by microscope (×40). $^{*}t$-test ($p < 0.05$).

slightly increased by the treatment of LSSE (Figure 4(b)). These results suggested that LSSE caused p53-mediated apoptosis through the intrinsic pathway in HCT116 cells. Additionally, we investigated the expression of p14/Arf and HDM2 as p53 regulators in HCT116 treated with LSSE (Figure 4(a)). The levels of p14 and HDM2 showed no difference between control and LSSE treatment. LSSE might induce p53 induction via the other pathway.

On the other hand, p53 deficiency was confirmed in HCT116 p53KO cells regardless of LSSE (Figure 4(a)). P21 as one of p53-targeted genes is known to be a cyclin-dependent kinase inhibitor and arrests cell cycle [15]. Also, p21 can cause apoptosis via the p53-independent pathway [16]. Thus, we investigated p21 levels in HCT116 p53 KO cells after treatment with LSSE, but no induction of p21 was observed (Figure 4(a)). Nevertheless, because LSSE had a greater apoptotic effect in HCT116 p53 KO cells, we further investigated the mechanism of cell death in these cells. As shown in Figure 4(c), *Lipastrotethya* sp. suppressed mTOR and Bcl-2 levels and increased LC3-II and Beclin-1 levels. As these factors

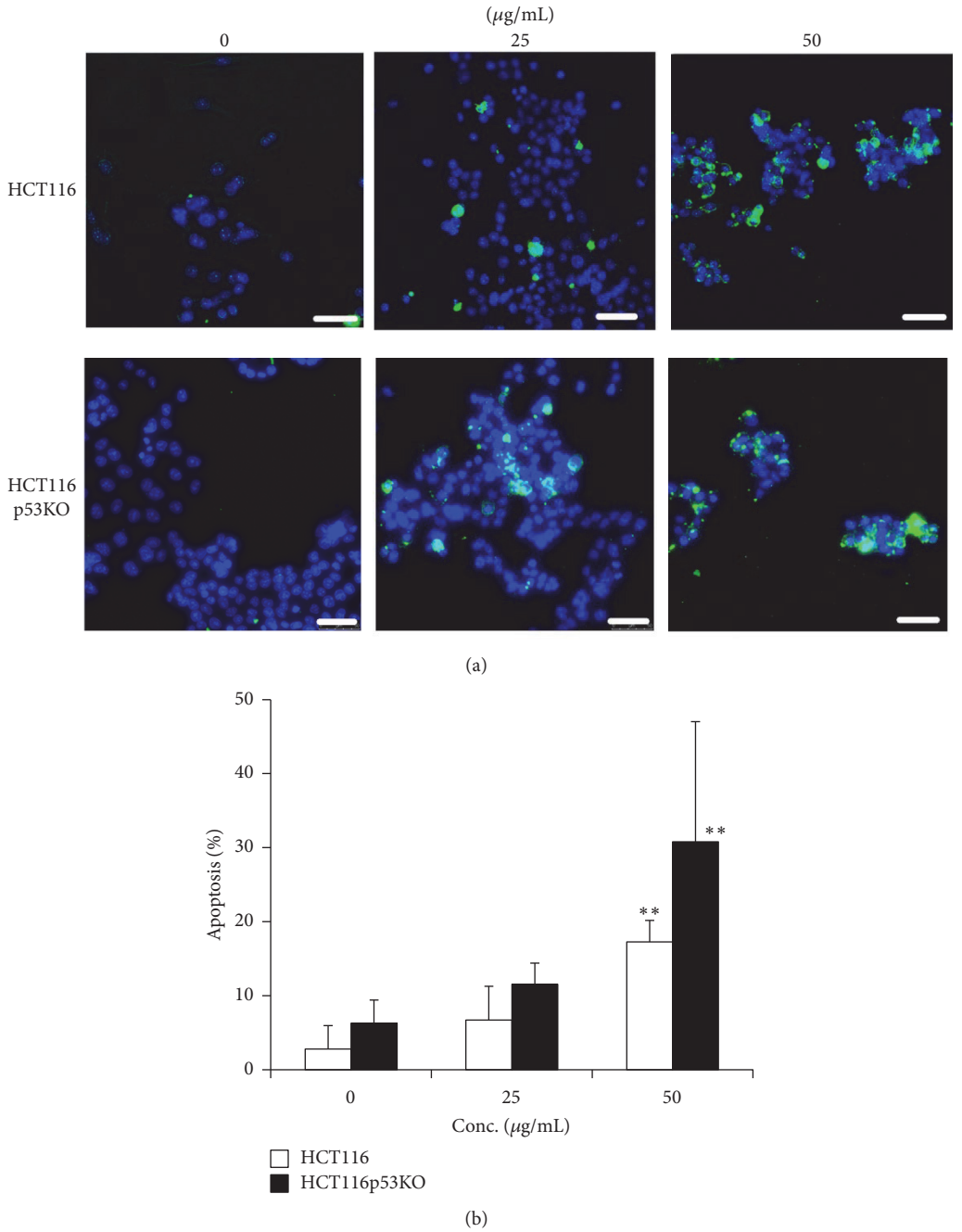


Figure 3: Induction of apoptosis by *Lipastrotethya* sp. extract. Cells treated with *Lipastrotethya* sp. extract were incubated for 24 h. Apoptotic cells were measured by Annexin V staining as described in Materials and Methods. (a) Green fluorescent cells show apoptotic cells. DAPI staining represents nucleus (Blue). Scale bar, 50 μm. (b) Data showed mean ± standard deviation ($n = 5$). $^{**}t$-test ($p < 0.01$).

are markers of autophagy, there results suggested that LSSE induced autophagy-related cell death in HCT116 p53KO cells.

4. Discussion

The present study was undertaken to investigate the mechanism responsible for the anticancer effect of the extract of *Lipastrotethya* sp. (LSSE) on wide-type p53 (WT) and p53 knockout (KO) HCT116 cells. LSSE had an antiproliferative effect on HCT116 cells regardless of p53 expression, and interestingly, the IC_{50} of LSSE in HCT116 p53KO cells was lower than in HCT116 cells (Figure 1). Usually, induction of p53 expression triggers apoptosis and increases anticancer effects because p53 is a tumor-suppressor gene. In the present study, LSSE increased the expressions of p53-target genes like p21, PUMA, and NOXA and induced apoptosis *via* intrinsic pathway in p53 expressing WT HCT116 cells (Figure 4(b)). Furthermore, we investigated whether LSSE activates apoptosis

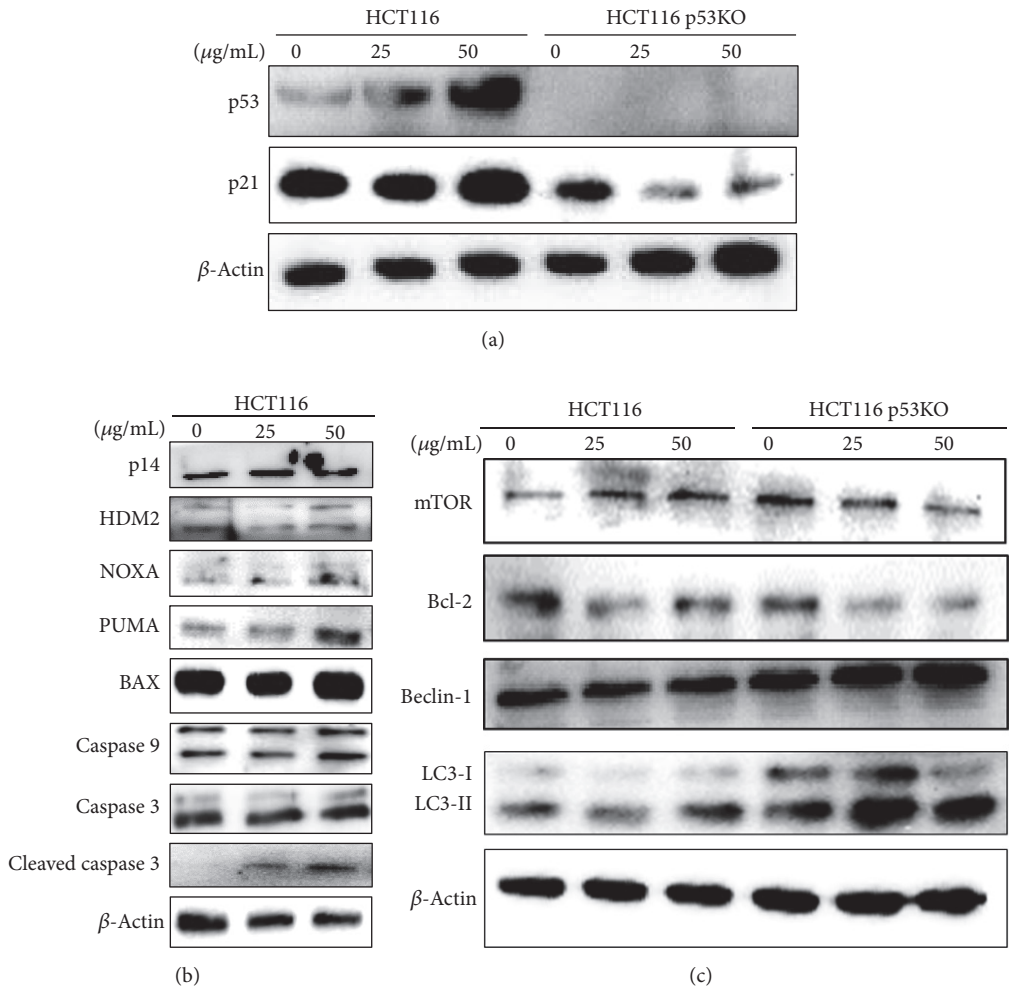


Figure 4: Effect of *Lipastrotethya* sp. extract on the expression of cell death-related proteins. Cells treated with *Lipastrotethya* sp. extract were incubated for 24 h. (a) p53 level and p21 level in HCT116 and HCT116 p53KO cells treated with *Lipastrotethya* sp. extract. (b) Apoptosis-related protein expression was analyzed by western blotting in HCT116 cells treated with *Lipastrotethya* sp. extract. (c) Autophagy-related protein expression was analyzed by western blotting in HCT116 and HCT116 p53KO cells treated with *Lipastrotethya* sp. extract.

via the Arf-HDM2/Mdm2-p53 tumor-suppressor pathway. P14/Arf is known to control the p53 activation *via* suppression of HDM2 [17]. However, our results showed no difference of p14 level between control and LSSE treatment as well as HDM2. In particular, HDM2 acts as both positive and negative regulator of p53 activity depending on stresses [18]. These results suggested that LSSE might induce the p53 level in response not to oncogenes stress but to DNA damage. Generally, deletion or loss of p53 is associated with chemoresistance and a poor prognosis [19]. Furthermore, cancer cells exhibiting p53 deletion or loss are aggressive and associated with poor survival [20]. Thus, new anticancer drugs are needed to treat aggressive or resistant cancers exhibiting p53 deletion or loss. In this study, LSSE triggered autophagy in HCT116 p53 KO cells by decreasing mTOR and increasing LC3-II protein levels (Figure 4(c)) and, thus, induced cell death (Figure 3). These results suggested that LSSE is good candidate treatment for p53-deficient colorectal cancer cells. Unfortunately, the active components of LSSE responsible for cell death mediated autophagy in p53 KO cells were not identified. Nevertheless, several components such as nortriterpene glycosides of sarasinoside have been previously isolated from *Lipastrotethya* sp. and shown to be toxic to A549 cells [21]. In addition, triterpene galactosides of pouoside have also been identified in *Lipastrotethya* sp. [22]. Several triterpenoids isolated from marine animals have been shown to have anticancer activities [23] and this structure has the benefits for several disease including cancers [24]. Moreover, our findings regarding the anticancer effects of *Lipastrotethya* sp. concur with those of previous studies.

5. Conclusions

Our results indicate that the mechanisms responsible for the anticancer effect of LSSE depend on p53 status: that is, in the HCT116 WT cells LSSE induced apoptosis via the intrinsic pathway but in HCT116 p53 KO cells, LSSE induced an autophagic response.

Competing Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

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References

[1] Y. Sumii, N. Kotoku, A. Fukuda, T. Kawachi, M. Arai, and M. Kobayashi, "Structure-activity relationship and *in vivo* antitumor evaluations of dictyoceratin-A and -C, hypoxia-selective growth inhibitors from marine sponge," *Marine Drugs*, vol. 13, no. 12, pp. 7419–7432, 2015.

[2] D. T. A. Youssef, L. A. Shaala, and K. Z. Alshali, "Bioactive hydantoin alkaloids from the Red Sea marine sponge *Hemimycale arabica*," *Marine Drugs*, vol. 13, no. 11, pp. 6609–6619, 2015.

[3] S.-Y. Wu, P.-J. Sung, Y.-L. Chang, S.-L. Pan, and C.-M. Teng, "Heteronemin, a spongean sesterterpene, induces cell apoptosis and autophagy in human renal carcinoma cells," *BioMed Research International*, vol. 2015, Article ID 738241, 13 pages, 2015.

[4] H.-Y. Lee, K. J. Chung, I. H. Hwang et al., "Activation of p53 with ilimaquinone and ethylsmenoquinone, marine sponge metabolites, induces apoptosis and autophagy in colon cancer cells," *Marine Drugs*, vol. 13, no. 1, pp. 543–557, 2015.

[5] M. R. Alison and C. E. Sarraf, "Apoptosis: a gene-directed programme of cell death," *Journal of the Royal College of Physicians of London*, vol. 26, no. 1, pp. 25–35, 1992.

[6] T. G. Cotter, S. V. Lennon, J. G. Glynn, and S. J. Martin, "Cell death via apoptosis and its relationship to growth, development and differentiation of both tumour and normal cells," *Anticancer Research*, vol. 10, no. 5, pp. 1153–1159, 1990.

[7] E. White, "The role for autophagy in cancer," *Journal of Clinical Investigation*, vol. 125, no. 1, pp. 42–46, 2015.

[8] J. Y. Guo, B. Xia, and E. White, "Autophagy-Mediated tumor Promotion," *Cell*, vol. 155, no. 6, pp. 1216–1219, 2013.

[9] A. Hjelmeland and J. Zhang, "Metabolic, autophagic, and mitophagic activities in cancer initiation and progression," *Biomedical Journal*, vol. 39, no. 2, pp. 98–106, 2016.

[10] E. Ratovitski, "Tumor protein (TP)-p53 members as regulators of autophagy in tumor cells upon marine drug exposure," *Marine Drugs*, vol. 14, no. 8, p. 154, 2016.

[11] L. Zhang, D. Xie, X. Chen et al., "p53 Mediates colistin-induced autophagy and apoptosis in PC-12 cells," *Antimicrobial Agents and Chemotherapy*, vol. 60, no. 9, pp. 5294–5301, 2016.

[12] K. M. Livesey, R. Kang, P. Vernon et al., "p53/HMGB1 complexes regulate autophagy and apoptosis," *Cancer Research*, vol. 72, no. 8, pp. 1996–2005, 2012.

[13] J.-W. Lee, H.-S. Lee, J. Shin et al., "Suvanine analogs from a *Coscinoderma* sp. marine sponge and their cytotoxicities against human cancer cell lines," *Archives of Pharmacal Research*, vol. 38, no. 6, pp. 1005–1010, 2015.

[14] H. K. Lim, W. Bae, H.-S. Lee, and J. Jung, "Anticancer activity of marine sponge *Hyrtios* sp. extract in human colorectal carcinoma RKO cells with different p53 status," *BioMed Research International*, vol. 2014, Article ID 413575, 5 pages, 2014.

[15] A. L. Gartel and A. L. Tyner, "The role of the cyclin-dependent kinase inhibitor p21 in apoptosis," *Molecular Cancer Therapeutics*, vol. 1, no. 8, pp. 639–649, 2002.

[16] H. Ko, J. M. Kim, S.-J. Kim, S. H. Shim, C. H. Ha, and H. I. Chang, "Induction of apoptosis by genipin inhibits cell proliferation in AGS human gastric cancer cells via Egr1/p21 signaling pathway," *Bioorganic and Medicinal Chemistry Letters*, vol. 25, no. 19, pp. 4191–4196, 2015.

[17] E. Kim and J. Shohet, "Targeted molecular therapy for neuroblastoma: the ARF/MDM2/p53 axis," *Journal of the National Cancer Institute*, vol. 101, no. 22, pp. 1527–1529, 2009.

[18] P.-J. Hamard and J. Manfredi, "Mdm2's dilemma: to degrade or to translate p53?" *Cancer Cell*, vol. 21, no. 1, pp. 3–5, 2012.

[19] D. Maglic, S. Zhu, E. A. Fry et al., "Prognostic value of the hDMP1-ARF-Hdm2-p53 pathway in breast cancer," *Oncogene*, vol. 32, no. 35, pp. 4120–4129, 2013.

[20] E. K. Markert, H. Mizuno, A. Vazquez, and A. J. Levine, "Molecular classification of prostate cancer using curated expression signatures," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 108, no. 52, pp. 21276–21281, 2011.

[21] J.-H. Lee, J.-E. Jeon, Y.-J. Lee et al., "Nortriterpene glycosides of the sarasinoside class from the sponge Lipastrotethya sp," *Journal of Natural Products*, vol. 75, no. 7, pp. 1365–1372, 2012.

[22] J.-H. Lee, K. H. Jang, Y.-J. Lee et al., "Triterpene galactosides of the pouoside class and corresponding aglycones from the sponge *Lipastrotethya* sp," *Journal of Natural Products*, vol. 74, no. 12, pp. 2563–2570, 2011.

[23] Y.-X. Li, S. W. A. Himaya, and S.-K. Kim, "Triterpenoids of marine origin as anti-cancer agents," *Molecules*, vol. 18, no. 7, pp. 7886–7909, 2013.

[24] J. M. R. Patlolla and C. V. Rao, "Triterpenoids for cancer prevention and treatment: current status and future prospects," *Current Pharmaceutical Biotechnology*, vol. 13, no. 1, pp. 147–155, 2012.

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Research Article

Spatholobus suberectus Column Extract Inhibits Estrogen Receptor Positive Breast Cancer via Suppressing ER MAPK PI3K/AKT Pathway

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Although Chinese herbal compounds have long been alternatively applied for cancer treatment in China, their treatment effects have not been sufficiently investigated. The Chinese herb *Spatholobus suberectus* is commonly prescribed to cancer patients. HPLC analysis has shown that the main components of *Spatholobus suberectus* are flavonoids that can be classified as phytoestrogens, having a structure similar to estrogen. This study was designed to investigate the effects of *Spatholobus suberectus* column extract (SSCE) on the estrogen receptor-positive (ER+) breast cancer cell line MCF-7 and its possible molecular mechanism. In our study, MTT assay was performed to evaluate cell viability. The results show that SSCE (80, 160, and 320 μg/ml) significantly decreased the viability of MCF-7 cells. SSCE also triggered apoptosis, arrested the cell cycle at the G0/G1 phase, and inhibited cell migration. A dual-luciferase reporter system showed that SSCE suppressed intranuclear p-ER activity; Western blot analysis confirmed the repressed expression of phosphorylated-ER alpha (p-ERα), ERK1/2, p-ERK1/2, AKT, p-AKT, p-mTOR, PI3K, and p-PI3K, indicating that SSCE suppressed the MAPK PI3K/AKT signaling pathway. Collectively, our results suggest that SSCE causes apoptosis, an arrest in the G0/G1 phase, and a decrease in migration in ER+ MCF-7 cells via hypoactivity of the ER and suppression of the MAPK PI3K/AKT pathway.

1. Introduction

Breast cancer is the most common malignancy and the leading cause of cancer-related morbidity and mortality among women worldwide [1]. Endocrine therapy represents a major treatment in all settings of the disease for estrogen receptor-positive breast cancers, which account for approximately 70% of mammary malignancies [2]. In this subgroup of breast cancers, endocrine therapies are effective both in the adjuvant and in the recurrent settings; however, resistance remains a major issue [3].

Spatholobus suberectus Dunn, belonging to the legume family (Fabaceae), is called "chicken blood vines" in China due to its red juice, similar in appearance to blood, that flows out when the vine is injured [4]. It has been used to treat a variety of diseases such as anemia [5], menoxenia, and rheumatism in traditional Chinese medicine [6, 7]. The Taiwanese National Health Insurance Research Database showed that *Spatholobus suberectus* is the 8th most common single herb in adjunctive Chinese herbal medicine therapy for chronic myeloid leukemia patients, and Chinese herbal medicine therapy improves the survival of patients with chronic myeloid leukemia [8]. A few studies have been conducted reporting various types of flavonoids as the principal characteristic components in this herb [9]. Flavonoids are phytoestrogens, which have a similar structure to estrogen and can bind to estrogen receptors.

Table 1: The components of SSCE detected by HPLC.

Peak	Components	Ratio in subfraction
1	Protocatechuic acid	0.704%
2	Unknown	1.183%
3	p-Hydroxybenzoic acid	0.486%
4	Epicatechin	0.927%
5	Puerarin	0.800%
6	Unknown	2.836%
7	Unknown	1.890%
8	Daidzein	9.504%
9	Glycyrrhizin	4.369%
10	Calycosin	4.555%
11	Unknown	5.340%
12	Genistein	7.204%
13	Formononetin	39.418%
14	Unknown	9.221%
15	Unknown	8.276%
16	Prunetin	3.288%

As an alternative therapy, traditional Chinese herb medicine is becoming accepted by more and more patients for controlling cancer [10], especially in the population who cannot tolerate increasingly intense conventional chemotherapy. Spatholobi Caulis is wildly used by mammary tumor patients in China; however, to date, its treatment effects and potential mechanisms have not been sufficiently investigated. It is of great importance to elucidate the effective mechanisms to support the clinical use of Spatholobi Caulis in ER-positive breast cancer patients. Our present research was designed to examine the effects of the extracts from *Spatholobus suberectus* (SSCE) on the functions of ER+ MCF-7 cells. The results show that SSCE inhibited cell growth via suppression of the MAPK PI3K/AKT pathway.

2. Material and Methods

2.1. Preparation of SSCE. *Spatholobus suberectus* column extract (SSCE) was prepared by the Beijing Institute of traditional Chinese Medicine. *Spatholobus suberectus* was obtained from Beijing Xinglin Pharmaceutical Co., Ltd. (place of production: Yunnan, Lot number: 11062601). *Spatholobus suberectus* (100 g) was extracted in 80% ethanol (500 ml) at room temperature over night followed by 2 h heating-refluxing extraction three times. After cooling down to room temperature, the mixture was filtered through a 180-mesh sieve, and the solvent was removed. The crude extract of *Spatholobus suberectus* was dissolved in deionized water and centrifuged (3500 r min^{-1}, 20 min). The supernatant was collected and analyzed on a polyamide resin column (Φ1 cm, column height 11 cm). The column was eluted with deionized water and the elute was extracted with three passes of diethyl ether (10, 10, and 5 ml) and pooled. The ether was evaporated and 276 mg of SSCE was obtained. The chemical composition of SSCE was determined by HPLC analysis (Beijing Institute of Traditional Chinese Medicine) as summarized in Table 1.

2.2. Chemicals, Reagents, and Antibodies. Bax and p-ERβ were purchased from Abcam (Cambridge, MA, USA). Other antibodies were all purchased from Cell Signaling Technology (Danvers, MA, USA). Cell culture medium (DMEM), penicillin-streptomycin, and fetal bovine serum (FBS) were purchased from Gibco (NY, USA). The plasmid was purchased from Promega. The dual-luciferase assay kit was purchased from Vigorous (Beijing, China). All primers were synthesized by Invitrogen™, Thermo Fisher Scientific.

2.3. Cell Culture. Human ER+ breast cancer cells (MCF-7) were obtained from The Basic Medical Research Institute of Medical Sciences Chinese cell resource center. The cells were cultured in high glucose DMEM medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin and incubated at 37°C in a humidified atmosphere with 5% CO_2. The media was changed to DMEM without phenol red (Gibco number 31053-028) and 10% charcoal-stripped fetal bovine serum (Gibco number 12676-029) with 1% penicillin-streptomycin 24 h prior to the experiments.

2.4. Cell Viability Assay. MTT assay was performed to quantify cell viability. MCF-7 cells were seeded into 96-well plates (2×10^3 cells per well) for 24 h and then exposed to various concentrations of E2 (10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8}, 10^{-9}, 10^{-10}, 10^{-11}, and 10^{-12} M), SSCE (0, 40, 80, 160, and 320 μg/ml), and E2 at 10^{-8} M coincubated with SSCE (0, 40, 80, 160, and 320 μg/ml). After 24, 48, and 72 h, the cells were incubated with 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) at 5 mg/ml and 10 μl/well for 4 h. The culture medium was then discarded, and cells were lysed in DMSO with gentle shaking for 15 sec. The optical density (OD) values were measured at 570 nm using a Multiskan Spectrum (Thermo Scientific, USA). Experiments were performed in triplicate.

2.5. Flow Cytometry Assay. Apoptosis was demonstrated and quantified by flow cytometry analysis using an apoptosis kit (Cat. number KGA7073 Nanjing KeyGen Biotech Co.). MCF-7 cells were seeded into 6-well plates (2×10^5/well). After treatment with SSCE (0, 160 μg/ml) for 24 h, MCF-7 cells were trypsinized by trypsin without EDTA, centrifuged at 1200 r for 3 min, washed twice with PBS, and stained with Annexin V-fluorescein isothiocyanate (FITC) and propidium iodide (PI) according to the manufacturer's instructions. The cells were then filtered with a 300-mesh filter. Apoptotic cells were detected with a Beckman flow cytometry system (EPICS XL, Beckman) and analyzed with FlowJo 7.6.1 software (Tree Star). Each assay included at least 10,000 gated events. The early apoptotic cells are Annexin V-FITC positive and PI negative, while late apoptotic cells are Annexin V-FITC and PI double-positive. Experiments were performed in triplicate.

The cell cycle was observed and quantified using propidium iodide (PI) staining for DNA by flow cytometry analysis. MCF-7 cells were seeded into 6-well plates (2×10^5/well) with phenol red-free DMEM media and charcoal-stripped fetal bovine serum. In addition, the cells were treated with 10^{-8} M E2 (as control), 10^{-8} M E2 and SSCE at the indicated

concentration (0, 160, and 320 μg/ml), 10^{-8} M E2 and 10^{-4} M ICI182780, and DMEM without E2 for 24 h. The cells were harvested and washed twice with ice-cold PBS. Cells were fixed in 70% ethanol overnight and washed once with PBS. After centrifugation at 1200 r for 3 min and resuspension in 400 μl PBS with 50 μg/ml propidium iodide (PI) and 20 μg/ml RNase A, the cells were incubated for 30 min at 37°C in the dark and filtered with a 300-mesh filter. PI fluorescence was detected using a Beckman flow cytometry system (EPICS XL, Beckman) and analyzed with FlowJo 7.6.1 software (Tree Star). Each assay included at least 10,000 gated events. Experiments were performed in triplicate.

2.6. Wound Healing Assays. Cells were seeded in 6-well tissue culture plates at 5×10^5 per well. When the cells grew into a monolayer, a scratch was created using a sterile p200 pipette. The scratch was created vertically in the middle of each well. The medium was removed, and the detached cells were removed by rinsing each well with 1 ml PBS. Formation of the wound was confirmed by inverted light microscopy. After addition of phenol red-free serum-free media, the cells were then incubated with SSCE (160 μg/ml), estradiol (10^{-8} M), and SSCE (160 μg/ml) + estradiol (10^{-8} M) and monitored by microscopic observation. Two vertical lines on each side of the scratch and one horizontal line, separating the wound in half, were placed with an indelible marker on the outside bottom of each well to ensure that the same field was identified during subsequent image acquisition. These markings served as reference points for photographic documentation. The change in the wound surface area was compared among groups over time. Digital photographic images were obtained at 0 h and 24 h using a motorized inverted microscope. Following the acquisition of all images, the surface area of each scratch was measured and outlined by two independent observers (blinded to the group situation) using Adobe Photoshop software. The surface area of each wounded region of the cell monolayer was then transformed into a square of equal surface area, and the linear mean length of each square was compared among groups. The rate of closure was quantified and compared between all groups for statistical analysis. Three replicate wells were used for each experimental condition. Experiments were performed in triplicate.

2.7. Western Blot Assay. MCF-7 cells were exposed to SSCE at the indicated concentration (0, 80, 160, and 320 μg/ml) and incubated for 24 h. The cells were washed once with PBS and collected. The cells were lysed for 30 min in lysis buffer (Beyotime, P0013K) containing cOmplete EDTA-free protease inhibitor (Roche, Cat. number 04693132001; 1 tablet suspended in 1 ml ultrapure water and added 1 : 25 to the lysis buffer) and PhosSTOP (Roche, Cat. number 04906845001; 1 tablet suspended in 1 ml ultrapure water and added 1 : 10 to lysis buffer). The lysates were subjected to shaking once every five min for the 30 min incubation. Then, the lysates were centrifuged at 12000 r for 15 min. The supernatants were transferred into new Eppendorf tubes, and the total protein was quantified. The samples were separated in a 10% gradient SDS-PAGE (the proteins were separated in an 8% gradient SDS-PAGE for the detection of mTOR and p-mTOR) and transferred onto nitrocellulose membranes. The membrane was blocked using a blocking buffer (5% milk, 20 mmol/l Tris-HCl, pH 7.6, 150 mmol/l NaCl, and 0.1% Tween 20) at room temperature for 1.5 h. The membranes were then incubated with antibodies against PCNA, ERK1/2, p-ERK1/2, PI3K, p-PI3K, AKT, p-AKT, mTOR, p-mTOR, cytochrome C, Bax, Bcl-2, phosphorylated ERα (Ser118), phosphorylated ERβ (Ser105), and β-actin, followed by incubation with secondary antibodies. Densitometric analysis of the immunoblots was performed using an Odyssey infrared imaging system (LI-COR Biosciences Company, USA). Experiments were performed in triplicate.

2.8. Dual-Luciferase Assay. MCF-7 cells were seeded into 24-well plates at the exponential phase of growth (approximately 2×10^5/well) and cultured for 18–24 h. Then, the serum containing medium was replaced by OPTI-MEM for transfection using Lipo2000 mixed with different plasmids, including 3X ERE TATA luc, pcDNA Flag ER beta, pEGFP-C1-ER alpha, pFLAG-CMV2, pEGFP-C1, and the Firefly luciferase plasmid: pGL3-Basic Vector and pRL-TK Vector at a 2000 : 1 ratio (1 μl lipo2000 : 0.5 μg plasmid). After 5 h, the cells were changed from the transfection medium to the normal medium with serum. The cells were cultured for 24 h before addition of SSCE. After a12 h incubation, the cells were collected, counted, and seeded onto 96-well plates with each sample in 3 wells. The luciferase activity was detected following the instructions in the dual-luciferase assay kit (Vigorous, Cat # T002). Briefly, the cells were washed with PBS, lysed in 30 μl/well 1x Universal Lysis Buffer (ULB), gently shaken for 10 min, and centrifuged at 1200 r for 0.5 min. The supernatants were placed in new Eppendorf tubes for luminescence detection. To detect the Firefly luciferase and the sea kidney luciferase, specific steps were carried out according to kit instructions.

2.9. Statistical Analysis. Data for cell proliferation were converted into percentages, where the values for the control condition were formulated as 100%. All data were presented in comparison to controls. All experiments were performed in triplicate. Statistical significance of the difference was calculated using Student's t-test for paired data with the level of significance selected at $P < 0.05$.

3. Results

SSCE inhibited the vitality of breast cancer MCF-7 cells both in the presence and in the absence of E2. We first examined the effect of different concentrations of E2 on the viability of ER+ MCF-7 cells using MTT assay and found that, at the concentration of 10^{-8} M E2, the cells exhibited the highest viability with approximately 35% proliferation (data not shown). SSCE inhibited the proliferation of the MCF-7 cells in a dose-dependent manner (Figure 1(a)) with an estimated IC50 value of 109 μg/ml after 24 h incubation. In the presence of E2 at the concentration of 10^{-8} M, the effect of SSCE was

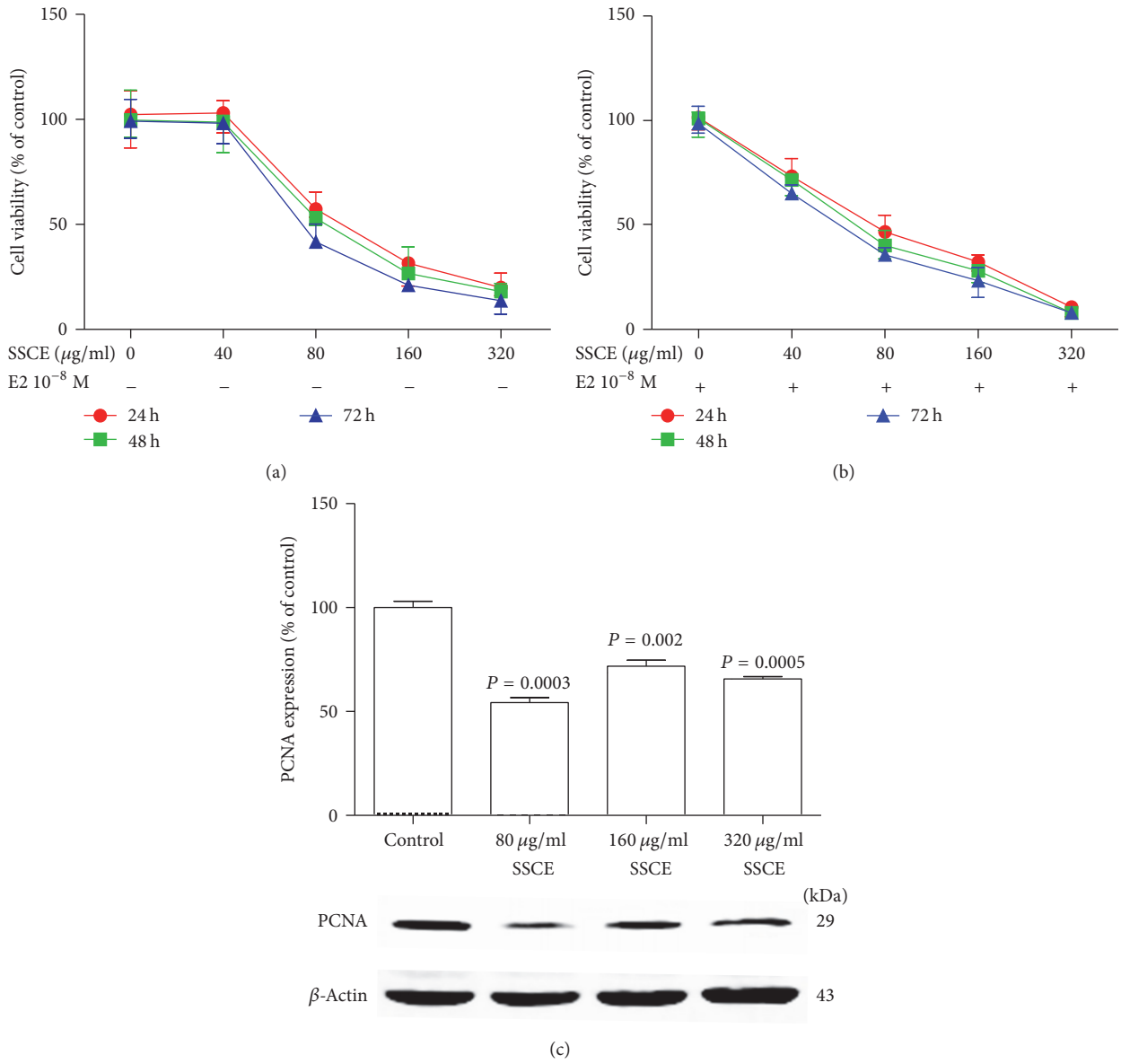

Figure 1: Inhibition of the viability of MCF-7 cells with SSCE treatment. (a) SSCE treatment in the absence of 17β-estradiol (E2) inhibited the proliferation of MCF-7 cells. The IC50 of SSCE treated for 24 h, 48 h, and 72 h was 109 μg/ml, 102 μg/ml, and 83 μg/ml, respectively. (b) Addition of 10^{-8} M E2 promoted the effect of SSCE. The IC50 of SSCE in the presence of 10^{-8} M E2 was 81 μg/ml, 68 μg/ml, and 60 μg/ml for a 24 h, 48 h, and 72 h incubation, respectively. (c) The proliferation-related protein (PCNA) level was decreased upon treatment with SSCE (80 μg/ml, 160 μg/ml, and 320 μg/ml) for 24 h compared to control.

enhanced (Figure 1(b)) with an IC50 value of 81 μg/ml after 24 h incubation. The inhibitory effect of SSCE was further demonstrated by the reduced level of the proliferation-related protein PCNA (Figure 1(c)).

3.1. SSCE Triggered Apoptosis of MCF-7 Cells. Apoptotic cells identified by a flow cytometry assay were significantly increased in the MCF-7 cells treated with SSCE at the concentration of 160 μg/ml for 24 h. Both early apoptotic cells and late apoptotic cells were increased and the differences compared to the control group were statistically significant (Figure 2(a)). Then, we assessed the apoptotic related proteins cytochrome C, Bax, and Bcl-2 by Western blot analysis. It showed that SSCE (80 μg/ml, 160 μg/ml, and 320 μg/ml) upregulated the levels of cytochrome C and Bax and down-regulated the level of Bcl-2 (Figure 2(b)).

3.2. SSCE Triggered a G0/G1 Phase Arrest of MCF-7 Cells. We analyzed the cell cycle of the MCF-7 cells cultured under different concentrations of SSCE (80 μg/ml, 160 μg/ml, and 320 μg/ml) in the presence of 10^{-8} M E2 for 24 h. The flow cytometry results showed that SSCE treatment induced a dose-dependent accumulation of cells arresting in the G0/G1 phase, with a reduction in the proportion of cells in the S

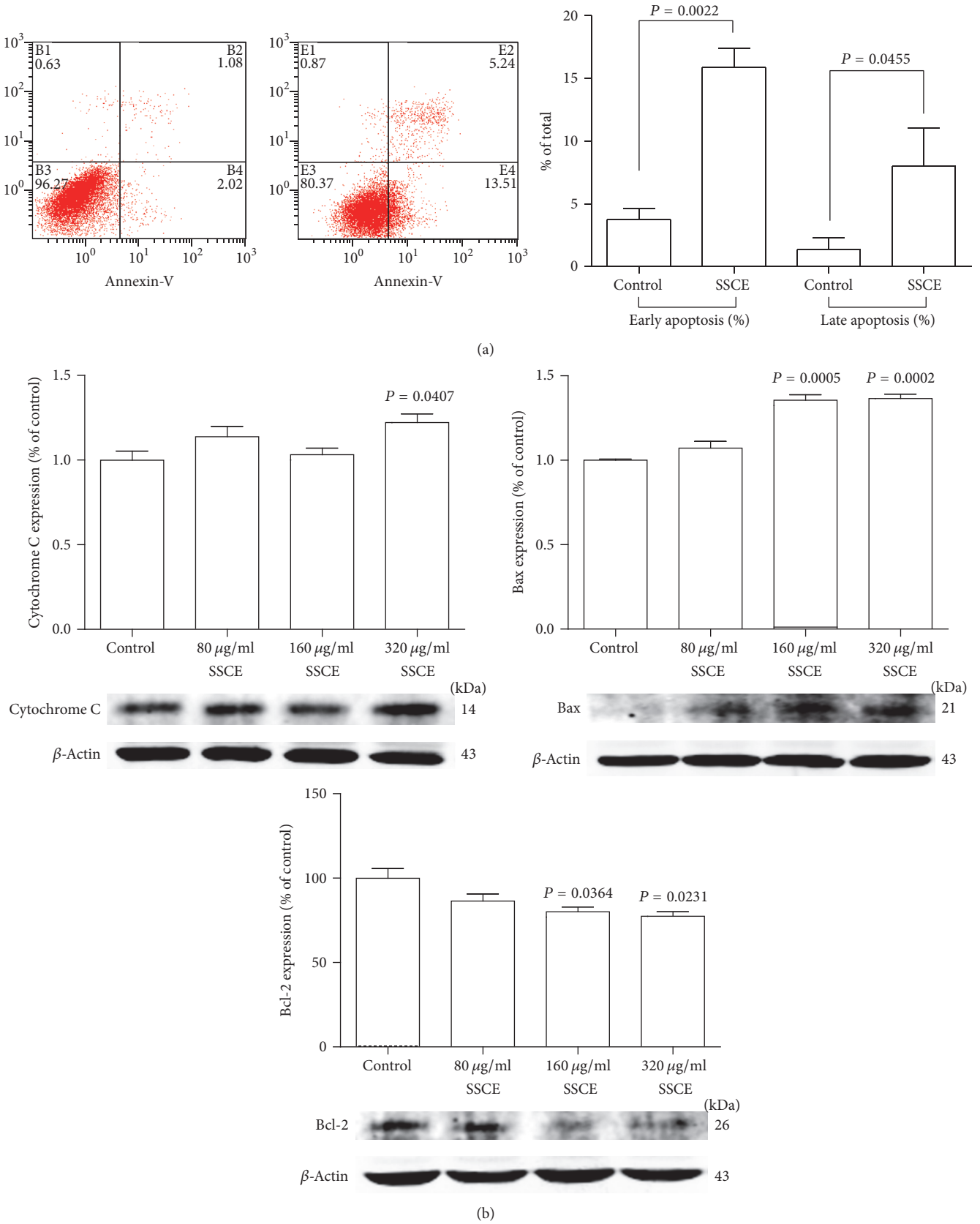

Figure 2: (a) SSCE (160 μg/ml) treatment for 24 h induced apoptosis of MCF-7 cells with a significant increase in the proportion of both early and late apoptotic cells analyzed by FACS. (b) Western blot analysis and quantification of the band intensity show that after SSCE (80 μg/ml, 160 μg/ml, and 320 μg/ml) treatment for 24 h, the expression levels of apoptosis-related proteins cytochrome C and Bax were elevated, and Bcl-2 expression was suppressed.

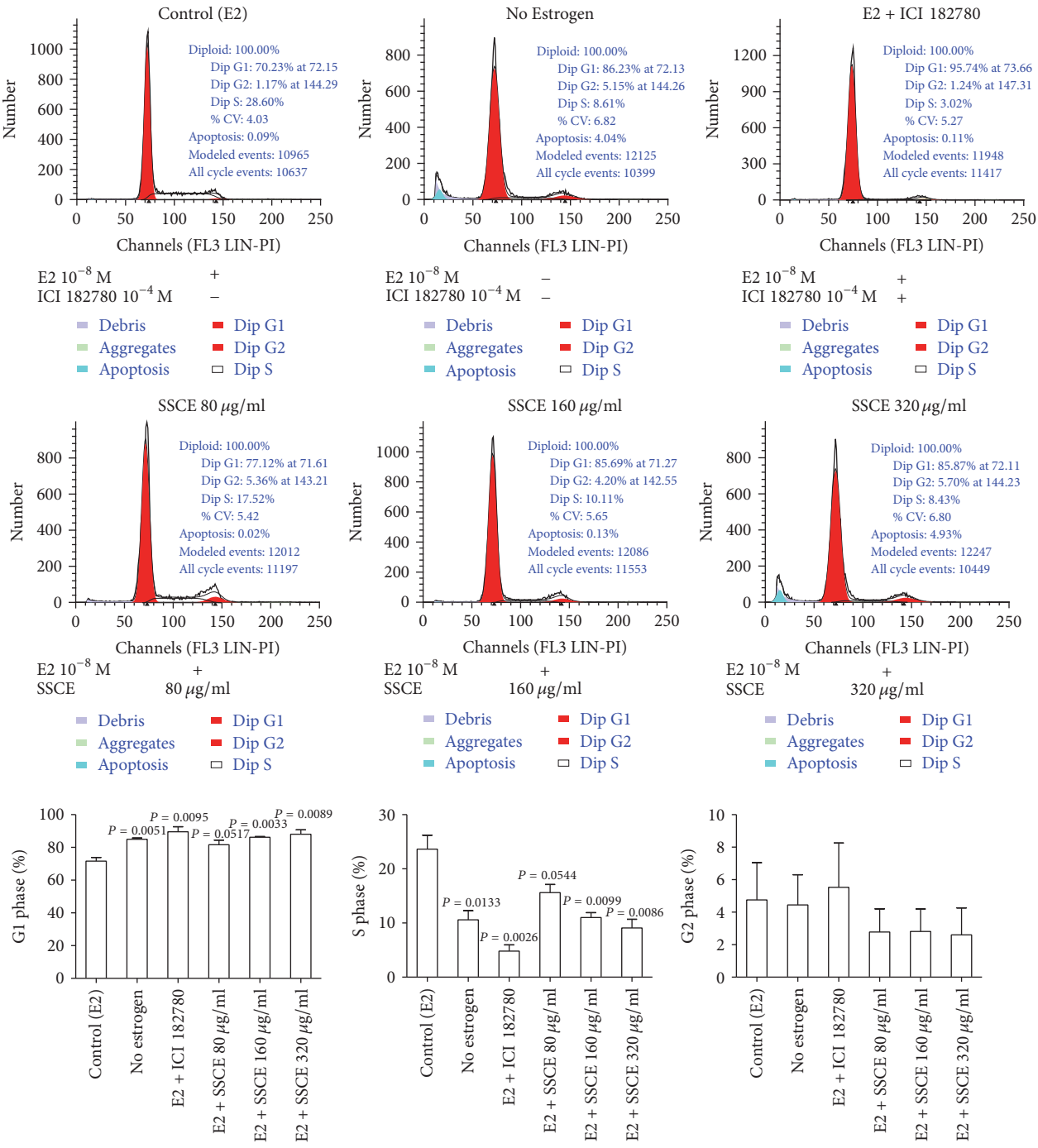

FIGURE 3: The control groups included MCF-7 cells cultured in DMEM with 10^{-8} M E2 and cells cultured in the absence of E2. When treated with SSCE (80 μg/ml, 160 μg/ml, and 320 μg/ml) in the presence of 10^{-8} M E2, the proportion of cells in the G1 phase of the cell cycle was exacerbated. The proportion of cells in the S phase was reduced in SSCE treated groups. There was no difference in the proportion of cells in the G2 phase between these groups. The cells were cultured in phenol red-free DMEM with charcoal-stripped FBS.

phase. Similar results were obtained for the cells cultured in the presence of ICI182780 (10^{-4} M) + E2 (10^{-8} M) or in the absence of E2 (the no-estrogen cultured group) (Figure 3).

3.3. SSCE Inhibited the Migration of MCF-7 Cells. Wound healing assays were used to assess the effect of SSCE on the invasion and migration ability of MCF-7 cells. The images of the scratches were documented at 0 and 24 h after the wounding (Figure 4(a)), and the closure of the wound was measured (Figure 4(b)). The healing assay indicated that, compared to the MCF-7 culture with no estrogen, E_2 (10^{-8} M) enhanced the invasion and migration of MCF-7 cells and

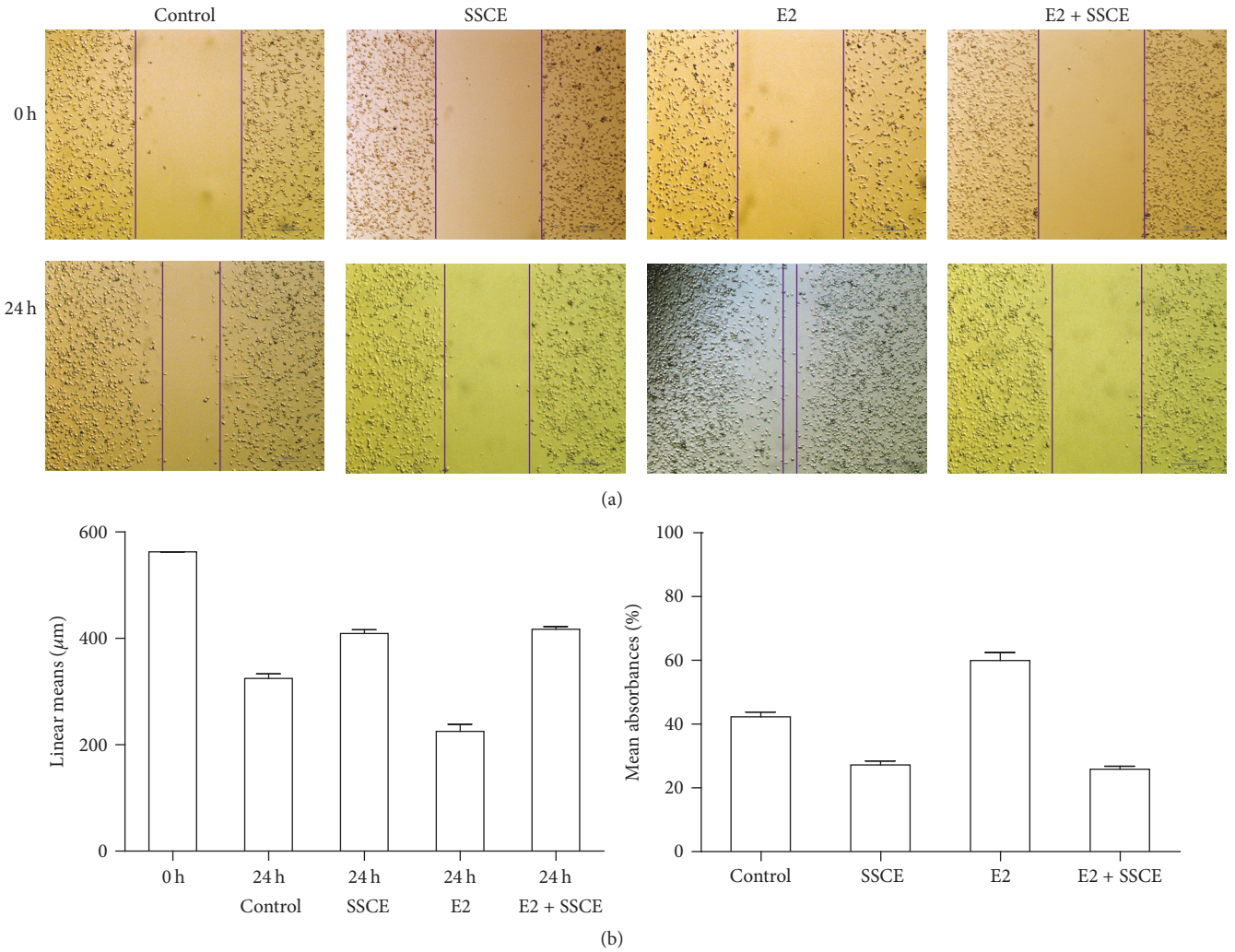

FIGURE 4: Wound healing assays to assess the effect of SSCE on migration ability. MCF-7 cells were cultured in a 6-well plate to approximately 90% confluency. A wound was generated by a scratch in the middle of the plate using a pipette. The wound closure was measured upon treatment with E2 (10^{-8} M) and SSCE (160 mg/ml) alone or E2 (10^{-8} M) + SSCE (160 mg/ml) for 24 h. (a) Representative images show the wound at 0 and 24 h. The migration of MCF-7 cells was promoted by E2 and inhibited by SSCE both in the presence and absence of E2. (b) The linear mean length and the confidence interval for each wounded area 24 h after the scratch are presented and compared to 0 h. The linear mean length of the control and the cells treated with SSCE and SSCE + E2 were significantly reduced compared to that in the other groups ($P < 0.001$). There was a significant difference noted in the linear means between the control and the E2-treated cells ($P < 0.001$). The rate of closure with the condition interval for each group 24 h after scratch is presented. The rates of closure of the SSCE group and SSCE + E2 group were significantly lower than that in the other groups.

SSCE (160 μg/ml) significantly suppressed the invasion and migration of MCF-7 cells both in the presence and in the absence of E_2 (10^{-8} M) (Figure 4).

3.4. SSCE Inhibited the Activity of the Estrogen Receptor (ERE). The cells were transiently transfected with the constructs as described in the Material and Methods section. The activity of intranuclear p-ER activity was detected using a dual-luciferase reporter system. The activity of p-ERα and p-Erβ in MCF-7 cells cultivated in the medium without estrogen is shown in Figure 5(a). The luciferase activity in p-ERα and p-ERβ is higher than that in the ERE-transfected cells. SSCE (80 μg/ml, 160 μg/ml, or 320 μg/ml) treatment for 12 h attenuated the luciferase activity in the p-ERα- and p-ERβ-transfected cells, especially in the group treated with 160 μg/ml SSCE (Figure 5(a)).

Expression of the receptors was analyzed by Western blot. The control group exhibited a strong phosphoestrogen receptor α (Ser118) protein band that was reduced in the cells cultured in the presence of SSCE (80 μg/ml, 160 μg/ml, or 320 μg/ml) for 24 h. Inhibition of expression of the receptors by SSCE was dose dependent (Figure 5(b)). In contrast, SSCE treatment did not affect the expression of phosphoestrogen receptor β (Ser105) (Figure 5(b)).

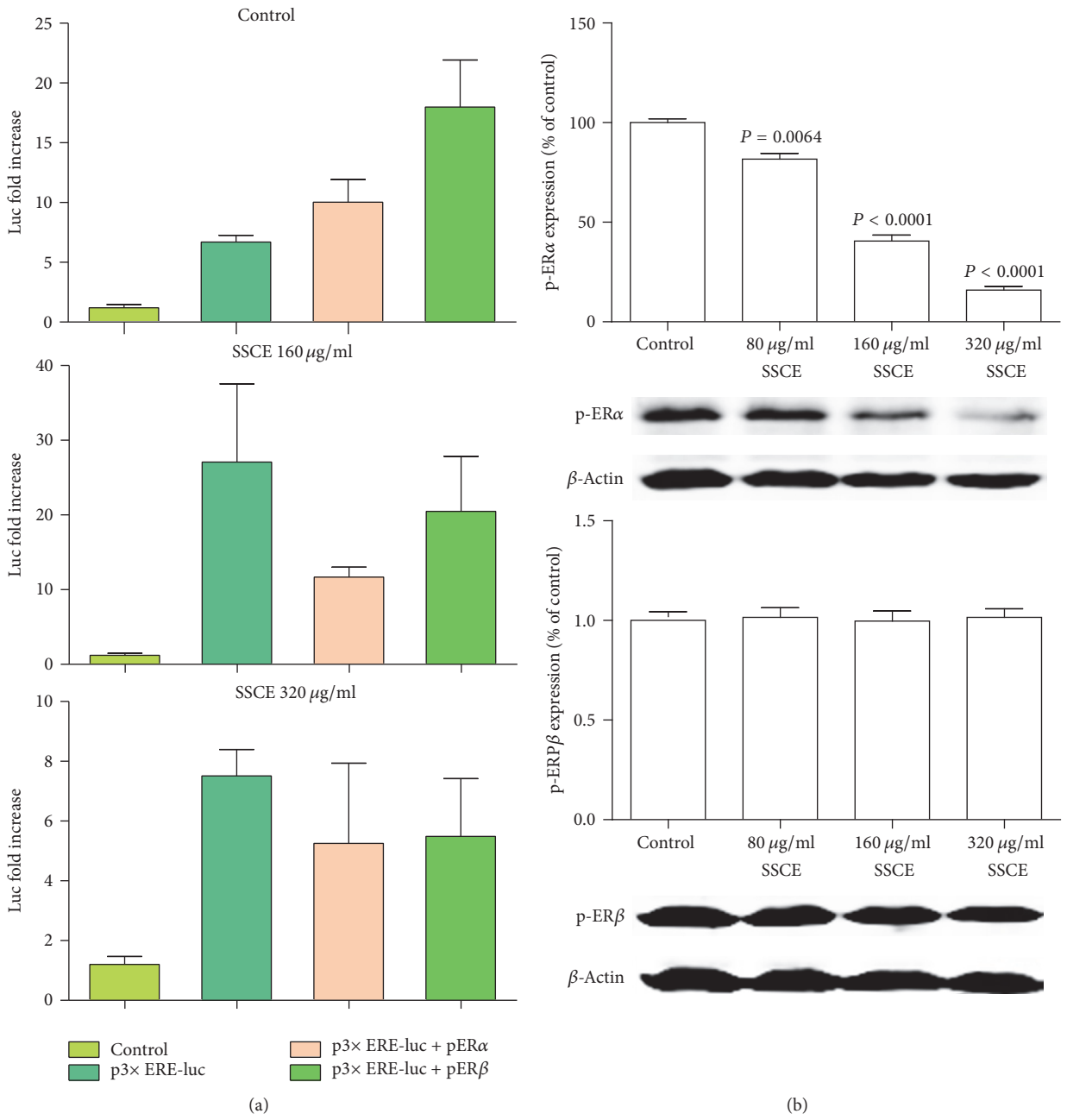

Figure 5: (a) The activity of nucleus ERE, p-ERα, and p-ERβ in MCF-7 cells cultured without estrogen detected by a double-fluorescence enzyme reporting system is shown. Relative to ERE, the activation of p-ERα and p-ERβ in the control group was significantly higher than that in MCF-7 cells cultured with SSCE (160 μg/ml and 320 μg/ml) for 12 h. (b) p-ERα and p-ERβ protein levels after treatment with SSCE (80 μg/ml, 160 μg/ml, and 320 μg/ml) cultured for 24 h were detected by Western blot. SSCE inhibited p-ERα expression in a concentration-dependent manner. However, SSCE did not affect the expression of p-ERβ.

3.5. SSCE Regulated the MAPK Signaling Pathway. Finally, we investigated the typical protein signaling events in the MAPK PI3K/AKT pathway by Western blot to determine whether SSCE affected the protein expression. The expression levels of ERK1/2, p-ERK1/2, AKT, p-AKT, p-mTOR, PI3K, and p-PI3K were significantly reduced in the MCF-7 cells treated with SSCE in a concentration-dependent manner. In comparison, there was no significant difference in the expression of mTOR between the different concentrations of SSCE (Figure 6).

4. Discussion

Earlier studies reported that Spatholobi Caulis extract inhibited proliferation of KB, K562, and HL60 cells with an IC50

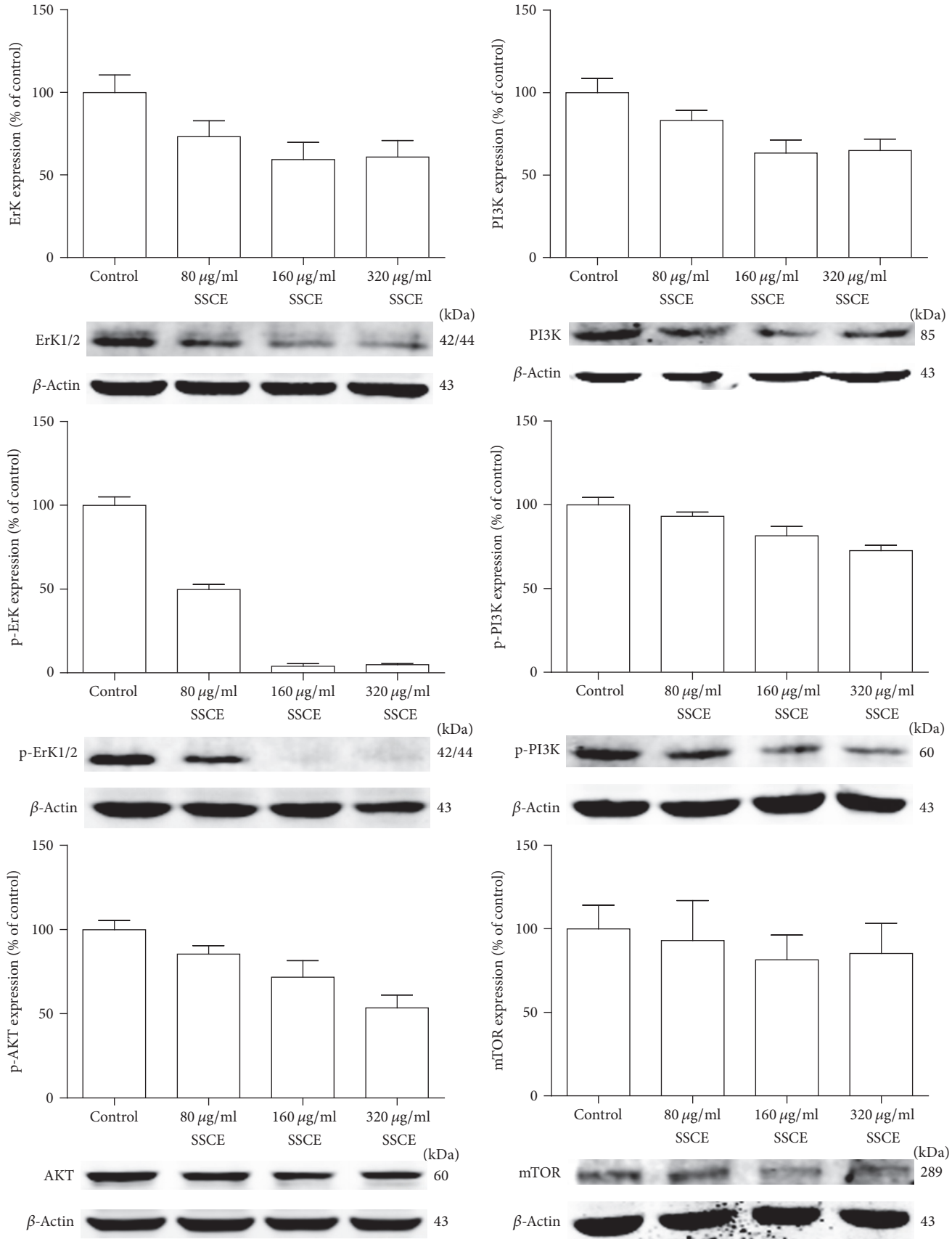

Figure 6: Continued.

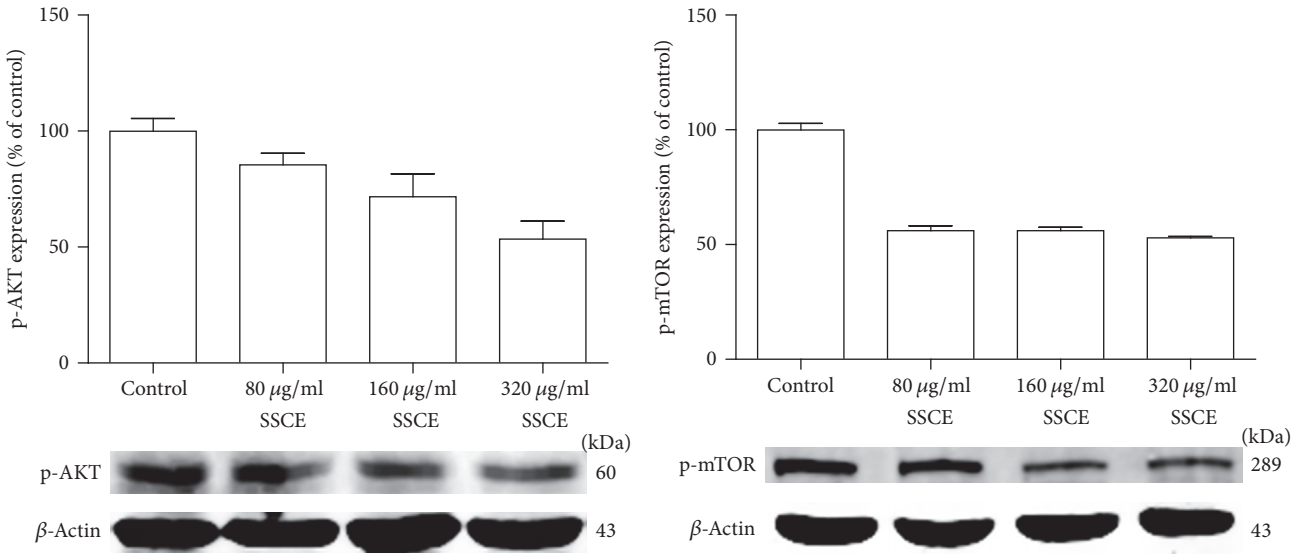

Figure 6: Western blot analysis showing that expression levels of ERK1/2, p-ERK1/2, AKT, p-AKT, PI3K, p-PI3K, and p-mTOR were significantly inhibited in the MCF-7 cells treated with SSCE (80 μg/ml, 160 μg/ml, and 320 μg/ml) in a concentration-dependent manner. The expression of mTOR had no obvious association with the SSCE concentration.

value of 17.6, 8.3, and 9.7 mg/ml, respectively [11]. Spatholobi Caulis was also demonstrated to inhibit platelet aggregation induced by B16BL6 melanoma cells with an IC50 of 50 mg/ml. It also inhibited HT1080 cancer cell invasion through a Matrigel-coated filter with an IC50 of 25 mg/ml [12]. SSCE was also found to induce apoptosis via the caspase-dependent pathway in U937 cells [13]. In line with the previous reports, we found similar antiproliferation effect of Spatholobi Caulis extract (SSCE) in MCF-7 cells with an IC50 value of 109 μg/ml. SSCE treatment resulted in inhibition of MCF-7 migration as observed in a scratch test, induced apoptosis, and arrested the cell cycle at the G0/G1 phase.

Estrogens are suggested to trigger breast cancer by stimulating cell proliferation through receptor-mediated processes and by way of their toxic metabolites [14, 15]. A 15-year randomized, placebo-controlled trial involving 161808 postmenopausal women revealed that long-term use of hormone replacement therapy (HRT) is associated with an increased risk of breast cancer [16]. Thus, steroidal was added to the list of known human carcinogens in the US in 2001 [17]. Spatholobi Caulis is wildly used for the treatment of ER+ mammary tumor patients in China. The main components of *Spatholobus suberectus* column extract (SSCE) from Spatholobi Caulis, analyzed by HPLC [18], include formononetin, genistein, calycosin, and daidzein, all of which are flavonoid phytoestrogens [18]. Phytoestrogens are biologically active compounds of plant origin that structurally mimic the mammalian steroid hormone 17β-estradiol (E2) and are mainly found in vegetables, fruits, and herbs [19]. Phytoestrogens bind with estrogen receptors and may modulate estrogen receptor activity [20, 21]. Studies have shown that women in the US have a three times higher risk for breast cancer than Asian women [22]. This difference has been partially associated with Asian diets that consist of a considerably large proportion of phytoestrogens [20, 21]. Some phytoestrogens can reduce the carcinogenic effect of bisphenol A by blocking the connection of bisphenol A and ER [23, 24]. Epidemiologic and experimental evidence suggest that phytoestrogens play a chemopreventive role in breast cancers. Our findings provided further biological evidence for the effect of phytoestrogen-type molecules in treatment of breast cancer.

There are two main pathways by which E2 plays its role. In the classical pathway, E2 diffuses into the nucleus and binds to the ER. Once activated by E2, the ER is able to be phosphorylated and translocates into the nucleus where it binds to specific sequences of DNA known as estrogen response elements (ERE) [25]. The DNA receptor complex then recruits other proteins that are responsible for the transcription of downstream DNA into mRNA and finally protein that results in a change in cell function. In addition, activated ER also interacts with activator protein 1 (AP-1) and transcription factor specificity protein 1 (Sp-1) to promote transcription via several coactivators such as PELP1 [25]. AP-1 activity can be regulated by the ERK pathway. This results in activated c-jun and its downstream targets such as RACK1 and cyclin D1 [26]. Cyclin D1 is required for the progression through the G1 phase of the cell cycle to the S phase and reduced cyclin D1 expression results in G1 arrest [27]. c-Jun is overexpressed in MCF-7 cells [28]. c-Jun can also protect cells from apoptosis [27] (Figure 7).

The activity of nucleus p-ERα and p-ERβ was suppressed by SSCE. SSCE may affect the activation of ERα, decreasing p-ERα. Inactivated ER cannot bind to ERE, preventing the transcription and translation of ERE and resulting in arrest of the cell cycle. It has been reported that phosphorylation of S105 ERβ in breast cancer is associated with improved survival. Even in endocrine-resistant breast tumors, S105-ERβ might be a useful additional prognostic marker [29]. Low S118 and

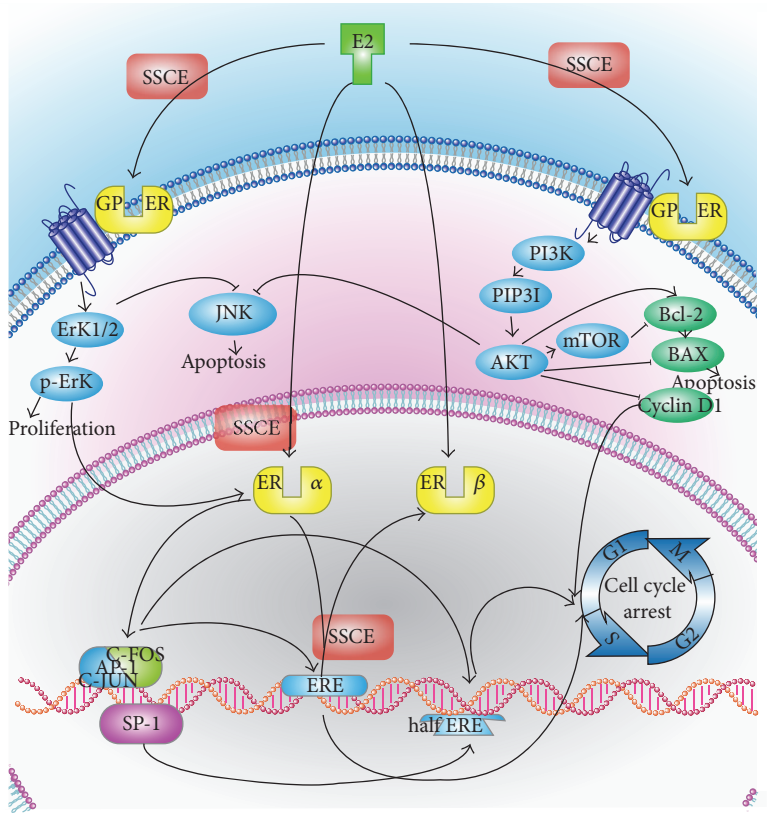

Figure 7: The classic mechanism for E2 action is E2 diffusing into the nucleus, binding to the nucleus estrogen receptor (ER), combining with ERE on DNA, starting transcription translation and inducing the cycle of multiplication in cancer cells. In addition, activated ERα can activate AP-1 and SP-1, which are transcription enhancers that can promote the transcription translation of ERE and enable incomplete ERE (half ERE) to transcribe and translate. SSCE may affect the activation of ERα, decreasing p-ERα, reducing the combination ability of ER and ERE, and stopping ERE from transcribing and translating. On the other hand, E2 can combine with GPER on the cell membrane, increase the expression of ERK, promote cell proliferation, activate the PI3K-AKT pathway, increase the activation of mTOR and the upregulation of Bcl-2 and cyclin D, downregulate Bax, reduce apoptosis, and start the cell cycle. Both ERK and AKT can inhibit JNK, reducing apoptosis. SSCE may downregulate the PI3K-AKT, ERK pathway by GPER, resulting in a downregulation of Bcl-2, upregulation of Bax, reduction in the inhibition of JNK, promotion of cell apoptosis, and downregulation of cyclin D, thereby blocking the cell cycle.

high S167 ER-phosphorylation are associated with a disease-free state and overall survival [30]. In the same line, our results show that SSCE treatment attenuated p-ERα (S118) expression but did not affect expression of p-ERβ (S105).

The second pathway of E2 is through binding to G protein-coupled ER on the cell membrane [31], increasing expression activity of ERK and activating the PI3K-AKT pathway [32]. As the mitogen-activated protein kinase (MAPK) cascades are major signaling transduction molecules in apoptosis [33] and MAPK signaling pathways have been identified as chemotherapeutic targets for sensitizing cancer cells to apoptosis [34], the reduction of ERK and p-ERK protein expression by SSCE treatment may have contributed to the inhibitory effect on the proliferation of the tumor cells. Further, the PI3K/AKT signal transduction pathway plays a pivotal role in cell survival and prevents cancer cells from apoptosis during tumorigenesis [35]. This is also in agreement with our findings that SSCE impaired the expression of AKT, p-AKT, PI3K, p-PI3K, and p-mTOR, repressing the PI3K/AKT pathway, downregulating Bcl-2, and upregulating Bax, which consequently induced cell cycle arrest and apoptosis. It appeared that SSCE downregulated the PI3K-AKT, ERK pathway by GPER; however, we cannot rule out whether SSCE has affected expression of other pathways, as it was reported that inhibition of ER signaling by tamoxifen enhanced HER2 expression [36].

5. Conclusion

In conclusion, our results showed that the *Spatholobus suberectus* column extract (SSCE) induced apoptotic cell cycle arrest of MCF-7 cells, inhibited activity of nucleus p-ERα and p-ERβ, and downregulated the expression of p-ERα, which resulted in suppression of the MAPK PI3K/AKT pathway. The results indicate that the herbal extract inhibited the proliferation of ER-positive MCF-7 cells, contributing to the suppression of ER activity. Our data support the use of Spatholobi

Caulis as a complementary medicine for management of breast cancer patients.

Competing Interests

The authors declare that there are no competing interests.

Acknowledgments

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References

[1] L. A. Torre, F. Bray, R. L. Siegel, J. Ferlay, J. Lortet-Tieulent, and A. Jemal, "Global cancer statistics, 2012," *CA: A Cancer Journal for Clinicians*, vol. 65, no. 2, pp. 87–108, 2015.

[2] R. L. Theriault, R. W. Carlson, C. Allred et al., "Breast cancer, version 3.2013: featured updates to the NCCN guidelines," *Journal of the National Comprehensive Cancer Network*, vol. 11, no. 7, pp. 753–761, 2013.

[3] T. De Marchi, J. A. Foekens, A. Umar, and J. W. Martens, "Endocrine therapy resistance in estrogen receptor (ER)-positive breast cancer," *Drug Discovery Today*, vol. 21, no. 7, pp. 1181–1188, 2016.

[4] S. Zhang and L. Xuan, "New phenolic constituents from the stems of Spatholobus suberectus," *Helvetica Chimica Acta*, vol. 89, no. 6, pp. 1241–1245, 2006.

[5] E.-Y. Su, Y.-H. Fang, and H.-S. Chen, "Clinical observation of treating 62 patients with severe aplastic anemia failing in immunosuppressive therapy by integrative medicine," *Zhongguo Zhong Xi Yi Jie He Za Zhi*, vol. 32, no. 12, pp. 1616–1620, 2012.

[6] *Chinese Pharmacopoeia*, China Medical Science Press, Beijing, China, 2010.

[7] M.-H. Lee, Y.-P. Lin, F.-L. Hsu, G.-R. Zhan, and K.-Y. Yen, "Bioactive constituents of *Spatholobus suberectus* in regulating tyrosinase-related proteins and mRNA in HEMn cells," *Phytochemistry*, vol. 67, no. 12, pp. 1262–1270, 2006.

[8] T. Fleischer, T. Chang, J. Chiang, C. Chang, C. Hsieh, and H. Yen, "Adjunctive Chinese Herbal Medicine therapy improves survival of patients with chronic myeloid leukemia: a nationwide population-based cohort study," *Cancer Medicine*, vol. 5, no. 4, pp. 640–648, 2016.

[9] Y. Fu, Y. Cheng, J.-P. Chen, and D.-M. Wang, "Advances in studies on chemical constituents in Spatholobi Caulis and their pharmacological activities," *Chinese Traditional and Herbal Drugs*, vol. 42, no. 6, pp. 1229–1234, 2011.

[10] H. Hatcher, R. Planalp, J. Cho, F. M. Torti, and S. V. Torti, "Curcumin: from ancient medicine to current clinical trials," *Cellular and Molecular Life Sciences*, vol. 65, no. 11, pp. 1631–1652, 2008.

[11] D. Lu, H. He, B. Wu, and S. Yao, "Cytotoxic effect on cancer cells and structural identification of phenols from Spatholobi caulis by HPLC-ESI-MS(n)," *Natural Product Communications*, vol. 4, no. 6, pp. 809–812, 2009.

[12] B.-J. Lee, I.-Y. Jo, Y. Bu et al., "Antiplatelet effects of *Spatholobus suberectus* via inhibition of the glycoprotein IIb/IIIa receptor," *Journal of Ethnopharmacology*, vol. 134, no. 2, pp. 460–467, 2011.

[13] E.-S. Ha, E.-O. Lee, T.-J. Yoon et al., "Methylene chloride fraction of *Spatholobi Caulis* induces apoptosis via caspase dependent pathway in U937 cells," *Biological & Pharmaceutical Bulletin*, vol. 27, no. 9, pp. 1348–1352, 2004.

[14] J. D. Yager and N. E. Davidson, "Estrogen carcinogenesis in breast cancer," *The New England Journal of Medicine*, vol. 354, no. 3, pp. 270–282, 2006.

[15] E. L. Cavalieri, D. E. Stack, P. D. Devanesan et al., "Molecular origin of cancer: catechol estrogen-3,4-quinones as endogenous tumor initiators," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 94, no. 20, pp. 10937–10942, 1997.

[16] W. Horst-Sikorska and A. Wawrzyniak, "The role of hormonal therapy in osteoporosis," *Endokrynologia Polska*, vol. 62, no. 1, pp. 61–64, 2011.

[17] L. N. Petz, Y. S. Ziegler, M. A. Loven, and A. M. Nardulli, "Estrogen receptor α and activating protein-1 mediate estrogen responsiveness of the progesterone receptor gene in MCF-7 breast cancer cells," *Endocrinology*, vol. 143, no. 12, pp. 4583–4591, 2002.

[18] H. Wang, Y. Liu, Z. Zenc, and W. He, "Study on HPLC chromatographic fingerprint of anti-tumor active site SSCE of *Caulis spatholobi*," *Zhongguo Zhongyao Zazhi*, vol. 36, no. 18, pp. 2525–2529, 2011.

[19] C. R. Sirtori, A. Arnoldi, and S. K. Johnson, "Phytoestrogens: end of a tale?" *Annals of Medicine*, vol. 37, no. 6, pp. 423–438, 2005.

[20] S. M. Mense, F. Remotti, A. Bhan et al., "Estrogen-induced breast cancer: alterations in breast morphology and oxidative stress as a function of estrogen exposure," *Toxicology and Applied Pharmacology*, vol. 232, no. 1, pp. 78–85, 2008.

[21] H. Adlercreutz, "Phytoestrogens and breast cancer," *Journal of Steroid Biochemistry and Molecular Biology*, vol. 83, no. 1–5, pp. 113–118, 2002.

[22] G. Ursin, L. Bernstein, and M. C. Pike, "Breast cancer," *Cancer Surveys*, vol. 19-20, pp. 241–264, 1994.

[23] B. D. Bernardo, J. Z. Brandt, T. F. Grassi, L. T. R. Silveira, W. R. Scarano, and L. F. Barbisan, "Genistein reduces the noxious effects of in utero bisphenol A exposure on the rat prostate gland at weaning and in adulthood," *Food and Chemical Toxicology*, vol. 84, pp. 64–73, 2015.

[24] J. Wang, S. Jenkins, and C. A. Lamartiniere, "Cell proliferation and apoptosis in rat mammary glands following combinational exposure to bisphenol A and genistein," *BMC Cancer*, vol. 14, article 379, 2014.

[25] E. R. Levin, "Integration of the extranuclear and nuclear actions of estrogen," *Molecular Endocrinology*, vol. 19, no. 8, pp. 1951–1959, 2005.

[26] P. Lopez-Bergami, C. Huang, J. S. Goydos et al., "Rewired ERK-JNK signaling pathways in melanoma," *Cancer Cell*, vol. 11, no. 5, pp. 447–460, 2007.

[27] R. Wisdom, R. S. Johnson, and C. Moore, "c-Jun regulates cell cycle progression and apoptosis by distinct mechanisms," *The EMBO Journal*, vol. 18, no. 1, pp. 188–197, 1999.

[28] L. M. Smith, S. C. Wise, D. T. Hendricks et al., "cJun overexpression in MCF-7 breast cancer cells produces a tumorigenic, invasive and hormone resistant phenotype," *Oncogene*, vol. 18, no. 44, pp. 6063–6070, 1999.

[29] W. Hamilton-Burke, L. Coleman, M. Cummings et al., "Phosphorylation of estrogen receptor β at serine 105 is associated with good prognosis in breast cancer," *The American Journal of Pathology*, vol. 177, no. 3, pp. 1079–1086, 2010.

[30] H. Yamashita, M. Nishio, T. Toyama et al., "Low phosphorylation of estrogen receptor α (ERα) serine 118 and high phosphorylation of ERα serine 167 improve survival in ER-positive breast cancer," *Endocrine-Related Cancer*, vol. 15, no. 3, pp. 755–763, 2008.

[31] D. Zivadinovic, B. Gametchu, and C. S. Watson, "Membrane estrogen receptor-alpha levels in MCF-7 breast cancer cells predict cAMP and proliferation responses," *Breast Cancer Research*, vol. 7, no. 1, pp. R101–112, 2005.

[32] S. Kato, H. Endoh, Y. Masuhiro et al., "Activation of the estrogen receptor through phosphorylation by mitogen-activated protein kinase," *Science*, vol. 270, no. 5241, pp. 1491–1494, 1995.

[33] M. Qi and E. A. Elion, "MAP kinase pathways," *Journal of Cell Science*, vol. 118, no. 16, pp. 3569–3572, 2005.

[34] C.-C. Su, J. Y.-F. Chen, Z.-H. Din et al., "13-Acetoxysarcocrassolide induces apoptosis on human gastric carcinoma cells through mitochondria-related apoptotic pathways: P38/JNK activation and PI3K/AKT suppression," *Marine Drugs*, vol. 12, no. 10, pp. 5295–5315, 2014.

[35] C. A. Castaneda, H. Cortes-Funes, H. L. Gomez, and E. M. Ciruelos, "The phosphatidyl inositol 3-kinase/AKT signaling pathway in breast cancer," *Cancer and Metastasis Reviews*, vol. 29, no. 4, pp. 751–759, 2010.

[36] S. Massarweh, C. K. Osborne, C. J. Creighton et al., "Tamoxifen resistance in breast tumors is driven by growth factor receptor signaling with repression of classic estrogen receptor genomic function," *Cancer Research*, vol. 68, no. 3, pp. 826–833, 2008.

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Research Article

Anticancer Activity, Antioxidant Activity, and Phenolic and Flavonoids Content of Wild *Tragopogon porrifolius* Plant Extracts

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Tragopogon porrifolius, commonly referred to as white salsify, is an edible herb used in folk medicine to treat cancer. Samples of *Tragopogon porrifolius* plant grown wild in Palestine were extracted with different solvents: water, 80% ethanol, and 100% ethanol. The extracts were analyzed for their total phenolic content (TPC), total flavonoid content (TFC), and antioxidant activity (AA). Four different antioxidant assays were used to evaluate AA of the extracts: two measures the reducing power of the extracts (ferric reducing antioxidant power (FRAP) and cupric reducing antioxidant power (CUPRAC)), while two other assays measure the scavenging ability of the extracts (2,2-azino-di-(3-ethylbenzothialozine-sulphonic acid (ABTS)) and 2,2-diphenyl-1-picrylhydrazyl (DPPH)). Anticancer activity of the plant extracts were also tested on HOS and KHOS osteosarcoma cell lines. The results revealed that the polarity of the extraction solvent affects the TPC, TFC, and AA. It was found that both TPC and AA are highest for plant extracted with 80% ethanol, followed by water, and finally with 100% ethanol. TFC however was the highest in the following order: 80% ethanol > 100% ethanol > water. The plant extracts showed anticancer activities against KHOS cancer cell lines; they reduced total cell count and induced cell death in a drastic manner.

1. Introduction

Tragopogon porrifolius, commonly known as salsify, oyster plant, and vegetable oyster belonging to Asteraceae family, is one type of *Tragopogon* species. *Tragopogon porrifolius* is grown in the wild and cultivated in the Mediterranean regions [1]. Every part of the plant including the roots, leafy shoots, and open flowers are edible [1]. The nutritional value of this plant has been attributed to its monounsaturated and essential fatty acids, vitamins, and polyphenols [2, 3]. It has been recorded since ancient times for traditional medicine. It is currently widely spread over the Middle Easter area, Europe, and North America [4].

Tragopogon porrifolius is a vegetable that is high in antioxidants which help in the process of preventing diseases by eliminating peroxides and free radicals from diets [5]. Phenolic compounds, which are plant secondary metabolites, are well-known antioxidants and so play important roles in disease resistance [6, 7].

Antioxidant enzymes constitute the first line of defense against oxidative stress and damage caused by free radicals. When there is an imbalance between oxidative stress and antioxidant enzymes then there is a chance that diseases such as cancer, autoimmune disorders, aging, cardiovascular, and neurodegenerative diseases may develop [8, 9]. The body will defend itself by synthesizing antioxidants or intake of food or supplements that contain antioxidants such as *Tragopogon porrifolius* [10].

Until now, the scientific literature does not report about the antioxidant activity and phenolic content or flavonoid content of *Tragopogon* plant from Palestine. Abundant literature dealing with total phenolic content and antioxidant

activity was published from different countries including those of the Middle East. Tenkerian et al. 2015 studied TPC, TFC, AA, and hepatoprotective and anticancer activities in vitro and in vivo of methanolic extract of *Tragopogon porrifolius* from Lebanon. Also, the extract of the aerial part of the plant was tested on rats with normal and damaged liver. Results were progressive on both rats on the levels of the liver antioxidants enzymes [1]. Mojarrab et al. 2014 [11] studied antioxidant activity and TPC of hydroethanolic extract of *Tragopogon buphthalmoides* from Iran. Asadi-Samani et al. 2015 studied medicinal plants with hepatoprotective activity in Iranian folk medicine and including *Tragopogon porrifolius* [12].

Investigation of the effects of water extract of *Tragopogon porrifolius* shoot on inflammation, oxidative stress, and hepatotoxicity using a rat model showed that after one month of *Tragopogon porrifolius* water extract intake, a significant decrease in the levels of serum cholesterol, triglyceride, glucose, and liver enzyme was observed [13–15]. In addition, *Tragopogon porrifolius* revealed effective antioxidant capability owing to its remarkable scavenging activity [13–15]. The objectives of the current work are to determine the AA, TPC, TFC, and anticancer activity of different extracts from *Tragopogon porrifolius* plant growing wild in Palestine. Antioxidants contents were assayed using FRAP, CUPRAC, DPPH, and ABTS colorimetric methods. TPC and TFC of the extracts were evaluated using Folin-Ciocalteu and aluminum chloride colorimetric methods, respectively. Anticancer activity was tested on HOS and KHOS osteosarcoma cell lines.

2. Materials and Methods

2.1. Plant Material. Tragopogon porrifolius plant was collected from the middle part of the West Bank, Palestine in February 2015. The plant was air-dried in dark at room temperature for two weeks, then milled to a powdered plant material, and then stored in fridge until extraction.

2.2. Chemicals and Reagents. 2,4,6-Tripyridyl-S-triazine (TPTZ), hydrochloric acid 37% (w/w), sodium hydroxide, ferric chloride trihydrate, ferrous sulfate heptahydrate, potassium persulphate, sodium acetate, sodium carbonate, sodium nitrite, aluminum chloride, methanol, Folin-Ciocalteu reagent, Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), gallic acid, cupper chloride, neocuproine, 99.9% ethanol, ammonium acetate, DPPH, methanol, ABTS (2,2-azino-di-(3-ethylbenzothialozine-sulphonic acid)), and potassium persulphate were all obtained from Sigma-Aldrich, Germany. All chemicals and reagents were of analytical grade. RPMI 1640, fetal bovine serum, antibiotics, and glutamine were purchased from Gibco.

FRAP reagent was prepared according to Benzie and Strain [16] by the addition of 2.5 mL of a 10 mM tripydyltriazine (TPTZ) solution in 40 mM HCl plus 2.5 mL of 20 mM $FeCl_3 \cdot 6H_2O$ and 25 mL of 0.3 M acetate buffer at pH 3.6. Acetate buffer (0.3 M) was prepared by dissolving 16.8 g of acetic acid and 0.8 g of sodium hydroxide in 1000 mL of distilled water.

2.3. Extraction of the Plant. Dry powder of plant material (five grams) was extracted separately with 50 mL of three extraction solvents (water, 80% ethanol, and 100% ethanol) in water bath at 37°C for three hours. The extracts were then filtered and the filtrate was stored at 4° C until used for analysis (TPC, TFC, and AA).

2.4. Measurement of Antioxidant Activity

2.4.1. FRAP Assay. The antioxidant activity of the extracts was determined using a modified method of the assay of ferric reducing/antioxidant power (FRAP) of Benzie and Strain [16]. Freshly prepared FRAP reagent (3.0 mL) was warmed at 37°C and mixed with 40 μL of the extract and the reaction mixtures were later incubated at 37°C. Absorbance at 593 nm was read with reference to a reagent blank containing distilled water which was also incubated at 37°C for up to 1 hour instead of 4 min, which was the original time applied in FRAP assay. Aqueous solutions of known Fe^{+2} concentrations in the range of 2–5 mM were used for calibration, and results were expressed as mmol Fe^{+2}/g.

2.4.2. Cupric Reducing Antioxidant Power (CUPRAC Assay). The cupric ion reducing antioxidant capacity of the extracts was determined according to the method of Apak et al. [17]. 100 μL of sample extract was mixed with 1 mL each of 10 mM of cupper chloride solution, 7.5 mM of neocuproine alcoholic solution (99.9% ethanol), 1 M (pH 7.0) of ammonium acetate buffer solution, and 1 mL of distilled water to make final volume 4.1 mL. After 30 min, the absorbance was recorded at 450 nm against the reagent blank. Standard curve was prepared using different concentrations of Trolox. The results were expressed as μmol Trolox/g.

2.4.3. Free Radical-Scavenging Activity Using DPPH (DPPH Assay). DPPH assay is based on the measurement of the scavenging ability of antioxidants towards the stable DPPH radical, and the procedure was done according to Brand-Williams et al. [18]. A 3.9 mL aliquot of a 0.0634 mM of DPPH solution in methanol (95%) was added to 100 μL of each extract. The mixture was vortexed for 5–10 sec. The change in the absorbance of the sample extract was measured at 515 nm for 30 min till the absorbance reached a steady state. The percentage inhibition of DPPH of the test sample and known solutions of Trolox were calculated by the following formula:

$$\%\ \text{inhibition} = \frac{(A^\circ - A)}{A^\circ} \times 100, \quad (1)$$

where A° is the absorbance of a solution of 100 μL methanol 95% and 3.9 mL of DPPH at 515 nm and A is the absorbance of the sample extract at 515 nm. Methanol (95%) was used as a blank. Standard curve was prepared using different concentrations of Trolox. The results were expressed as μmol Trolox/g.

2.4.4. Free Radical-Scavenging Activity Using ABTS (ABTS Assay). A modified procedure using ABTS (2,2-azino-di-(3-ethylbenzothialozine-sulphonic acid)) as described by

Table 1: Total phenolic content (TPC as mg gallic acid/g DW*), total flavonoids contents (TFC as mg catechin/g DW), FRAP (mmol Fe^{+2}/g DW), CUPRAC (μmol Trolox/g DW), DPPH (μmol Trolox/g DW), ABTS (μmol Trolox/g DW), DPPH % inhibition, and ABTS % inhibition of *Tragopogon porrifolius* plant extracted with water, 80% ethanol, and 100% ethanol.

	TPC** (mg/g)	TFC (mg/g)	FRAP (mmol/g)	CUPRAC (μmol/g)	DPPH (μmol/g)	ABTS (μmol/g)	DPPH % inhibition	ABTS % inhibition
Water	$102.9^{b} \pm 3.9$	$4.8^{c} \pm 0.3$	$5.4^{b} \pm 0.5$	$1945^{b} \pm 33$	$226^{b} \pm 7.1$	$44.1^{b} \pm 2.2$	$77.3^{b} \pm 1.3$	$80.2^{b} \pm 1.4$
Ethanol (80%)	$145.3^{a} \pm 3.1$	$28.5^{a} \pm 0.2$	$12.1^{a} \pm 0.8$	$4522^{a} \pm 42$	$324^{a} \pm 3.2$	$84.2^{a} \pm 2.6$	$83.2^{a} \pm 2.4$	$88.0^{a} \pm 1.2$
Ethanol (100%)	$87.3^{c} \pm 1.8$	$14.7^{b} \pm 0.3$	$2.1^{c} \pm 0.7$	$1232^{c} \pm 41$	$117^{c} \pm 2.5$	$29.4^{c} \pm 1.2$	$70.5^{c} \pm 1.6$	$78.3^{c} \pm 2.0$

*DW: dry weight

**Results are expressed as average of three samples of *T.* shoots. Different small letters within column indicate significant difference ($p < 0.05$, $n = 3$).

Re et al. [19] was used. The ABTS stock solution (7 mM) was prepared through reaction of 7 mM ABTS and 2.45 mM of potassium persulphate as the oxidant agent. The working solution of $ABTS^{+\bullet}$ was obtained by diluting the stock solution in 99.9% ethanol to give an absorption of 0.70 ± 0.02 at 734 nm. 200 μL sample extract was added to 1800 μL of $ABTS^{+\bullet}$ solution and absorbance readings at 734 nm were taken at 30°C exactly 10 min after initial mixing (A). The percentage inhibition of $ABTS^{+\bullet}$ of the test sample and known solutions of Trolox were calculated by the following formula.

% inhibition $= ((A^{\circ} - A)/A^{\circ}) \times 100$, where A° is the absorbance of a solution of 200 μL of distilled water and 1800 μL of $ABTS^{+\bullet}$ at 734 nm and A is the absorbance of the test sample at 734 nm. The calibration curve between % inhibition and known solutions of Trolox (50–1000 μM) was then established. The radical-scavenging activity of the test samples was expressed as Trolox equivalent antioxidant capacity TEAC (μmol Trolox/g sample).

2.5. Total Phenolic Content (Folin-Ciocalteu Assay). Total phenolics were determined using Folin-Ciocalteu reagents [20]. *Tragopogon porrifolius* plant extracts or gallic acid standard (40 μL) was mixed with 1.8 mL of Folin-Ciocalteu reagent (prediluted 10-fold with distilled water) and allowed to stand at room temperature for 5 min, and then 1.2 mL of sodium bicarbonate (7.5%, w/v) was added to the mixture. After standing for 60 min at room temperature, absorbance was measured at 765 nm. Aqueous solutions of known gallic acid concentrations in the range of 10–500 mg/L were used for calibration. Results were expressed as mg gallic acid equivalents (GAE)/g sample.

2.6. Total Flavonoid Content. The determination of total flavonoids was performed according to the colorimetric assay of Kim et al. [21]. Distilled water (4 mL) was added to 1 mL of the extract in a test tube. Then, 0.3 mL of 5% sodium nitrite solution was added, followed by 0.3 mL of 10% aluminum chloride solution. Test tubes were incubated at ambient temperature for 5 minutes, and then 2 mL of 1 M sodium hydroxide was added to the mixture. Immediately, the volume of reaction mixture was made to 10 mL with distilled water. The mixture was thoroughly mixed using test tube shaker and the absorbance of the pink color developed was determined at 510 nm. Aqueous solutions of known catechin concentrations in the range of 50–100 mg/L were used for calibration and the results were expressed as mg catechin equivalents (CEQ)/g sample.

2.7. Cell Culture. HOS and KHOS human osteosarcoma cell lines were grown in RPMI 1640 medium supplemented 10% fetal bovine serum (FBS), 100 μg/mL streptomycin, and 100 U/mL penicillin. Cells were maintained at 37°C in a humidified atmosphere of 5% CO_2.

2.8. Anticancer Activity. The anticancer activity of the plant extract was evaluated over 3 days after treating osteosarcoma cell lines KHOS with either 2 mg/mL or 4 mg/mL of the crude plant extract. Additionally anticancer activity of the plant extract was evaluated over 2 days after treating osteosarcoma cell lines HOS with 4 mg/mL of the crude plant extract. Cell death was evaluated using trypan blue exclusion assay where cells were trypsinized, suspended, and counted using hemocytometer. Cell growth was measured by counting the cells over 3 days posttreatment.

2.9. Statistical Analyses. Three samples of *Tragopogon porrifolius* plant were independently analyzed and all of the determinations were carried out in triplicate. The results are expressed as means ± standard deviations.

3. Results and Discussion

3.1. Total Phenolic Contents (TPC). TPC of *Tragopogon porrifolius* plant extracts using three different solvents is shown in Table 1. As it is obvious from this table, the extraction solvent has an effect on the TPC of the plant extracts where significant differences ($p < 0.05$) between the TPC of the three extracts are indicated by different small letters (a, b, and c). The highest TPC was found for the plant material when extracted with 80% ethanol (145.3 ± 3.1 mg/g), followed by plant material extracted with water (102.9 ± 3.9 mg/g) and finally with 100% ethanol (87.3±1.8 mg/g). These results show that TPC were only 68% and 42% when the plant material was extracted by distilled water and 100% ethanol, respectively, as compared with the TPC extracted with 80% ethanol indicating the higher solubility of the phenolic compounds in 80% ethanol.

The results showed that *Tragopogon porrifolius* plant investigated in this study are richer with phenolic compounds (87.3 to 145.3 mg/g) than that from Turkey (from 63.4 to

68.9 mg/g caffeic acid) [4] or from Lebanon (37.0 mgGAE/g) [1].

3.2. Total Flavonoid Content (TFC). The results of ferric chloride colorimetric test for determining flavonoids content are presented in Table 1. The same statistical analyses as for TPC were performed for total flavonoids content (TFC), and the results (Table 1) showed that significant differences between total flavonoids content of the plant materials extracted with the three solvents were obtained, where significant differences ($p < 0.05$) are indicated by small letters (a, b, and c). The highest TFC was found for the plant material when extracted with 80% ethanol (28.5 ± 0.2 mg/g) which is about two times significantly higher than that extracted with 100% ethanol (14.7 ± 0.3 mg/g) and the latter was significantly about three times higher than the TFC extracted with water (4.8 ± 0.3 mg/g). Different trend of solvent effect on TFC and TPC was obtained where the highest content of TPC and TFC was obtained when the plant was extracted with 80% ethanol, while the lowest was with 100% ethanol for TPC and for water for TFC; see Table 1. Apparently, mixed solvents of intermediate polarities (80% ethanol) are the most suitable extracting solvent for recovering the highest amounts of phenolic and flavonoid compounds which have both polar and nonpolar functional groups.

It was interesting to compare TFC of *Tragopogon porrifolius* plant analyzed in this study (range: 4.8–28.5 mg/g) with that from other countries (4 to 210 mg/g quercetin for *Tragopogon porrifolius* from Turkey [4] and 16.6 mg/g quercetin for *Tragopogon porrifolius* from Lebanon [1]).

3.3. Antioxidant Activity. AA accounts for the presence of efficient oxygen radical scavengers, such as phenolic compounds [22]. The antioxidant activity of phenolics is mainly due to their redox properties, which make them act as reducing agents, hydrogen donors, and singlet oxygen quenchers [22].

3.3.1. Reducing Potential of Plant Extracts

(1) FRAP Assay. FRAP assay measures the reducing potential of an antioxidant reacting with a ferric tripyridyltriazine (Fe^{3+}-TPTZ) complex and producing a colored ferrous tripyridyltriazine (Fe^{2+}-TPTZ).

The antioxidant tests based on FRAP assay of *Tragopogon porrifolius* plant extracts using three different solvents are presented in Table 1 (expressed as mmol Fe^{+2}/g of dry plant material). Statistical analyses showed that there are significant differences between FRAP values as a function of extraction solvent (Table 1), where significant differences ($p < 0.05$) are indicated by different small letters (a, b, and c).

Table 1 revealed that antioxidant activity (FRAP) of the *Tragopogon porrifolius* plant increased as the polarity of solvent changes (80% ethanol > water > 100% ethanol), where FRAP values were found to be about two and six times significantly higher when extracted with 80% ethanol compared to water and 100% ethanol, respectively.

The trend of extraction solvent on the FRAP values was found to be the same as for TPC but different from TFC. This suggests that there is a correlation between AA (expressed as FRAP) and TPC, reflecting the fact that total phenolics are the major determinant of AA.

As in the case of TPC and TFC, 80% ethanol gives higher amounts of AA (FRAP) compared with water as extraction solvent of *Tragopogon porrifolius*.

It is interesting to compare AA (FRAP) of Palestinian *Tragopogon porrifolius* with that from other countries. For example, *Tragopogon porrifolius* from Lebanon [1] was found to have 0.659 mmol Fe^{+2}/g which is much lower than the FRAP value for *Tragopogon porrifolius* plant analyzed in this study (2.1–12.1 mmol Fe^{+2}/g).

(2) CUPRAC Assay. Although FRAP antioxidant assay has been very popular among researchers, CUPRAC assay is a relatively new assay developed by Apak et al. [17]. It utilizes the copper(II)-neocuproine [Cu(II)-Nc] reagent as the chromogenic oxidizing agent and is based on the cupric reducing ability of reducing compounds to cuprous.

Table 1 shows the CUPRAC antioxidant activity (expressed as μmole Trolox/g) of *Tragopogon porrifolius* plant extracts using three different solvents. Statistical analyses showed that there are significant differences between AA using the three extraction solvents, where significant differences ($p < 0.05$) are indicated by different small letters (a, b, and c).

Results showed that CUPRAC antioxidant activity of the *Tragopogon porrifolius* plant increased in the following order: 80% ethanol > water > 100% ethanol which is the same trend as FRAP antioxidant activity and TPC but different from TFC, which suggests that there is a correlation between CUPRAC AA and TPC.

3.3.2. Free Radical-Scavenging Ability of Plant Extracts

(1) DPPH Assay. DPPH is a free radical compound and has been widely used to test the free radical-scavenging ability of various samples [23]. It is a stable free radical with a characteristic absorption at 517 nm that was used to study the radical-scavenging effects of extracts. As antioxidants donate protons to this radical, the absorption decreases. Antioxidants, on interaction with DPPH, transfer either an electron or hydrogen atom to DPPH, thus neutralizing its free radical character [24]. The color changed from purple to yellow and the absorbance at wavelength 517 nm decreased.

DPPH assay is based on the ability of the stable free radical 2,2-diphenyl-1-picrylhydrazyl to react with hydrogen donors including phenolics. The bleaching of DPPH solution increases linearly with increasing amount of extract in a given volume.

Table 1 shows the % inhibition of DPPH free radicals by the *Tragopogon porrifolius* plant extracted with the three solvents. Statistical analyses showed that there are significant differences between % inhibitions using the three extraction solvents, where significant differences ($p < 0.05$) are indicated by different small letters (a, b, and c); see Table 1.

Table 2 shows the % inhibition of DPPH at different concentrations of the crude extract (from 20 to 120 μg/mL). This data shows that the extracts exhibited a dose dependent scavenging activity (a linear relationship between % of DPPH

Table 2: % inhibition of DPPH and ABTS free radicals by different concentrations of *Tragopogon porrifolius* plant extract.

Concentration of DPPH (μg/mL)	% inhibition of DPPH*,°	Concentration of ABTS (μg/mL)	% inhibition of ABTS*,°
20	13.3 ± 0.5	20	16.9 ± 0.3
40	26.7 ± 1.2	40	34.1 ± 0.7
60	41.4 ± 1.0	60	50.3 ± 0.9
80	54.6 ± 1.4	80	67.2 ± 1.1
120	83.2 ± 2.1	100	81.4 ± 1.5

*Results are expressed as average ± standard deviation of three samples.
°EC50 for DPPH and ABTS are 73 and 60.8 μg/mL, respectively.

inhibition and concentration ($y = 0.6995x - 0.9297$, with R^2 of 0.9998), where y is the % of inhibition and x is the concentration). From this linear relationship, EC50 which is the concentration required to quench 50% of the DPPH free radicals was determined and was found to be 73 μg/mL.

DPPH antioxidant activity of *Tragopogon porrifolius* plant extracts using three different solvents was expressed as μmole Trolox/g (Table 1) and EC50 (Table 2). Statistical analyses showed that there are significant differences between AA using the three extraction solvents, where significant differences ($p < 0.05$) are indicated by different small letters (a, b, and c).

Results showed that DPPH antioxidant activity of the *Tragopogon porrifolius* plant increased in the following order: 80% ethanol > water > 100% ethanol which is the same trend as TPC, FRAP, and CUPRAC antioxidant activity.

(2) ABTS Assay. The ABTS assay measures the relative antioxidant ability of extracts to scavenge the radical-cation $ABTS^{+\bullet}$ produced by the oxidation of 2,2′-azinobis-3-ethylbenzothiazoline-6-sulphonate.

Table 1 shows the % inhibition of ABTS free radicals by the plant extracted with the three solvents. Statistical analyses showed that there are significant differences between AA using the three extraction solvents, where significant differences ($p < 0.05$) are indicated by different small letters (a, b, and c); see Table 1.

Table 2 shows the % inhibition of ABTS at different concentrations of the crude extract (from 20 to 100 μg/mL). This data shows that the extracts showed a dose dependent scavenging activity (a linear relationship between % of ABTS inhibition and concentration ($y = 0.8105x + 1.35$, with R^2 of 0.999), where y is the % of inhibition and x is the concentration). From this linear relationship, IC50 was determined and was found to be about 60.8 μg/mL.

ABTS antioxidant activity of *Tragopogon porrifolius* plant extracts using three different solvents was expressed as μmol Trolox/g (Table 1) and EC50 (Table 2). Statistical analyses showed that there are significant differences between AA using the three extraction solvents, where significant differences ($p < 0.05$) are indicated by different small letters (a, b, and c).

Results showed that ABTS antioxidant activity of the *Tragopogon porrifolius* plant increased in the following order:

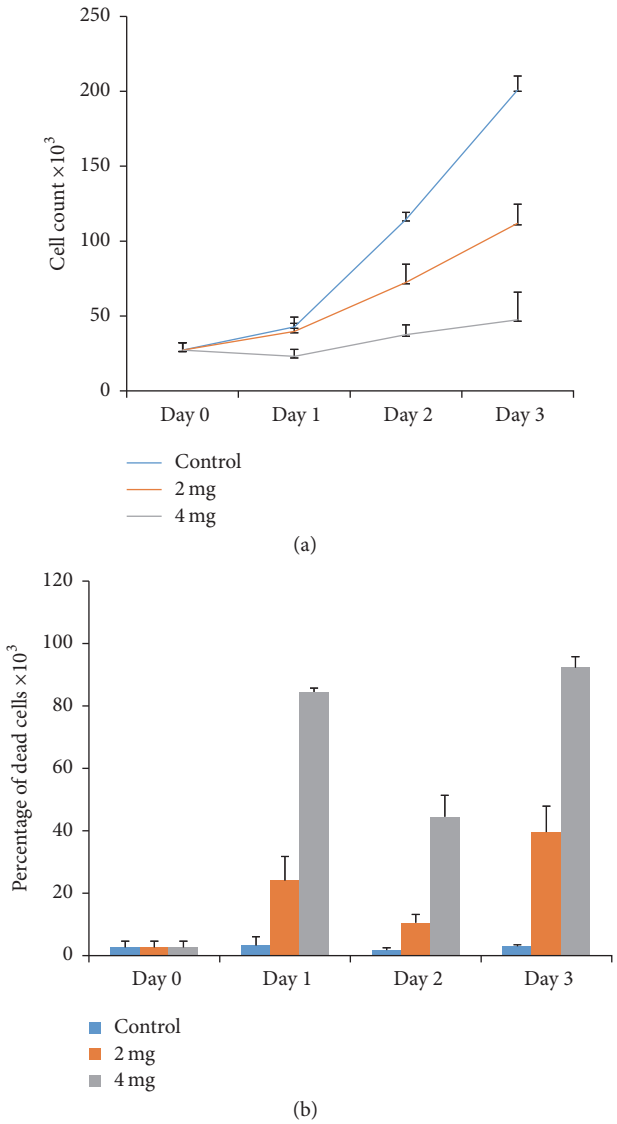

Figure 1: Cytotoxicity of *Tragopogon porrifolius* ethanolic extract on KHOS cancer cell lines. (a) Effect of the extract on cell growth after treating the cells with the indicated concentrations for the indicated time points. (b) Effect of the extract on cell viability after treating the cells with the indicated concentrations for the indicated time points. Error bars represent standard deviation calculated from three different experiments carried out in triplicate.

80% ethanol > water > 100% ethanol which is the same trend as FRAP, CUPRAC, and DPPH antioxidant activities. Additionally this trend is the same as TPC but different from TFC, which suggests that there is a correlation between ABTS and TPC.

3.4. Anticancer Activity. In order to test the anticancer activity of the crude extract of the Palestinian *Tragopogon porrifolius,* human osteosarcoma cells, HOS and KHOS, were treated with different concentrations of the extract (2 mg/mL and 4 mg/mL for KHOS and 4 mg/mL for HOS cells) and counted them and scored the percentage of dead cells. As shown in Figures 1(a) and 2(a), ethanolic extract

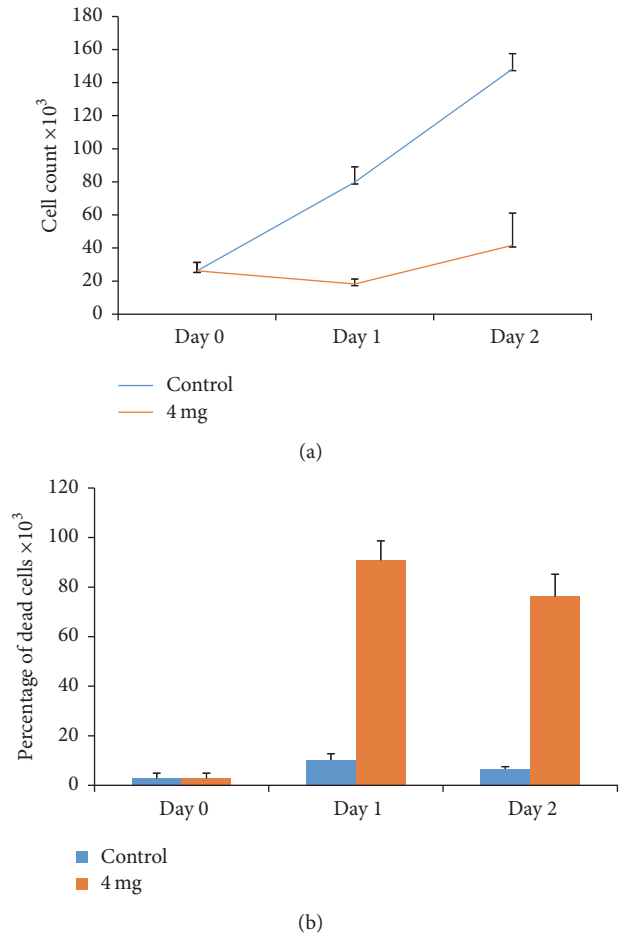

Figure 2: Cytotoxicity of *Tragopogon porrifolius* ethanolic extract on HOS cells. (a) Effect of the extract on cell growth after treating the cells with the indicated concentrations for the indicated time points. (b) Effect of the extract on cell viability after treating the cells with the indicated concentrations for the indicated time points. Error bars represent standard deviation calculated from three different experiments carried out in triplicate.

decreased cell growth rate to less than 30% after three days for KHOS cells or two days for HOS cells. Moreover, the extract increased the percentage of dead cells by many folds as compared to control untreated cells (Figures 1(b) and 2(b)). Indeed our results are consistent with previous results that showed that *Tragopogon porrifolius* has a strong anticancer activity [1]. While it has been shown that *Tragopogon porrifolius* has a strong anticancer activity against both breast and colon cancer cell lines [1], we here succeeded to demonstrate an anticancer activity against two very aggressive osteosarcoma cell lines.

4. Conclusions

Tragopogon porrifolius plant is rich with phenolic compounds and flavonoids, and it has anticancer activities against two very aggressive osteosarcoma cancer cell lines (KHOS and HOS). Total phenolics and flavonoid contents as well as antioxidant activities are highest for plant extracted with 80% ethanol. Mixture of ethanol and water (80% ethanol) are the best solvent for extraction of phenolic and flavonoid compounds. There is a correlation between antioxidant activity and total phenolic content but not with total flavonoid content. *Tragopogon porrifolius* plant constitutes a natural source of potent antioxidants that may prevent many diseases and could be potentially used in food, cosmetics, and pharmaceutical industries.

Competing Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

References

[1] C. Tenkerian, M. El-Sibai, C. F. Daher, and M. Mroueh, "Hepatoprotective, antioxidant, and anticancer effects of the tragopogon porrifolius methanolic extract," *Evidence-Based Complementary and Alternative Medicine*, vol. 2015, Article ID 161720, 10 pages, 2015.

[2] C. Formisano, D. Rigano, F. Senatore, M. Bruno, and S. Rosselli, "Volatile constituents of the aerial parts of white salsify (*Tragopogon porrifolius* L., Asteraceae)," *Natural Product Research*, vol. 24, no. 7, pp. 663–668, 2010.

[3] M. Konopiński, "Influence of intercrop plants and varied tillage on yields and nutritional value of salsify (*Tragopogon porrifolius* L.) roots," *Acta Scientiarum Polonorum, Hortorum Cultus*, vol. 8, no. 2, pp. 27–36, 2009.

[4] B. A. Özlem, S. C. Gulcin, and Ç. Tülay, "Evaluation of antioxidant properties of some tragopogon species growing in Turkey," *Turkish Journal of Pharmaceutical Sciences*, vol. 10, no. 3, pp. 377–384, 2013.

[5] L. A. Pham-Huy, H. He, and C. Pham-Huy, "Free radicals, antioxidants in disease and health," *International Journal of Biomedical Science*, vol. 4, no. 2, pp. 89–96, 2008.

[6] M. Servili and G. Montedoro, "Contribution of phenolic compounds in virgin olive oil quality," *European Journal of Lipid Science and Technology*, vol. 104, no. 9-10, pp. 602–613, 2002.

[7] S. Silva, L. Gomes, F. Leitão, A. V. Coelho, and L. V. Boas, "Phenolic compounds and antioxidant activity of Olea europaea L. Fruits and leaves," *Food Science and Technology International*, vol. 12, no. 5, pp. 385–396, 2006.

[8] G. Waris and H. Ahsan, "Reactive oxygen species: role in the development of cancer and various chronic conditions," *Journal of Carcinogenesis*, vol. 5, article 14, 2006.

[9] M. D. Evans, M. Dizdaroglu, and M. S. Cooke, "Oxidative DNA damage and disease: induction, repair and significance," *Mutation Research*, vol. 567, no. 1, pp. 1–61, 2004.

[10] A. Prakash, F. Rigelhof, and E. MIller, Antioxidant Activity, World Wide Web, 2011, http://www.medlabs.com/downloads/antiox_acti_.pdf.

[11] M. Mojarrab, A. Khan Mohammadi, L. Hosseinzadeh, and Z. M. Siavash-Haghighi, "Antioxidant activity and safety assessment of Tragopogon buphthalmoides hydroethanolic extract: acute and subchronic toxicities," *Research in Pharmaceutical Sciences*, vol. 9, no. 5, pp. 359–366, 2014.

[12] M. Asadi-Samani, N. Kafash-Farkhad, N. Azimi, A. Fasihi, E. Alinia-Ahandani, and M. Rafieian-Kopaei, "Medicinal plants with hepatoprotective activity in Iranian folk medicine," *Asian*

Pacific Journal of Tropical Biomedicine, vol. 5, no. 2, pp. 146–157, 2015.

[13] N. Zeeni, C. F. Daher, L. Saab, and M. Mroueh, "*Tragopogon porrifolius* improves serum lipid profile and increases short-term satiety in rats," *Appetite*, vol. 72, pp. 1–7, 2014.

[14] M. Mroueh, C. Daher, M. El Sibai, and C. Tenkerian, "Antioxidant and hepatoprotective activity of *Tragopogon porrifolius* methanolic extract," *Planta Medica*, vol. 77, article PF72, 2011.

[15] P. Govind, "Medicinal plants against liver diseases," *International Research Journal of Pharmacy*, vol. 2, pp. 115–121, 2011.

[16] I. F. F. Benzie and J. J. Strain, "Ferric reducing/antioxidant power assay: direct measure of total antioxidant activity of biological fluids and modified version for simultaneous measurement of total antioxidant power and ascorbic acid concentration," *Methods in Enzymology*, vol. 299, pp. 15–27, 1998.

[17] R. Apak, K. Güçlü, M. Özyürek, and S. E. Çelik, "Mechanism of antioxidant capacity assays and the CUPRAC (cupric ion reducing antioxidant capacity) assay," *Microchimica Acta*, vol. 160, no. 4, pp. 413–419, 2008.

[18] W. Brand-Williams, M. E. Cuvelier, and C. Berset, "Use of a free radical method to evaluate antioxidant activity," *LWT—Food Science and Technology*, vol. 28, no. 1, pp. 25–30, 1995.

[19] R. Re, N. Pellegrini, A. Proteggente, A. Pannala, M. Yang, and C. Rice-Evans, "Antioxidant activity applying an improved ABTS radical cation decolorization assay," *Free Radical Biology and Medicine*, vol. 26, no. 9-10, pp. 1231–1237, 1999.

[20] V. L. Singleton and J. A. Rossi, "Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents," *American Journal of Enology and Viticulture*, vol. 16, pp. 144–158, 1965.

[21] D.-O. Kim, S. W. Jeong, and C. Y. Lee, "Antioxidant capacity of phenolic phytochemicals from various cultivars of plums," *Food Chemistry*, vol. 81, no. 3, pp. 321–326, 2003.

[22] L. Scalfi, V. Fogliano, A. Pentangelo, G. Graziani, I. Giordano, and A. Ritieni, "Antioxidant activity and general fruit characteristics in different ecotypes of Corbarini small tomatoes," *Journal of Agricultural and Food Chemistry*, vol. 48, no. 4, pp. 1363–1366, 2000.

[23] S. Sakanaka, Y. Tachibana, and Y. Okada, "Preparation and antioxidant properties of extracts of Japanese persimmon leaf tea (kakinoha-cha)," *Food Chemistry*, vol. 89, no. 4, pp. 569–575, 2005.

[24] G. H. Naik, K. I. Priyadarsini, J. G. Satav et al., "Comparative antioxidant activity of individual herbal components used in ayurvedic medicine," *Phytochemistry*, vol. 63, no. 1, pp. 97–104, 2003.

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Research Article

The Effects of Guizhi Fuling Capsule Drug Serum on Uterine Leiomyoma Cells and Its Mechanism

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Aims. To observe the effects of Guizhi Fuling Capsule (GZFLC) drug serum on uterine leiomyoma cells and explore its mechanism. *Main Methods*. Sixty Sprague Dawley rats were randomly divided into two groups (normal saline lavage group and GZFLC lavage group), then, respectively, blank serum and GZFLC drug serum were collected, and finally human uterine leiomyoma cells were treated. Human leiomyoma tissues were collected from 20 patients who underwent uterine leiomyomas operations, and leiomyoma cells were primary cultured. The leiomyoma cells were treated by GZFLC drug serum in different concentrations (10%, 20%, and 30%) and variable treatment time (12 h, 24 h, 36 h, 48 h, and 72 h). Cell proliferation was observed using CCK8 assay. Flow cytometry and Annexin V/PI were used to assay the effects of GZFLC drug serum on cell apoptosis. Western blot analysis was used to assay the effects of GZFLC drug serum on TSC2, FOXO, and 14-3-3γ expression in uterine leiomyoma cells. *Key Findings*. In the concentrations of 10%~30%, GZFLC drug serum could inhibit proliferation of leiomyoma cells in dose-dependent manner; at the time of 36 h, cell inhibition rate was at the peak; GZFLC drug serum could induce apoptosis of leiomyoma also in a dose-dependent manner, and apoptosis rate quickly achieved maximum at 12 h time points, and then second apoptosis peak appeared at 36 h. Compared to nontreatment group, TSC2, FOXO, and 14-3-3γ expressions in drug serum group were significantly changed after 12 h treatment. *Significance*. GZFLC drug serum can efficiently inhibit the proliferation and induce apoptosis of leiomyoma cells, which is related to the 14-3-3γ pathway.

1. Introduction

Uterine leiomyomas are the most frequent neoplasm, affecting 20–40% of reproductive-age women [1]. Although frequently asymptomatic, leiomyomas can cause menorrhagia, abnormal uterine bleeding, pelvic pain or pressure, infertility, and miscarriage. Current treatments include surgical approaches and medical therapies (such as gonadotropin-releasing hormone agonists and hormonal therapies) [2]. However, surgery has some risks and is not suitable for patients who refuse operation [3]. Medical therapy also has some limitations. Gonadotropin-releasing hormone agonists can relieve leiomyoma-related symptoms but might have significant menopausal side effects [4, 5]. Progesterone antagonists, such as mifepristone, significantly reduce uterine and leiomyoma volume and alleviate leiomyoma-related symptoms without major adverse events. However, progesterone antagonists and other hormonal therapies which change progesterone and estrogen production could affect women's fertility function [6]. Therefore, safer and more effective therapy is needed for uterine leiomyomas.

Guizhi Fuling Capsule (GZFLC), as a famous traditional Chinese herbal formula, consisted of five herbs: *Cortex Moutan*, *Radix Paeoniae*, *Ramulus Cinnamomi*, *Poria cocos*, and *Semen Persicae* [7]. About 1800 years ago, it was first presented in *Jin Gui Yao Lue* (*Essential Prescriptions from the Golden Cabinet*) by the Chinese doctor Zhang Zhongjing. It has been widely used for the treatment of different diseases such as gynecological diseases and atherosclerosis [8, 9] and is involved in multiple pharmacological activities, such as stimulating the proliferative lesion soft and absorption, enhancing immune response, and preventing cancer cell growth [10]. In recent years, a clinical research confirmed that GZFLC was effective in uterine leiomyomas [11, 12]. GZFLC, the clinical recommended regimen, is taken orally as 3 capsules (in total 0.93 g) at one time, 3 times per day, 3 to 6 months, used alone

or combined with mifepristone, GnRH-a, or other drugs to treat uterine leiomyomas. However, the treatment mechanism is not clear so far.

14-3-3 proteins, as a highly conserved phosphoserine/threonine-binding proteins family, comprised 7 isoforms (β, γ, ε, η, σ, τ/θ, and ξ) and participate in many physiological processes including cycle progression, transcriptional regulation, cell apoptosis, and proliferation [13–15]. In our previous study, via proteomics, we found that the expression of 14-3-3γ was reduced in uterine leiomyoma compared with normal myometrium [16]. Besides, 14-3-3γ is the receptor of FOXO and TSC2, playing an important role in cell proliferation and apoptosis via preventing them from dephosphorylation to control the proportion of cytoplasmic and nuclear proteins [15, 17]. However, the mechanism explaining how GZFLC modulates 14-3-3γ, FOXO, and TSC2 is poorly understood.

We undertook this study to observe the effects of GZFLC drug serum on proliferation and apoptosis of leiomyoma cells and to explore its mechanism. We hope to provide a new thread for effective treatment on uterine leiomyoma clinically.

2. Materials and Methods

2.1. Tissue Collection. Between October 2013 and July 2014, 20 patients who were diagnosed by B-ultrasound of uterine leiomyoma were recruited for the study. Patients' age ranged from 30 to 55 years. The types of surgery were uterine myomectomy and subtotal or total hysterectomy. No patients received any medication or hormonal therapy for at least 3 months prior to operation. Patients complicated with chronic diseases (such as hypertension and diabetes), infection, uterine malignancy, and adenomyosis (on the basis of tissue pathology) were excluded from the present study. The study protocol was approved by the Research Ethical Committee of the Second Affiliated Hospital of Wenzhou Medical University and written consent was obtained from patients before the collection of samples. The study met the standards of the Declaration of Helsinki.

2.2. Cell Primary Culture. Fresh tissues were thoroughly washed with phosphate-buffered saline (PBS) to remove blood. Then they were cut into small pieces (1 mm^3) and placed into dissociation solution (Dulbecco's modified Eagle's medium (DMEM) and 0.2% v/v collagenase II (Invitrogen, Carlsbad, CA, USA)), followed by incubation for 4 h at 37°C in a water bath with continuous shaking to dissociate uterine leiomyoma cells. The dispersed cells were centrifuged at 100 ×g for 5 min. The resultant deposit was mixed with complete culture medium (DMEM, 10% fetal bovine serum, 100 IU/mL of penicillin G, and 100 μg/mL streptomycin) and centrifuged at 100 ×g for 5 minutes. The resultant cells were plated at a density of 1×10^5 cells per 60 mm dishes (Corning, USA) under 5% CO_2 at 37°C in the complete culture medium. Culture medium was changed every other day. Cells were passaged as 1 : 2 every 4 to 6 days. Cells from third passage to the fifth one were used for all the experiments.

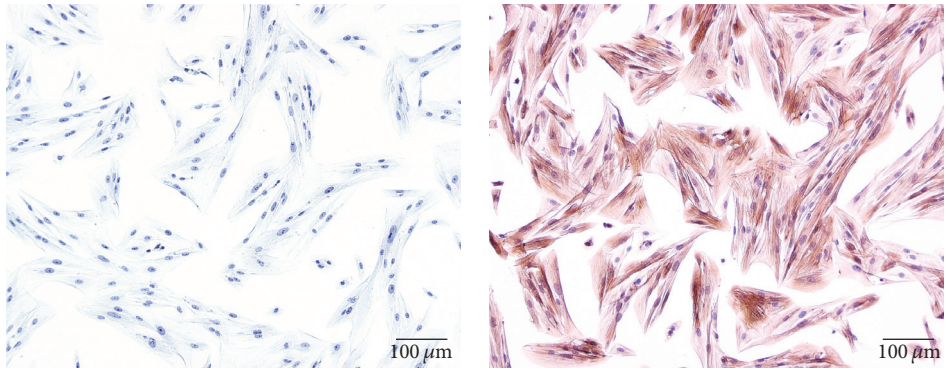
2.3. Preparation of Serum Containing the Tested Drugs. Subsequent to obtaining approval from the Ethics Committee of Wenzhou Medical University (Wenzhou, China), 60 male Sprague Dawley rats weighing 180–220 g were provided by the Experimental Animal Center of Wenzhou Medical University and housed in a room with a temperature of 21–25°C, relative humidity of 50–60%, and a 12-hour light/dark cycle. The recommended daily allowance of GZFLC tablet is 2.79 g for 60 kg body weight. The conversion ratio from a 70 kg man to a 200 g rat is 0.018 [18]; the corresponding dose of GZFLC tablet for rats was 0.293 g/kg per day. Therefore, rats were randomly divided into two groups, the experimental group was intragastrically given GZFLC (0.146 g/kg) twice daily (days 1–7) and the negative control group was intragastrically given same frequency with normal saline. Blood was acquired from the abdominal aorta of the rats 1 h after the last time of administration. The serum was collected by centrifugation (720 ×g for 20 min) and then filtered through a 0.22 μm cellulose acetate membrane. Next, the serum was inactivated in 56°C water for 30 min and stored at −20°C until use.

In order to explain the method to make different concentrations of GZFLC serum, for example, 10 mL 20% GZFLC serum (complete culture medium containing 20% GZFLC serum) consisted of 7.9 mL DMEM, 2 mL rat serum with GZFLC treatment, 0.1 mL penicillin G, and streptomycin.

2.4. Cell Counting Kit-8 (CCK-8) Assay. The uterine leiomyoma cells were seeded in 96-well plates at 1×10^4 cells/well and incubated in a 37°C, 5% CO_2 incubator. After 24 h of seeding, the culture medium was removed and cells were accordingly treated. Subsequently, CCK-8 (Dojindo, Japan) at 10 μL was added to each well and incubated for additional 2 h. The quantification was done by using spectrophotometry at a 450 nm wavelength, and the viability percentage was calculated as follows: (treated cells absorbent/nontreated cells absorbent) × 100.

2.5. Immunocytochemistry. Cells were washed with PBS and fixed in PBS containing 4% paraformaldehyde for 15 min, washed extensively with PBS after fixing and permeabilized in PBS containing 0.2% Triton X-100 for 15 min, and blocked in a serum-free blocking solution for 15 min at room temperature. Cells were then incubated with anti-α-smooth muscle actin antibody (1 : 100 dilution; Zhongshan Golden Bridge Biotechnology Co., Ltd., Beijing, China) overnight at 4°C. After PBS washing, cells were incubated with biotinylated rabbit anti-human IgG as secondary antibody. After incubation, the binding sites were visualised using 3,3′-diaminobenzidine. Finally, nuclei were stained with hematoxylin. Negative control slides, where primary antibody was replaced with PBS, were also included.

2.6. Annexin V-FITC/PI Assay. For apoptosis analysis, Annexin V-FITC/PI staining was performed using flow cytometry according to the manufacturer's guidelines. Briefly, cells were washed twice with cold PBS and then resuspended cells in ×1 binding buffer at concentration 1×10^6 cells/mL. 10 μL of the solution (1×10^5 cells) was transferred to a 5 mL culture



(a) (b)

Figure 1: Staining of α-actin in human leiomyoma cells (SP staining, ×100). (a) Negative control; (b) α-actin positive staining.

tube. 5 μL of FITC Annexin V and 5 μL PI were added. The cells were gently vortexed and incubated for 15 min at room temperature (25°C) in the dark. 40 μL of ×1 binding buffer was added to each tube. Cells were analyzed by flow cytometry within 1 h.

2.7. Cell Lysate Preparation and Western Blot Analysis. Whole-cell lysates were prepared using ice-cold cell lysis buffer. Cells were collected and washed after indicated treatments. The protein from each experimental group was quantified by bicinchoninic acid (Beyotime, China). Cellular proteins (8 μg) were solubilized in sample buffer (4% SDS, 30 mm dithiothreitol, 0.25 m sucrose, 0.01 m EDTA-Na_2, and 0.075% bromophenol blue) and heated at 100°C for 5 min to denature proteins. The lysates were separated using electrophoresis on 12% sodium dodecyl sulfate-polyacrylamide gel and then electroblotted onto polyvinylidene fluoride membranes (Millipore, USA). The membranes were blocked for 2 h at room temperature in 0.05 M Tris-buffered saline with 0.5% triton X-100 (TBS-T, pH 7.4) containing 5% skimmed milk and then incubated with the appropriate primary antibody (anti-14-3-3γ (1 : 1000 dilution; Santa Cruz, USA), anti-FOXO (1 : 1000 dilution; CST, USA), anti-TSC2 (1 : 1000 dilution; CST, USA), and anti-α-Tubulin (1 : 2000 dilution; Beyotime, China)) in TBS-T overnight at 4°C. After washing with TBS-T, horseradish peroxidase-conjugated secondary antibodies (1 : 2000 for anti-rabbit IgG or anti-mouse IgG) for 1 h at room temperature, blots were developed by enhanced chemiluminescence. The expression levels of 14-3-3γ, FOXO, and TSC2 were quantified with densitometry and normalized by corresponding levels of α-Tubulin, respectively. Each experiment was repeated at least three times.

2.8. Statistical Methods. All statistical analyses were performed with SPSS17.0 software. If each group data was of normal distribution and homogeneity of variance, quantitative data was presented as the mean ± standard deviation; difference between two groups was analyzed by Student's t-test.

Difference among multiple groups was analyzed by one-way ANOVA, if variances were homogeneous; then least significance difference (LSD) method was used to compare between two groups. If variances were nonhomogeneous, Dunnett's T3 method was applied to compare between two groups.

Two-tailed P value < 0.05 was considered statistically significant.

3. Results

3.1. The Identification of Primary Culture Human Uterine Leiomyoma Cells. To identify primary culture human uterine leiomyoma cells, immunocytochemistry staining of the cells with α-actin antibody was performed. As shown in Figure 1, positive brown signals of α-actin antibody mainly stained in cytoplasm, indicating that cell cultures derived from uterine leiomyoma tissues were identified correctly and retained their smooth muscle characteristics.

3.2. The Effect of GZFLC Drug Serum on Cell Proliferation of Human Uterine Leiomyoma Cells. As shown in Table 1, the negative control group compared with the blank control group (culture without serum); the negative control group at the saline serum concentration of 10%, 20%, and 30% and at time point of 24 h, 36 h, 48 h, and 72 h had higher optical density (OD) values ($P < 0.05$), indicating that rats serum promoted uterine leiomyoma cells proliferation. Compared with the negative control group, the GZFLC group showed that the OD value of 10% GZFLC serum at 36 h and 48 h was significantly lower ($P < 0.05$), the OD value of 20% GZFLC serum at 24 h, 36 h, and 48 h time point was obviously lower ($P < 0.05$), and at 72 h time point the inhibition phenomenon disappeared ($P < 0.05$); the OD value of 30% GZFLC serum at 12 h, 24 h, 36 h, and 48 h time point was significantly lower ($P < 0.05$) and at 72 h time point the inhibition phenomenon disappeared ($P < 0.05$), indicating that 30% GZFLC serum quickly inhibited cells proliferation and was effectively maintained for a long time. As the time point (12 h, 24 h, 36 h, and 48 h) increased, the OD value decreased with the drug

Table 1: The effect of GZFLC drug serum on cell proliferation of human uterine leiomyoma cells.

Groups	Concentration (%)	Cases	OD value				
			12 h	24 h	36 h	48 h	72 h
GZFLC	10	6	0.60 ± 0.05	0.75 ± 0.04	0.86 ± 0.04$^{\#}$	1.06 ± 0.02$^{\#}$	1.27 ± 0.04*
	20	6	0.60 ± 0.025	0.72 ± 0.02$^{\#}$	0.84 ± 0.02$^{\#}$	1.00 ± 0.06$^{\#}$	1.39 ± 0.022$^{\#*}$
	30	6	0.58 ± 0.02$^{\#}$	0.69 ± 0.02$^{\#}$	0.78 ± 0.02$^{\#}$	0.91 ± 0.02$^{\#}$	1.49 ± 0.02$^{\#*}$
Negative control	10	6	0.63 ± 0.05	0.80 ± 0.03*	0.99 ± 0.05*	1.19 ± 0.02*	1.25 ± 0.03*
	20	6	0.66 ± 0.03	0.89 ± 0.03*	1.11 ± 0.05*	1.28 ± 0.07*	1.30 ± 0.02*
	30	6	0.67 ± 0.03	0.92 ± 0.02*	1.20 ± 0.01*	1.32 ± 0.01*	1.33 ± 0.01*
Blank control	—	6	0.61 ± 0.03	0.70 ± 0.04	0.83 ± 0.05	0.90 ± 0.09	0.85 ± 0.08

*Compared with blank control, $P < 0.05$.
$^{\#}$Compared with negative control, $P < 0.05$.

concentration rise, but, at 72 h time point, the difference was not obvious, indicating that, within 48 hours, GZFLC serum inhibited human uterine leiomyoma cells proliferation was concentration-dependent and inhibitory effect weakened beyond that time.

3.3. The Effect of GZFLC Drug Serum on Cell Apoptosis of Human Uterine Leiomyoma Cells. As shown in Figures 2 and 3, the negative control group compared with the blank control group at the concentration of 10% and 20% (12 h) had no significant difference in cells apoptosis rate ($P > 0.05$), but at 30% it had increased apoptosis rate ($P < 0.05$), indicating that 30% rats blank serum promoted uterine leiomyoma cells apoptosis. Compared with the negative control group, the GZFLC group with different concentration (10%, 20%, and 30%) had higher cells apoptosis rate ($P < 0.05$). In addition, with the increased GZFLC serum concentration and cell apoptosis rate increasing, the different concentration within the apoptosis rate had significant difference ($P < 0.05$), indicating that GZFLC serum promoted human uterine leiomyoma cells apoptosis was concentration-dependent.

As shown in Figures 4 and 5, the negative control group compared with the blank control group at different time point (20% drug concentration) had no significant difference in cells apoptosis rate ($P > 0.05$), indicating that 20% rats blank serum had no obvious effects on cells apoptosis. Compared with the negative control group, the GZFLC group with 20% drug concentration had the most obvious apoptosis rate difference at 12 h, and then at 24 h it decreased, and after 36 h, apoptosis rate increased again ($P < 0.05$) and finally decreased.

GZFLC serum promoted human uterine leiomyoma cells apoptosis was concentration-dependent, 30% GZFLC serum had 34% apoptosis rate, but 30% rats blank serum also obviously promoted cells apoptosis (15% apoptosis rate); thus we chose 20% GZFLC serum as the optimal concentration to avoid the interference effect from rat blank serum. As the 20% drug concentration had the highest apoptosis rate at 12 h, it was considered as the optimal time point.

3.4. Effect of GZFLC Drug Serum on the Expression of TSC2, FOXO, and 14-3-3γ in the Human Leiomyoma Cells. The proteins were collected at the optimal concentration and time point above. As shown in Figure 6, there was no significant difference in the expression of TSC2, FOXO, and 14-3-3γ proteins between the negative control group and the blank control group ($P > 0.05$). However, the GZFLC group had higher expression of TSC2, FOXO, and 14-3-3γ proteins compared with the negative control group ($P < 0.05$).

These results suggest that 14-3-3γ signal transduction pathway might be involved in GZFLC drug serum which inhibited the proliferation and induced apoptosis of uterine leiomyoma cells.

4. Discussion

In the present study, CCK-8 analysis and flow cytometry assay showed that GZFLC drug serum efficiently inhibited the proliferation and induced apoptosis of human uterine leiomyoma cells. In addition, Western blot revealed that this function may be related to the 14-3-3γ, TSC2, and FOXO pathway.

GZFLC is widely applied for uterine leiomyomas in China. Its traditional effects are invigorating blood, resolving masses, and dissolving stasis [8]. To assess the efficacy and safety of GZFLC for the treatment of uterine leiomyomas, Chen et al. identified 38 randomized controlled trials involving 3816 participants by meta-analyses [11] and found that GZFLC plus mifepristone was more effective than mifepristone alone in reducing the volume of uterine leiomyoma. GZFLC significantly improved symptoms of uterine leiomyoma, particularly in dysmenorrhea, when it was used alone or in combination with mifepristone. No serious adverse events were reported. However, the exact mechanism of GZFLC treatment for uterine leiomyomas is unclear.

Hu et al. reported that GZFLC attenuated endometriosis in rats via induction of apoptosis and inhibition of cell proliferation and metastasis [19]. In terms of its anticancer effect, this phenomenon was also found in human hepatocellular

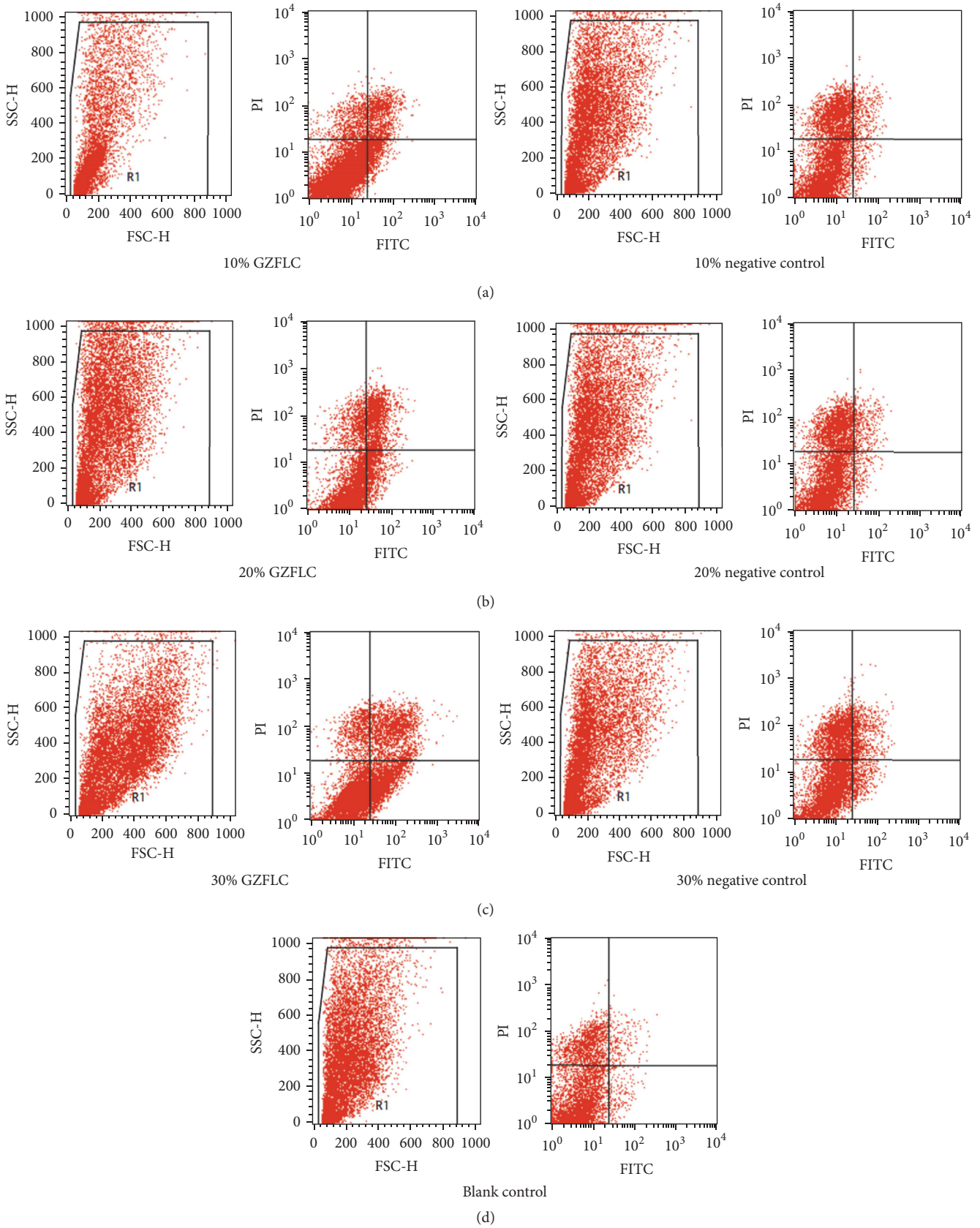


Figure 2: The cell apoptosis rate of different concentrations of GZFLC drug serum on the human leiomyoma cells via flow cytometry. (a) 10% GZFLC serum and 10% negative control. (b) 20% GZFLC serum and 20% negative control. (c) 30% GZFLC serum and 30% negative control. (d) Blank control.

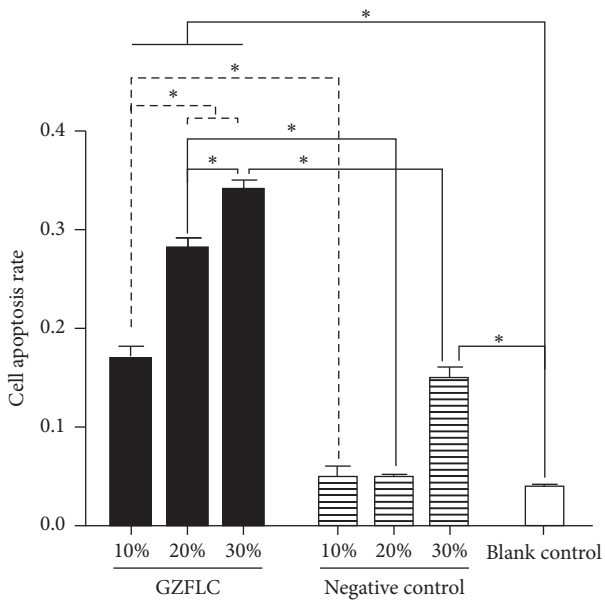


Figure 3: The cell apoptosis rate of different concentrations of GZFLC drug serum on the human leiomyoma cells. $*$ indicated $P < 0.05$.

carcinoma [20, 21], bladder cancer [22], and cervical cancer [23]. As female rats have physiological cycle and the hormone levels and serum enzyme activity are relatively stable in male rats, male rats were selected in our experiments [24]. In this study, drug serum was chosen to simulate the drug effects on patients local tissue after medication; thus drug serum from rats after intragastric administration was applied to the primary culture cells, while normal saline serum was a negative control. In in vitro cell experiments with serum pharmacology, it is better to use the same species serum, as serums from different sources may interact with each other. In order to avoid the interference from fetal bovine serum, in this experiment, the rats serums were used to prepare different concentration with DMEM, which served as cell nutrients provider and drug carrier. GZFLC drug serum showed the inhibition of proliferation and induction of apoptosis on human uterine leiomyoma cells, which may explain that GZFLC could reduce the volume of leiomyomas and relieve the symptoms of leiomyomas. Compared to other time points, 12 h of GZFLC drug serum treatment was shown to be most effective in inducing cell apoptosis, indicating that the GZFLC serum has rapid efficacy. But the effects lasted a short time, at 24 h time point, the apoptosis rate decreased, and the possible explanation could be the increased amount of cell necrosis resulting in decreasing apoptosis rate. However, at 36 h, cell apoptosis rate rose again; the possibility that the second metabolic products of the compound traditional Chinese medicine worked could not be ruled out. Further research is needed.

In our previous study, 14-3-3γ was significantly downregulated in uterine leiomyoma compared to normal myometrium [16]; these results were then confirmed by several studies [25, 26], indicating that 14-3-3γ may play a role in the origin or growth of uterine leiomyomas. Wei et al. found that TSC2 was downregulated in uterine leiomyoma compared to normal myometrium from 60 hysterectomy specimens [27]. Kovács et al. reported that total FOXO1 protein exhibited nonsignificant difference, but phosphorylation FOXO1 protein was increased in leiomyoma compared with normal myometrium [25]. TSC2 and FOXO are regulated by specific interactions with 14-3-3 proteins [15]. In this study, we found that 14-3-3γ signal transduction pathway might be involved in GZFLC drug serum which inhibited the proliferation and induced apoptosis of uterine leiomyoma cells, but more researches are needed to confirm this link and elucidate the mechanisms.

5. Conclusions

The present study demonstrated that GZFLC drug serum inhibited the proliferation and induced apoptosis of uterine leiomyoma cells, which might be regulated by 14-3-3γ signal transduction pathway. These results may support further evaluation of a new thread for effective treatment on uterine leiomyoma clinically.

Competing Interests

The authors have declared that no competing interests exist.

Authors' Contributions

Xueqiong Zhu designed and coordinated the study. Qi Shen, Weijing Ye, Xiaoli Hu, Chuchu Zhao, and Lulu Zhou

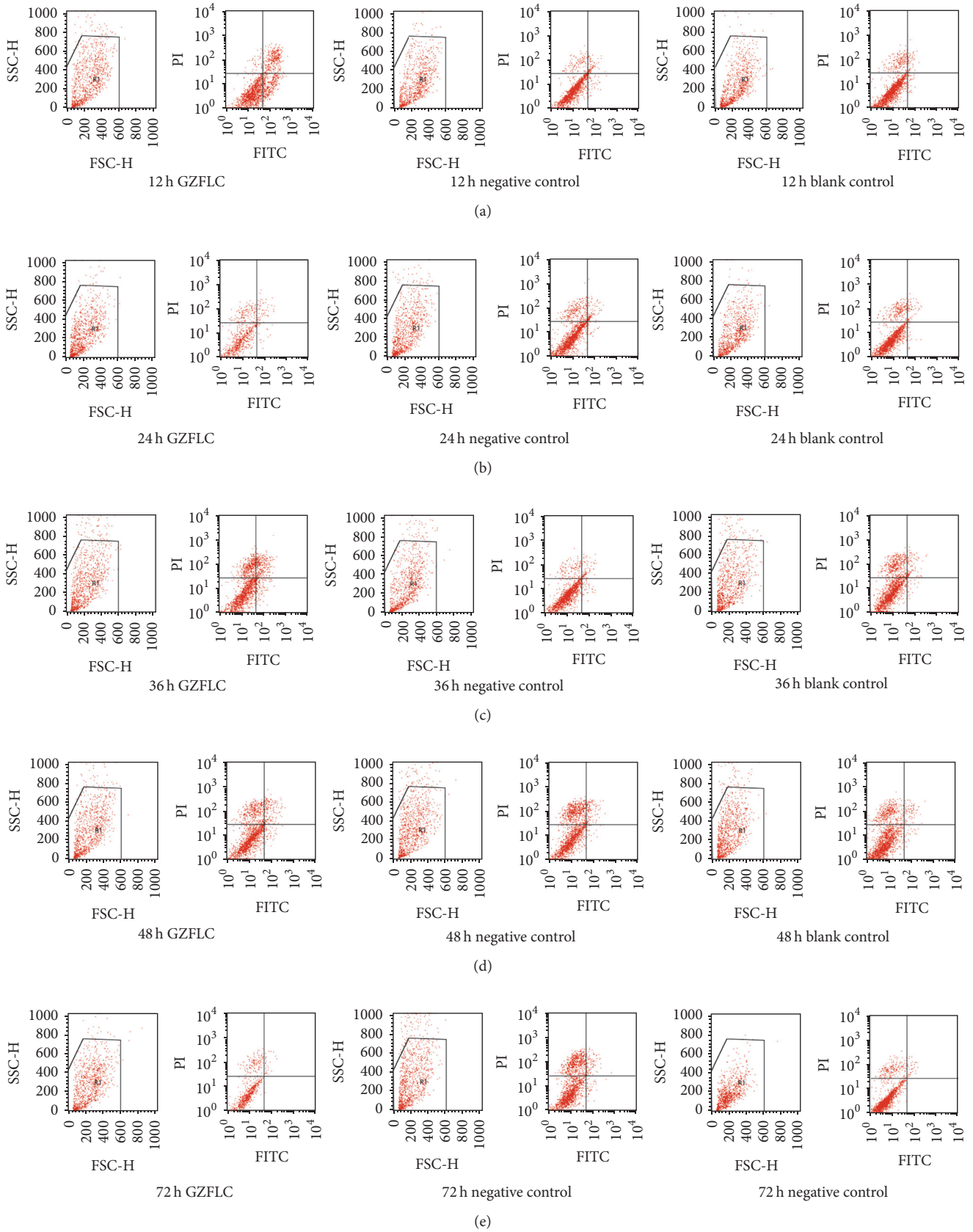


Figure 4: The cell apoptosis rate of GZFLC drug serum in different treatment points on the human leiomyoma cells via flow cytometry. (a) 12 h; (b) 24 h; (c) 36 h; (d) 48 h; and (e) 72 h.

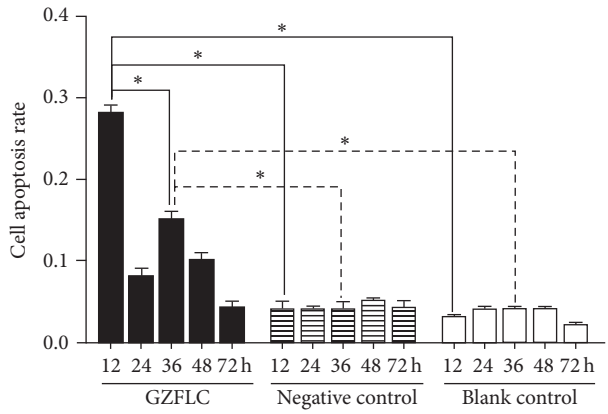


Figure 5: The cell apoptosis rate of GZFLC drug serum in different treatment points on the human leiomyoma cells (20% GZFLC serum concentration). ∗ indicated $P < 0.05$.

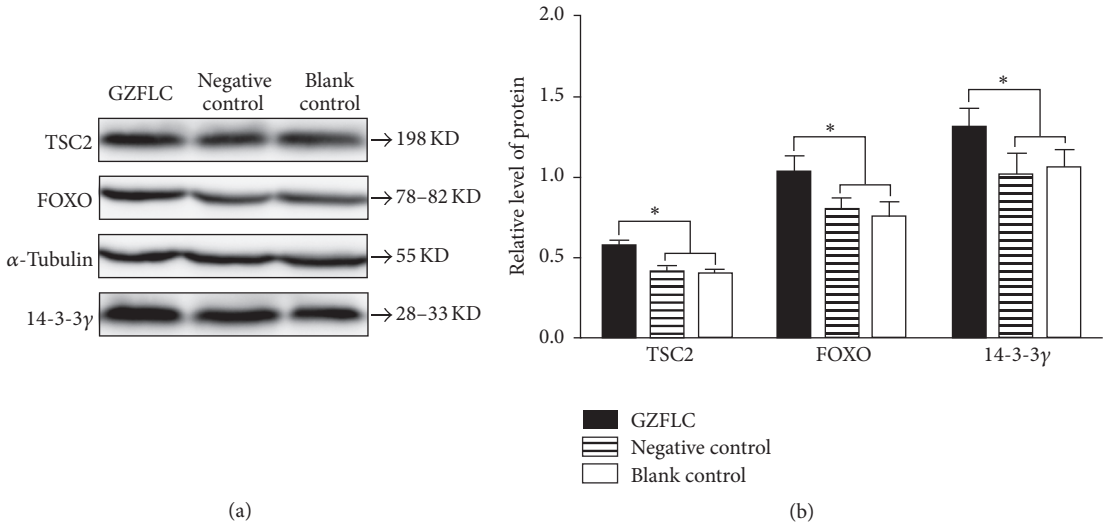


Figure 6: Effect of GZFLC drug serum on the expression of TSC2, FOXO, and 14-3-3γ in the human leiomyoma cells (12 h, 20% GZFLC serum concentration). (a) Western blot result. (b) Histogram result. ∗ indicated $P < 0.05$.

conducted the experiments and data analysis. Qi Shen and Xueqiong Zhu drafted the manuscript. All authors critically reviewed the manuscript and approved the final version for publication. Qi Shen and Weijing Ye contributed equally to this work and should be considered co-first authors.

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References

[1] E. E. Wallach and N. F. Vlahos, "Uterine myomas: an overview of development, clinical features, and management," *Obstetrics and Gynecology*, vol. 104, no. 2, pp. 393–406, 2004.

[2] B. S. Levy, "Modern management of uterine fibroids," *Acta Obstetricia et Gynecologica Scandinavica*, vol. 87, no. 8, pp. 812–823, 2008.

[3] M.-A. Lumsden, "Modern management of fibroids," *Obstetrics, Gynaecology and Reproductive Medicine*, vol. 20, no. 3, pp. 82–86, 2010.

[4] C. L. Walker and E. A. Stewart, "Uterine fibroids: the elephant in the room," *Science*, vol. 308, no. 5728, pp. 1589–1592, 2005.

[5] A. Lethaby, B. Vollenhoven, and M. Sowter, "Efficacy of preoperative gonadotrophin hormone releasing analogues for women with uterine fibroids undergoing hysterectomy or myomectomy: a systematic review," *BJOG: An International Journal of Obstetrics and Gynaecology*, vol. 109, no. 10, pp. 1097–1108, 2002.

[6] S. Sankaran and I. T. Manyonda, "Medical management of fibroids," *Best Practice and Research: Clinical Obstetrics and Gynaecology*, vol. 22, no. 4, pp. 655–676, 2008.

[7] L. Chen, D. Wang, J. Wu, B. Yu, and D. Zhu, "Identification of multiple constituents in the traditional Chinese medicine

formula GuiZhiFuLing-Wan by HPLC-DAD-MS/MS," *Journal of Pharmaceutical and Biomedical Analysis*, vol. 49, no. 2, pp. 267–275, 2009.

[8] K.-J. Wu, Y.-F. Chen, H.-Y. Tsai, C.-R. Wu, and W. G. Wood, "*Guizhi-Fuling-Wan*, a traditional Chinese Herbal Medicine, ameliorates memory deficits and neuronal apoptosis in the streptozotocin-induced hyperglycemic rodents via the decrease of Bax/Bcl2 ratio and caspase-3 expression," *Evidence-Based Complementary and Alternative Medicine*, vol. 2012, Article ID 656150, 11 pages, 2012.

[9] Y. Nagata, H. Goto, H. Hikiami et al., "Effect of keishibukuryogan on endothelial function in patients with at least one component of the diagnostic criteria for metabolic syndrome: a controlled clinical trial with crossover design," *Evidence-Based Complementary and Alternative Medicine*, vol. 2012, Article ID 359282, 10 pages, 2012.

[10] F. Xiao, Q. Li, K. Liang et al., "Comparative pharmacokinetics of three triterpene acids in rat plasma after oral administration of *Poria* extract and its formulated herbal preparation: GuiZhi-FuLing capsule," *Fitoterapia*, vol. 83, no. 1, pp. 117–124, 2012.

[11] N.-N. Chen, M. Han, H. Yang et al., "Chinese herbal medicine Guizhi Fuling Formula for treatment of uterine fibroids: a systematic review of randomised clinical trials," *BMC Complementary and Alternative Medicine*, vol. 14, article 2, 2014.

[12] J. P. Liu, H. Yang, Y. Xia, and F. Cardini, "Herbal preparations for uterine fibroids," *The Cochrane Database of Systematic Reviews*, vol. 4, Article ID CD005292, 2013.

[13] A. Aitken, "14-3-3 Proteins: a historic overview," *Seminars in Cancer Biology*, vol. 16, no. 3, pp. 162–172, 2006.

[14] S. Wang, X. Li, Z.-G. Li et al., "Gene expression profile changes and possible molecular subtypes in differentiated-type nonkeratinizing nasopharyngeal carcinoma," *International Journal of Cancer*, vol. 128, no. 4, pp. 753–762, 2011.

[15] D. K. Morrison, "The 14-3-3 proteins: integrators of diverse signaling cues that impact cell fate and cancer development," *Trends in Cell Biology*, vol. 19, no. 1, pp. 16–23, 2009.

[16] J. Q. Lv, X. Q. Zhu, K. Dong, Y. Lin, Y. Hu, and C. Zhu, "Reduced expression of 14-3-3 gamma in uterine leiomyoma as identified by proteomics," *Fertility and Sterility*, vol. 90, no. 5, pp. 1892–1898, 2008.

[17] J. Du, W. Liao, Y. Wang, C. Han, and Y. Zhang, "Inhibitory effect of 14-3-3 proteins on serum-induced proliferation of cardiac fibroblasts," *European Journal of Cell Biology*, vol. 84, no. 10, pp. 843–852, 2005.

[18] M. Yang, C. Xiao, Q. Wu et al., "Anti-inflammatory effect of Sanshuibaihu decoction may be associated with nuclear factor-κB and p38 MAPKα in collagen-induced arthritis in rat," *Journal of Ethnopharmacology*, vol. 127, no. 2, pp. 264–273, 2010.

[19] C. Hu, Z. Wang, Z. Pang et al., "Guizhi Fuling capsule, an ancient Chinese formula, attenuates endometriosis in rats via induction of apoptosis," *Climacteric*, vol. 17, no. 4, pp. 410–416, 2014.

[20] W.-H. Park, S.-T. Joo, K.-K. Park, Y.-C. Chang, and C.-H. Kim, "Effects of the Geiji-Bokryung-Hwan on carrageenan-induced inflammation in mice and cyclooxygenase-2 in hepatoma cells of HepG2 and Hep3B," *Immunopharmacology and Immunotoxicology*, vol. 26, no. 1, pp. 103–112, 2004.

[21] W.-H. Park, S.-K. Lee, H.-K. Oh, J.-Y. Bae, and C.-H. Kim, "Tumor initiation inhibition through inhibition COX-1 activity of a traditional Korean herbal prescription, Geiji-Bokryung-Hwan, in human hepatocarcinoma cells," *Immunopharmacology and Immunotoxicology*, vol. 27, no. 3, pp. 473–483, 2005.

[22] C.-C. Lu, M.-Y. Lin, S.-Y. Chen et al., "The investigation of a traditional chinese medicine, Guizhi Fuling Wan (GFW) as an intravesical therapeutic agent for urothelial carcinoma of the bladder," *BMC Complementary and Alternative Medicine*, vol. 13, article 44, 2013.

[23] Z. Yao and Z. Shulan, "Inhibition effect of Guizhi-Fuling-decoction on the invasion of human cervical cancer," *Journal of Ethnopharmacology*, vol. 120, no. 1, pp. 25–35, 2008.

[24] D. Zurawek, A. Faron-Górecka, M. Kuśmider et al., "Mesolimbic dopamine D2 receptor plasticity contributes to stress resilience in rats subjected to chronic mild stress," *Psychopharmacology*, vol. 227, no. 4, pp. 583–593, 2013.

[25] K. A. Kovács, F. Lengyel, F. Wilhelm, Z. Vértes, B. Sumegi, and M. Vértes, "Involvement of FKHR (FOXO1) transcription factor in human uterus leiomyoma growth," *Fertility and Sterility*, vol. 94, no. 4, pp. 1491–1495, 2010.

[26] L. Wang, H. Huang, D. Liu et al., "Evaluation of 14-3-3 protein family levels and associated receptor expression of estrogen and progesterone in human uterine leiomyomas," *Gynecological Endocrinology*, vol. 28, no. 8, pp. 665–668, 2012.

[27] J. Wei, L. Chiriboga, M. Mizuguchi, H. Yee, and K. Mittal, "Expression profile of tuberin and some potential tumorigenic factors in 60 patients with uterine leiomyomata," *Modern Pathology*, vol. 18, no. 2, pp. 179–188, 2005.

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Research Article

The Zuo Jin Wan Formula Induces Mitochondrial Apoptosis of Cisplatin-Resistant Gastric Cancer Cells via Cofilin-1

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Despite the status of cisplatin (DDP) as a classical chemotherapeutic agent in the treatment of cancer, the development of multidrug resistance often leads to a failure of DDP therapy. Here we found that phosphorylated cofilin-1 (p-cofilin-1) was overexpressed in the DDP-resistant human gastric cancer cell lines SGC7901/DDP and BGC823/DDP, relative to the respective parent cell lines (SGC7901 and BGC823), and that DDP induced the dephosphorylation of p-cofilin-1 in both parent lines but not in the DDP-resistant lines. However, we noted that the traditional Chinese medicine formula Zuo Jin Wan (ZJW) could induce the dephosphorylation of p-cofilin-1 and promote cofilin-1 translocation from the cytoplasm into the mitochondria in both SGC7901/DDP and BGC823/DDP cells. This mitochondrial translocation of cofilin-1 was found to induce the conversion of filamentous actin to globular-actin, activate mitochondrial damage and calcium overloading, and induce the mitochondrial apoptosis pathway. We further observed that these effects of ZJW on DDP-resistant human gastric cancer cell lines could be reversed via transfection with cofilin-1-specific siRNA, or treatment with a PP1 and PP2A inhibitor. These results suggest that ZJW is an effective drug therapy for patients with DDP-resistant gastric cancer.

1. Introduction

Gastric cancer is the fourth most common type of cancer worldwide; approximately 989,600 new gastric cancer cases and 738,000 gastric cancer-related deaths were estimated worldwide in 2008 [1]. Surgical resection is currently the treatment of choice for gastric cancer; chemotherapy, radiotherapy, and gene therapy are considered the main adjuvant therapy methods. Efficacy rates of 50% have been reported for chemotherapy drugs, such as cisplatin (DDP), which are widely used in clinical settings [2, 3]. Although chemotherapy is among the primary methods used to treat gastric cancer, the development of multidrug resistance (MDR) commonly leads to a failure of chemotherapy. Therefore, studying the mechanism of MDR and exploring effect MDR-reversing drug are necessary to overcome the bottleneck of cancer chemotherapy.

Recently studies had shown that phosphorylated cofilin-1 (p-cofilin-1) and cofilin-1 played an important role in MDR of cancer. One study had found that p-cofilin-1 was high-expressed in taxol-resistant cells and chemoresistant primary human ovarian cancer tissues [4]. In another study, p-cofilin-1 also showed high-expression levels in vincristine-resistant human osteosarcoma cell line MG63/VCR, which was upregulated by overexpression of LIMK1 [5]. The cofilin-1 was showed higher expression levels in DDP-resistant non-small cell lung cancer (NSCLC) cell ICR-A549 [6]. Cofilin-1 belongs to the actin-binding protein family, the members of which regulate actin depolymerisation. The main functions of cofilin-1 are the decomposition of actin microfilaments

and elevation of the rate of actin depolymerisation, both of which influence actin cytoskeletal remodelling. Moreover, cofilin-1 can induce the transformation of filamentous actin (F-actin) to globular-actin (G-actin), activate mitochondrial damage and calcium overloading, and induce the mitochondrial apoptosis pathway. Notably, p-cofilin-1 must be dephosphorylated to participate in actin depolymerisation and translocation into the mitochondria [7, 8].

In recent years, traditional Chinese medicine (TCM) as adjuvant chemotherapy of cancer drugs in China has been widely used in cancer treatment. TCM has the function of strengthening the effect of chemotherapy, reducing the toxic and side effects, reversing the drug resistance of the tumor. Zuo Jin Wan (ZJW), a TCM formula, showed better therapeutic effects in adjuvant treatment of tumors [9–12]. Besides, ZJW has also the effect of reversing drug resistance in gastric cancer and colorectal cancer cell [13–15]. By pharmacodynamics experiments, the effects of ZJW reversing drug resistance in gastric cancer was proved, but its exact mechanism was still unclear.

In the present study, we identified a novel molecular mechanism by which ZJW inhibits DDP-resistance by inducing the mitochondrial translocation of cofilin-1.

2. Materials and Methods

2.1. Cell Lines and Cultures. BGC823 and SGC7901 human gastric cancer cells were purchased from the Shanghai Cell Collection (Shanghai, China). Cells were cultured in RPMI 1640 medium (Gibco Laboratories, USA) supplemented with 10% (v/v) foetal bovine serum (Gibco Laboratories, USA), 100 μg/mL streptomycin, and 100 U/mL penicillin at 37°C and 5% CO_2. DDP was purchased from Sigma (St. Louis, MO, USA).

DDP-resistant SGC7901/DDP and BGC823/DDP cells were induced from SGC7901 and BGC823 cells, respectively, using a concentration gradient method to increase the half maximal inhibitory concentration (IC_{50}) of DDP. At first, SGC7901 and BGC823 cells were treated with the culture medium containing 0.05 μg/mL DDP for 24 h. Then, the culture medium containing DDP was substituted for the fresh culture medium. When the cell density reached 80%, cells were digested and passage. The cells were treated with 0.05 μg/mL DDP time and again until the cells can be stable passage in such concentration of DDP. Subsequently, the cells were treated with higher concentration of DDP in turn, until the final concentration of DDP reached 1 μg/mL. The cells were cultured in the culture medium containing 1 μg/mL DDP to maintain its drug resistance. Before every experiment, both DDP-resistant gastric cell lines were cultured in drug-free RPMI 1640 medium for 2 weeks.

2.2. Preparation of the ZJW Extracts. The ZJW formula composed of two herbs as Rhizoma Coptidis and Fructus evodiae in a 6 : 1 ratio (w : w). Rhizoma coptidis and Fructus evodiae were from TCM pharmacy of Putuo Hospital, Shanghai University of Traditional Chinese Medicine (Shanghai, China). ZJW extracts were prepared as previously described [13–15]. ZJW was extracted through two 1-hour reflux procedures in ethanol (1 : 8, v : v). The extracted mixtures were subsequently filtered, concentrated, and vacuum-dried at 60°C. The preparation of ZJW extracts was standardised and quality controlled according to the guidelines of the Chinese State Food and Drug Administration.

2.3. Antibodies. Anti-cofilin-1 (#5175), anti-p-cofilin-1 (#3313), anti-GAPDH (#2118), anti-β-actin (8456), anti-cleaved caspase-9 (#7237), anti-cleaved caspase-3 (#9664) anti-cytochrome c (#11940), anti-PARP (#9532), anti-cox IV (#11967), horseradish-peroxidase- (HRP-) conjugated anti-rabbit (7075), and anti-mouse secondary antibodies (7076) were obtained from Cell Signaling Technology, Inc. (Danvers, MA, USA). Anti-slingshot homolog 1 (ab76943), anti-gamma actin (ab194952), and anti-F-actin antibodies (ab205) were obtained from Abcam (Cambridge, MA, USA). An anti-PP1 (E-9) antibody (sc-7482) was obtained from Santa Cruz Biotechnology (Dallas, TX, USA).

2.4. Knockdown of the Cofilin-1 Gene. Three siRNAs, specific for the human cofilin-1 gene (Gene ID: 49472823), were synthesised by Biomics Biotechnologies (Nantong, China). The three siRNA and control sequences were as follows: sequence 1: 5′-GAGUGAGGACAAGAAGAACAU-3′; sequence 2: 5′-CGCCACCTTTGTCAAGATGCT-3′; sequence 3: 5′-GAUUUAUGCCAGCUCCAAGGA-3′; control sequence: 5′-UAAGGCUAUGAAGAGAUAC-3′. For knockdown experiments, 5×10^5 cells were plated per well, in a six-well plate. After 24 h, cells were transfected with the above siRNA at the concentration of 50 nM using Lipofectamine 3000 (Life Technologies, Carlsbad, CA, USA) for 48 h.

2.5. Quantitative Real-Time PCR Assay. Total cellular RNA was isolated using Plus RNAiso (Takara Bio, Shiga, Japan); subsequently, a PrimeScript RT reagent kit (Takara Bio) was used to synthesise cDNA. Quantitative real-time PCR (Q-RTPCR) to detect cofilin-1 gene expression was performed according to Tang et al. [8]. The following cofilin-1 primer sequences were used: forward, 5′-AAGGCGGTGCTCTTCTGC-3′; reverse, 5′-TTGACAAAGGTGGCGTAG-3′; TaqMan probe, 5′-FAM-CATCCTGGAGGAGGGCAAGGAGAT-TAMRA-3′. The following GAPDH primer sequences were used as controls: forward, 5′-GGTGGTCTCCTCTGACTTCAACA-3′; reverse, 5′-CCAAATTCGTTGTCATACCAGGAAATG-3′; TaqMan probe, 5′-FAM-CGACACCCACTCCTCCACCTTTGACGC-TAMRA-3′. These primers and probes were purchased from Sangon Biotech (Shanghai, China).

2.6. Apoptosis Rate Assay. Apoptosis rates were determined using an Annexin V-FITC/propidium iodide (PI) Apoptosis Detection Kit (BD Biosciences, San Jose, CA, USA). Cells were harvested by trypsinisation, washed twice with cold phosphate-buffered serum (PBS), and incubated with Annexin V-FITC, followed by PI. Flow cytometric analysis was performed on a FACSCalibur system (Becton Dickinson, Franklin Lakes, NJ, USA).

2.7. Immunofluorescence Assay. HCT116 cells were seeded on cover slips precoated with 0.01% poly-lysine at a density of 5,000 cells per well in a 24-well chamber. Following treatment with ZJW, the cells were treated in the following sequence: 4% paraformaldehyde for 20 min, 0.1% Triton X-100 for 10 min, 5% bovine serum albumin (BSA) for 60 min, and primary antibodies overnight at 4°C. Subsequently, the cells were washed three times using PBS and incubated with an Alexa Fluor 488-conjugated anti-mouse IgG antibody or Alexa Fluor 555-conjugated anti-rabbit IgG antibody (Life Technologies) for 1 h prior to observation with a fluorescence microscope (Leica, Wetzlar, Germany).

2.8. Cell Viability Assay. Cells were plated in 10% FBS RPMI 1640 medium at a density of 5,000 cells/well, in a 96-well plate. Cell viability was analysed using a Cell Counting Kit-8 (CCK-8, Dojindo Laboratories, Kumamoto, Japan), according to the manufacturer's protocol. Absorbances of the wells at 450 nm were read using a plate reader (Bio-Rad, Hercules, CA, USA). Each sample was analysed in sextuplicate, and experiments were repeated three times.

2.9. Mitochondrial Membrane Potential Assay and Intracellular Calcium Assay. Mitochondrial membrane potential ($\Delta\Psi m$) was analysed using JC-1 (Beyotime, Haimen, China) in both flow cytometric and cell fluorescence assays. JC-1, a fluorescent probe that detects mitochondrial membrane potential, forms a red fluorescent polymer upon accumulation in the mitochondrial matrix under conditions of high membrane potential; at a lower membrane potential, JC-1 exists as a green fluorescent monomer. Mitochondrial membrane potential was, therefore, measured as the degree of change in the red/green fluorescence ratio. The intracellular calcium concentration was measured using the Fluo-3 AM assay (Beyotime, Haimen, China). In this assay, the intensity of green fluorescence was used to determine the relative concentration of intracellular calcium. The increasing of intracellular calcium concentration was thought to be the results of mitochondrial membrane potential damage and proapoptosis marker. Both flow cytometric and cell fluorescence assays were also used to measure the intracellular calcium concentration.

2.10. Analysis of Caspase-3 and Caspase-9 Protease Activity. Caspase-3 protease activity was measured using a Caspase-3 Activity Assay Kit (Beyotime, Haimen, China), according to the manufacturer's instructions. Similarly, Caspase-9 Activity Assay Kit was used to detect caspase-9 protease activity. The kit was used to generate a standard curve from which the caspase-3/ caspase-9 protease activity of each sample was calculated, after which the relative fold changes in activity were calculated using the control value.

2.11. Western Blotting and Coimmunoprecipitation (co-IP) Assay. Total cell proteins and cytoplasmic and mitochondrial protein fractions were extracted using the Cell Lysis Buffer for Western, IP, and Cell Mitochondria Isolation Kit, respectively (Beyotime, Haimen, China). Following separation using sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis, proteins were transferred onto polyvinylidene fluoride membranes, blocked with 5% BSA, and incubated with primary antibodies and corresponding HRP-conjugated secondary antibodies in sequence. Labelled membranes were visualised using an Enhanced Chemiluminescent Western Blotting Detection System (Millipore, Billerica, MA, USA). GAPDH and COX IV were used as loading controls for total/cytoplasmic and mitochondrial proteins, respectively. A Dynabeads® Coimmunoprecipitation Kit (Pierce Biotechnology, Rockford, IL, USA) was used for the co-IP analysis. Total cell, cytoplasmic, and mitochondrial proteins were incubated in anti-cofilin-1 antibodies and normal mouse IgG at 4°C overnight. Subsequently, co-IP samples were subjected to western blotting as described above, using the appropriate antibodies.

2.12. G/F-Actin Ratio Assay. The cellular G/F-actin ratio assay was referenced to that previously described [16, 17]. Briefly, cells were washed in ice-cold PBS and then suspended in lysis buffer (50 mm HEPES, pH 6.4, 1 mm $MgCl_2$, 10 mm EDTA, and 1% Triton X-100) for 5 min and centrifuged for 30 min at 18,000 ×g to isolate the supernatant (containing G-actin). The remaining pellet containing F-actin was washed in PBS and resuspended in an equal volume lysis buffer under vigorous agitation. The G-actin protein from the supernatant and the F-actin protein from the pellet were checked by western blot. The ratio of G-actin to F-actin was quantified by the proteins expression level.

2.13. Statistical Analysis. Using the SPSS 13.0 software package (SPSS, Inc., Chicago, IL, USA), data were subjected to a single-factor analysis of variance and Student's t-test. The results are presented as means ($X \pm$ SDs). $P < 0.05$ was considered statistically significant.

3. Results

3.1. ZJW Increases the Sensitivity of DDP-Resistant Gastric Cancer Cells to DDP. We established the DDP-resistant cell lines BGC823/DDP and SGC7901/DDP by chronic exposure of the DDP-sensitive parent gastric cancer cell lines BGC823 and SGC7901 to low-dose DDP. A CCK8 cell viability assay was then used to detect the inhibitory effects of DDP on BGC823, BGC823/DDP, SGC7901, and SGC7901/DDP gastric cancer cells for 48 h. As shown in Figures 1(a) and 1(b), DDP had significantly lower inhibitory effects on BGC823/DDP (IC_{50} = 10.26 μg/mL) and SGC7901/DDP (IC_{50} = 7.84 μg/mL), relative to their respective DDP-sensitive parent cell lines (BGC823, IC_{50} = 0.89 μg/mL; SGC-7901, IC_{50} = 0.93 μg/mL).

Previous studies have reported that ZJW can reverse the effect of multidrug resistance [13–15]. In the present study, we also observed the ability of ZJW to reverse DDP resistance in BGC823/DDP and SGC7901/DDP cells following treatment with different concentrations of ZJW for 48 h. For BGC823/DDP cells, IC_{50} of DDP decreased from 10.26 to 3.54 μg/mL after ZJW treatment; similarly, IC_{50} of DDP

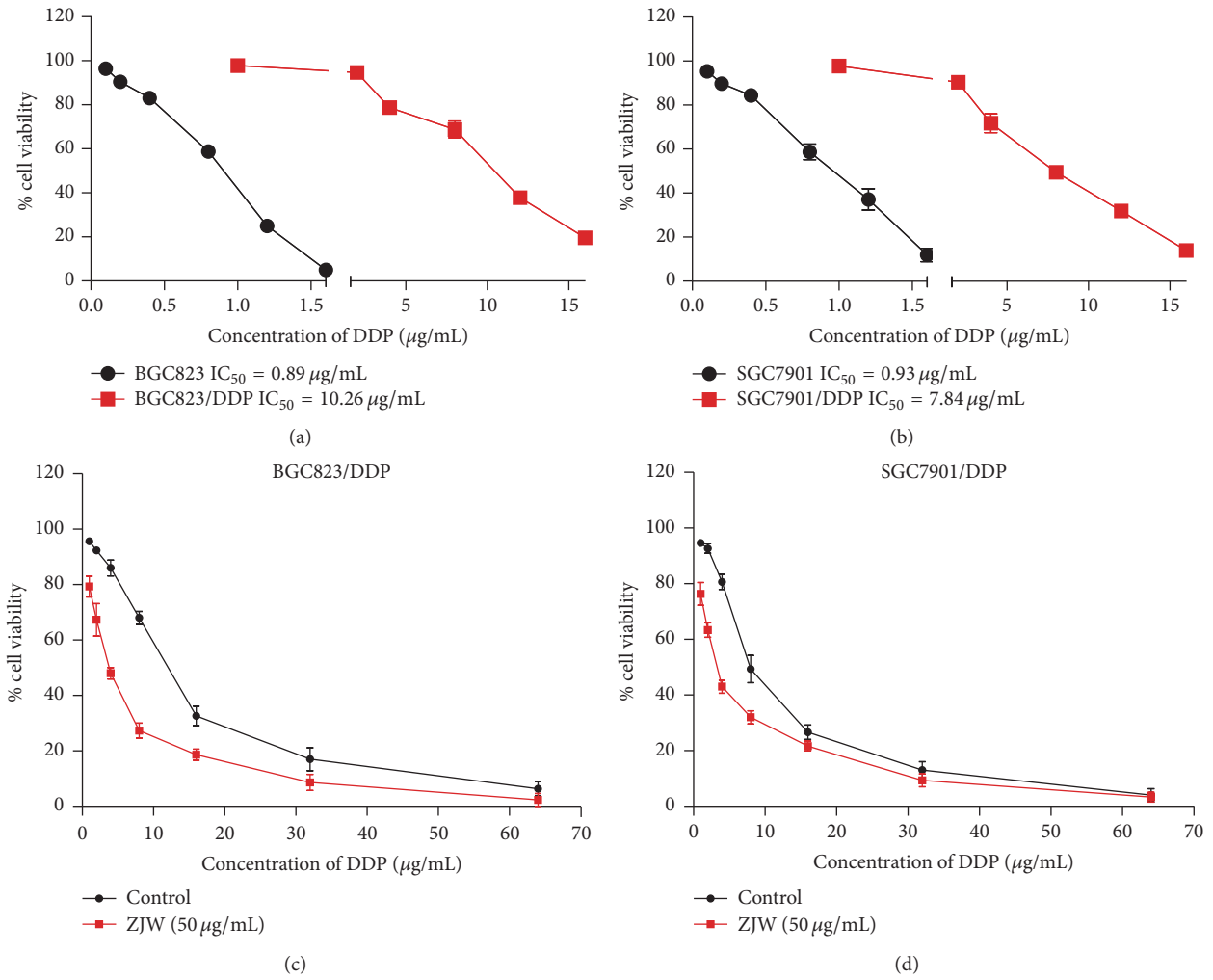

Figure 1: ZJW increases the sensitivity to DDP in BGC823/DDP and SGC7901/DDP cells. (a) The cell viabilities of DDP-resistant BGC823/DDP cells and BGC823 parent cells were measured following exposure to DDP for 48 h. (b) The cell viabilities of DDP-resistant SGC7901/DDP cells and SGC7901 parent cells were measured following the exposure to DDP. (c) and (d) CCK-8 assay was used to detect the cell viability of DDP in BGC823/DDP and SGC7901/DDP cells treated with ZJW (50 μg/mL) and DDP in different concentration for 48 h.

for SGC7901/DDP cells decreased from 7.84 to 2.92 μg/mL. These results indicate that ZJW can enhance the sensitivity of BGC823/DDP and SGC7901/DDP cells to DDP (Figures 1(c) and 1(d)).

3.2. Effects of ZJW on Mitochondrial Signalling Pathways. Given the role of reversing drug resistance, we hypothesised that ZJW could induce mitochondrial apoptosis in DDP-resistant gastric cancer cells. As the western blotting results in Figure 2(a), ZJW induced caspase-9 and caspase-3 activation and PARP degradation in a time- and dose-dependent manner in SGC7901/DDP cells. These events were accompanied by an increase of the apoptosis promoter BAX, decrease of the apoptosis inhibitor BCL-2, and the release of mitochondrial cytochrome C to the cytoplasm.

We also examined the activities of two key proteases caspase-3 and caspase-9 in the mitochondrial apoptotic signalling pathway by using a Caspase-3 Activity Assay Kit. As shown in Figure 2(b), ZJW induced the activation of caspase-9 and caspase-3 in a time- and dose-dependent manner in SGC7901/DDP cells. These results demonstrate that ZJW can trigger mitochondrial apoptosis in DDP-resistant gastric cancer cells.

3.3. ZJW Induces Mitochondrial Injury in SGC7901/DDP Cells. Mitochondrial injury represents an early stage in the mitochondrial apoptosis pathway, characterised by changes in $\Delta\Psi$m and cytosolic calcium concentration. Accordingly, we detected the $\Delta\Psi$m and cytosolic calcium concentration of SGC7901/DDP cells using flow cytometry and immunofluorescence, respectively. Using JC-1, we observed an increase in the FLH1 green fluorescence density (JC-1 monomer) and decrease in the FLH2 red fluorescence density (JC-1 polymer), indicating a relative decrease in the $\Delta\Psi$m (MeanFLH2/MeanFLH1, Figures 3(a) and 3(b)). Using the Fluo-3 AM reagent, we observed an increase in FLH1 green

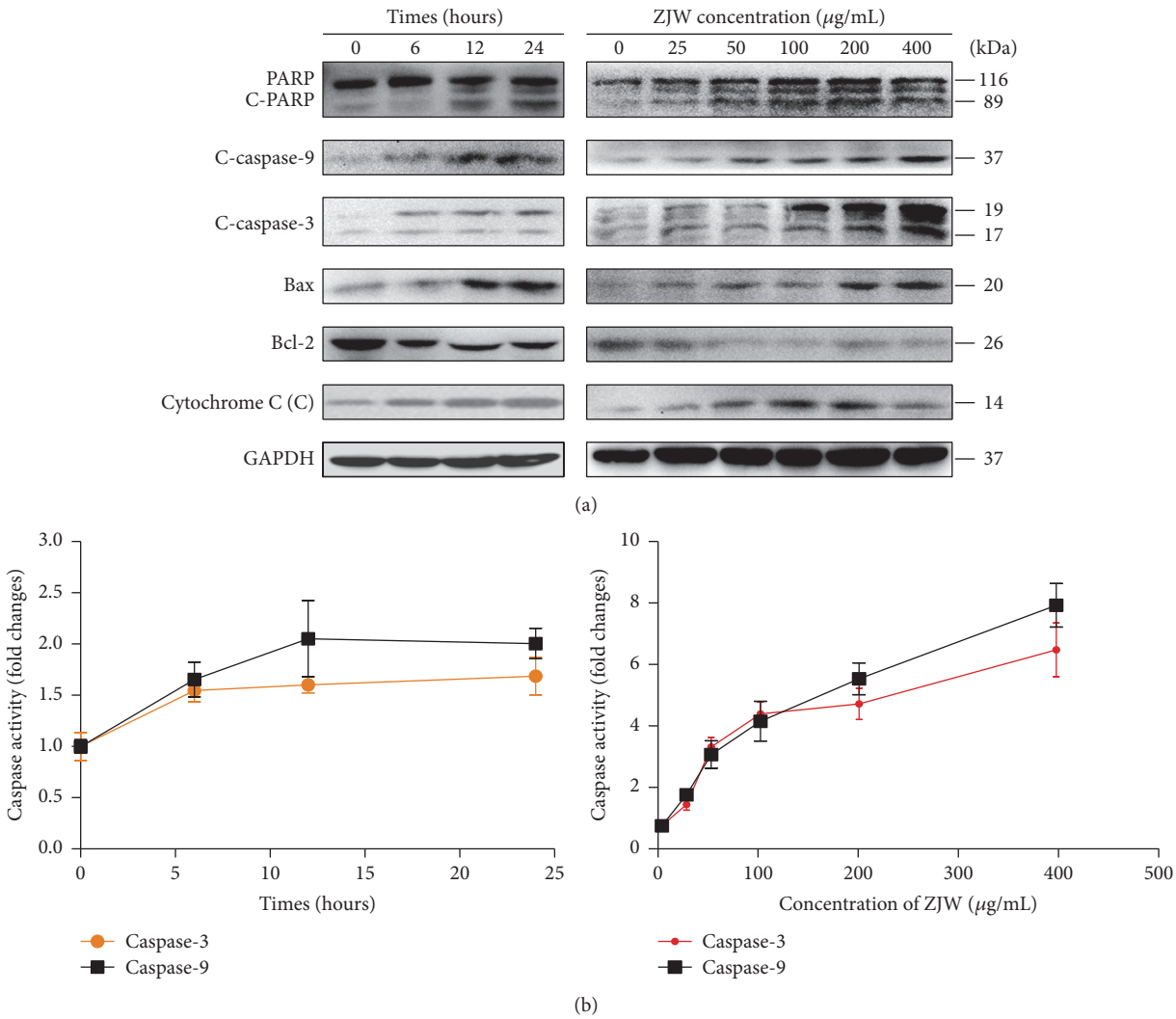

Figure 2: Effects of ZJW on mitochondrial apoptosis signalling pathways. (a) The levels of proteins in mitochondrial apoptosis signalling pathways were evaluated using western blotting in cisplatin (DDP)-resistant SGC7901/DDP cells following treatment with ZJW. Cytochrome C (C) represented the protein level of cytochrome C from cytoplasm. The results indicated dose- (treating time, 24 h) and time-dependent (ZJW, 50 μg/mL) effects. (b) After the same treating method as above, caspase-3 and caspase-9 activity were measured using a standard colorimetric assay (Bio-Rad). Relative caspase-3 and caspase-9 activity levels were normalized against control levels.

fluorescence density (calcium concentration) (Figures 3(c) and 3(d)). These results suggest that ZJW could induce mitochondrial injury in SGC7901 cells.

3.4. ZJW Induces the Dephosphorylation of p-Cofilin-1 in DDP-Resistant Gastric Cancer Cell Lines. Given the role of p-cofilin-1 and cofilin-1 in drug resistance of cancer [7, 8, 18, 19], we detected p-cofilin-1 and cofilin-1 protein expression levels in the four gastric cancer cell lines. As shown in Figure 4(a), the DDP-resistant cell lines BGC823/DDP and SGC7901/DDP exhibited significantly higher p-cofilin-1 expression levels, relative to their corresponding DDP-sensitive parent cell lines.

After treatment with DDP for 24 h, the expression of p-cofilin-1 decreased significantly in BGC823 and SGC7901 cells, suggesting that DDP could induce p-cofilin-1 dephosphorylation and cofilin-1 activation in DDP-sensitive gastric cancer cell lines (Figures 4(b) and 4(c)). In contrast, the levels of p-cofilin-1 in the DDP-resistant cell lines SGC7901/DDP and BGC823/DDP did not noticeably change, demonstrating that treatment with a lower concentration of DDP could not induce the dephosphorylation of p-cofilin-1. These results suggest that p-cofilin-1 plays an important role in DDP-resistance in gastric cancer cells.

Next, we used western blotting to evaluate the expression of p-cofilin-1 protein in BGC823/DDP and SGC7901/DDP cells treated with DDP and ZJW. Notably, p-cofilin-1 expression significantly decreased after treatment with a combination of DDP and ZJW, although the total cofilin-1 expression level was not significantly changed. These results suggest that

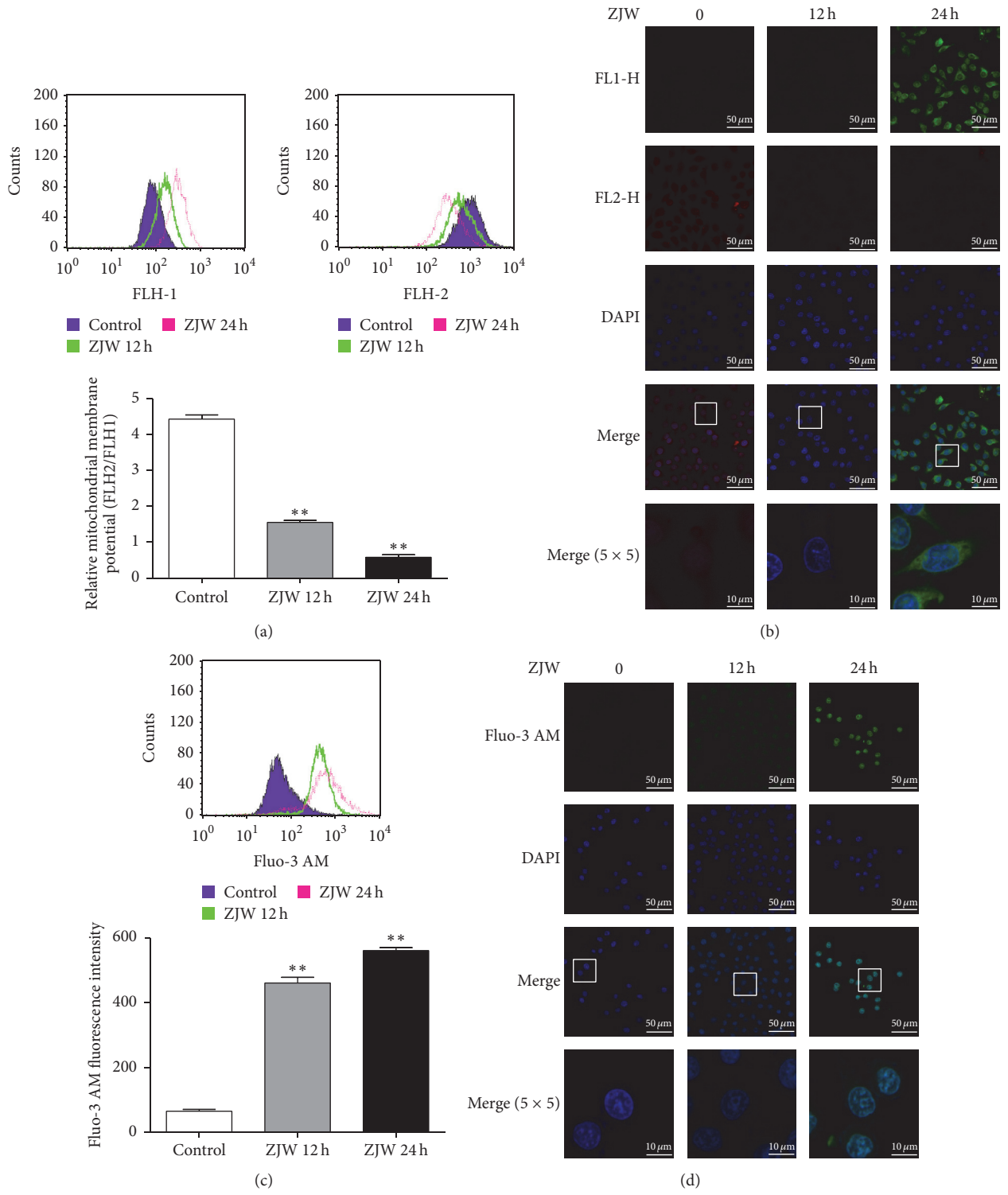

Figure 3: ZJW induces mitochondrial injury in DDP-resistant SGC7901/DDP cells. (a) ZJW decreased the mitochondrial membrane potential (△Ψm) of SGC7901/DDP cells. SGC7901/DDP cells were treated with 50 μg/mL ZJW for 0, 12, and 24 h. Then △Ψm was evaluated using flow cytometry with the fluorescent indicator JC-1 as the degree of change in the ratio of red (FLH-2) to green (FLH-1) fluorescence. $^{**}p < 0.01$, versus control groups treated with ZJW for 0 h. (b) A cell fluorescence assay detected the FLH-2 and FLH-1 fluorescence intensities of JC-1 in SGC7901/DDP cells after exposure to ZJW. (c) ZJW induced intracellular calcium overloading in SGC7901/DDP cells. The intracellular calcium was evaluated using flow cytometry with the fluorescent indicator Fluo-3 AM. $^{**}p < 0.01$, versus control groups treated with ZJW for 0 h. (d) A cell fluorescence assay detected the FLH-1 fluorescence intensity of Fluo-3 AM in SGC7901/DDP exposed to ZJW.

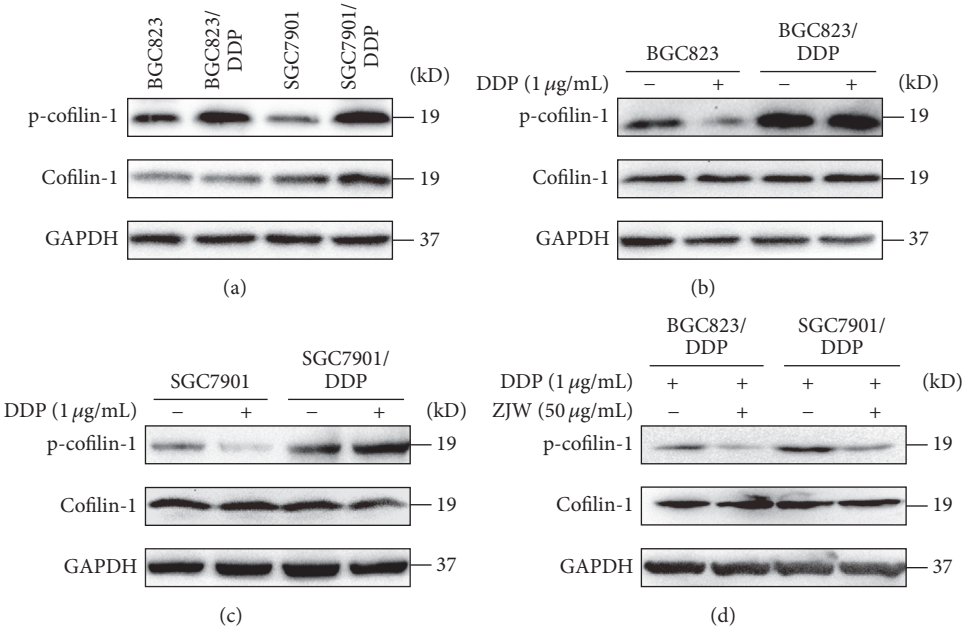

Figure 4: ZJW induces dephosphorylation of p-cofilin-1 in BGC823/DDP and SGC7901/DDP cells. (a) Western blot assayed p-cofilin-1 expressing level in DDP-sensitive cells and DDP resistant cells. (b) and (c) P-cofilin-1 expressing level changes after being treated with 1 μg/mL DDP in four gastric cancer cells for 24 h. (d) The combination of DDP and ZJW induces the dephosphorylation of p-cofilin-1 in BGC823/DDP and SGC7901/DDP cells.

the ability of ZJW to modulate DDP resistance correlates with the induced dephosphorylation of p-cofilin-1 (Figure 4(d)).

3.5. Translocation of Cofilin-1 and Actin to the Mitochondria Is Induced by ZJW in SGC7901/DDP Cells. Previous studies demonstrated that the dephosphorylation of p-cofilin-1 and degradation of F-actin to G-actin, followed by the translocation of actin with cofilin-1 to the mitochondria, were important initiating factors within mitochondrial apoptotic signalling pathways [7, 19]. Accordingly, we first examined the p-cofilin-1, cofilin-1, and actin protein levels in ZJW-treated SGC7901/DDP cells. As shown in Figure 5(a), the expression of p-cofilin-1 decreased after ZJW treatment; in addition, the expression pattern of cofilin-1 shifted from the cytoplasm to the mitochondria. Similarly, the expression pattern of gamma actin (instead of G-actin) also shifted from the cytoplasm to mitochondria. A co-IP assay, with an anti-cofilin-1 antibody, demonstrated an increased amount of actin associated with cofilin-1 in the mitochondria and a concomitant decrease in cytoplasm following ZJW treatment (Figure 5(b)).

Immunofluorescence analysis demonstrated that ZJW induced the degradation of F-actin and the translocation to and aggregation of actin and cofilin-1 in the mitochondria (Figures 5(c) and 5(d)). These findings indicate that ZJW dephosphorylates p-cofilin-1, leading to the degradation of F-actin to G-actin, translocation of actin and cofilin-1 to the mitochondria, and initiation of mitochondrial apoptosis.

3.6. Silencing of Cofilin-1 Protects Cells from ZJW-Mediated Apoptosis. To verify the role of cofilin-1 in the ability of ZJW to modulate DDP resistance in gastric cancer cell lines, we used RNA interference to reduce the expression of cofilin-1 in SGC7901/DDP cells and subsequently observed the effects of ZJW. We evaluated the expression of cofilin-1 protein and mRNA using western blotting and Q-RTPCR, respectively, at 72 h after transfection with three cofilin-1 siRNA constructs. The results (Figures 6(a) and 6(b)) demonstrated that all three siRNA constructs exerted good inhibitory effects on the expression of cofilin-1 protein and mRNA.

Flow cytometry was used to assess apoptosis in SGC7901/DDP cells treated with different concentrations of ZJW after cofilin-1 knockdown (Figure 6(c)). We observed no significant apoptosis in either cofilin-1 siRNA-treated or control siRNA-treated SGC7901/DDP in the absence of ZJW treatment. ZJW induced apoptosis in both cofilin-1 siRNA- and control siRNA-treated SGC7901/DDP cells in a dose-dependent manner. However, the apoptosis rate was significantly higher in SGC7901/DDP cells treated with control siRNA compared with those treated with cofilin-1 siRNA, indicating the importance of cofilin-1 in the process of ZJW-induced apoptosis.

A CCK8 assay was used to detect the viability of cofilin-1 siRNA-treated SGC7901/DDP cells following treatment with ZJW. As shown in Figure 6(d), ZJW had a significantly stronger inhibitory effect on control siRNA-treated SGC7901/DDP cells (IC_{50} = 134.37 μg/mL) than on cofilin-1 siRNA-treated SGC7901/DDP cells (IC_{50} = 287.43 μg/mL), indicating the importance of cofilin-1 on the ability of ZJW to induce apoptosis and regulate DDP resistance.

Changes in the expression levels and patterns of F-actin, G-actin, and cofilin-1 following ZJW treatment were

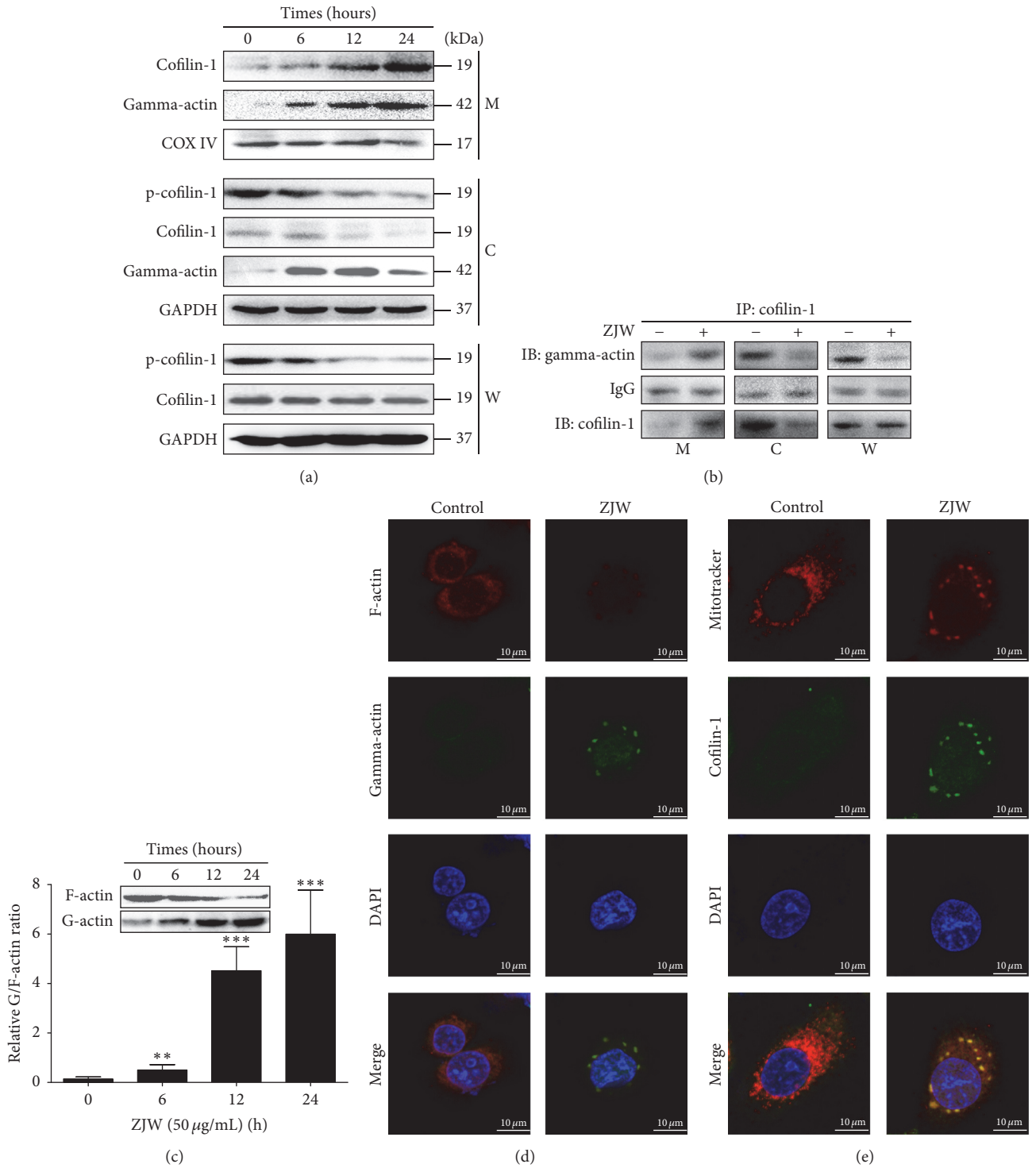

Figure 5: Translocation of cofilin-1 and actin to the mitochondria of SGC7901/DDP cells is induced by ZJW. (a) SGC7901/DDP cells were treated with 50 μg/mL ZJW for 0, 6, 12, and 24 h as indicated. Whole cell lysates and mitochondrial and cytosolic proteins were prepared for the detection of p-cofilin-1, cofilin-1, and gamma-actin using western blotting. The level of each protein was normalised against those of GAPDH (total and cytosolic proteins) or COX IV (mitochondrial proteins). (b) Treated with ZJW (50 μg/mL) for 24 h, a coimmunoprecipitation (co-IP) assay with an anti-cofilin antibody was used to detect the translocation of gamma-actin from the cytoplasm to the mitochondria in SGC7901/DDP cells. (c) Western blot analysis of the G/F-actin ratio changes induced by ZJW in SGC7901/DDP. SGC7901/DDP cells were treated with 50 μg/mL ZJW for 6, 12, and 24 h, respectively, isolating the F-actin extracts and G-actin extracts; the expression of F-actin and G-actin was detected using the anti-F-actin and anti-gamma-actin antibody. $^{**}P < 0.01$ or $^{***}P < 0.001$, versus control groups treated ZJW for 0 h. (d) Depolymerisation of F-actin and translocation of G-actin (gamma-actin) from the cytoplasm to the mitochondria was induced by ZJW for 24 h in SGC7901/DDP cells and detected using an immunofluorescence assay. (e) As the same treating method, the ZJW-induced translocation of cofilin-1 from the cytoplasm to the mitochondria (stained with Mitotracker Red) was detected in SGC7901/DDP cells using an immunofluorescence assay.

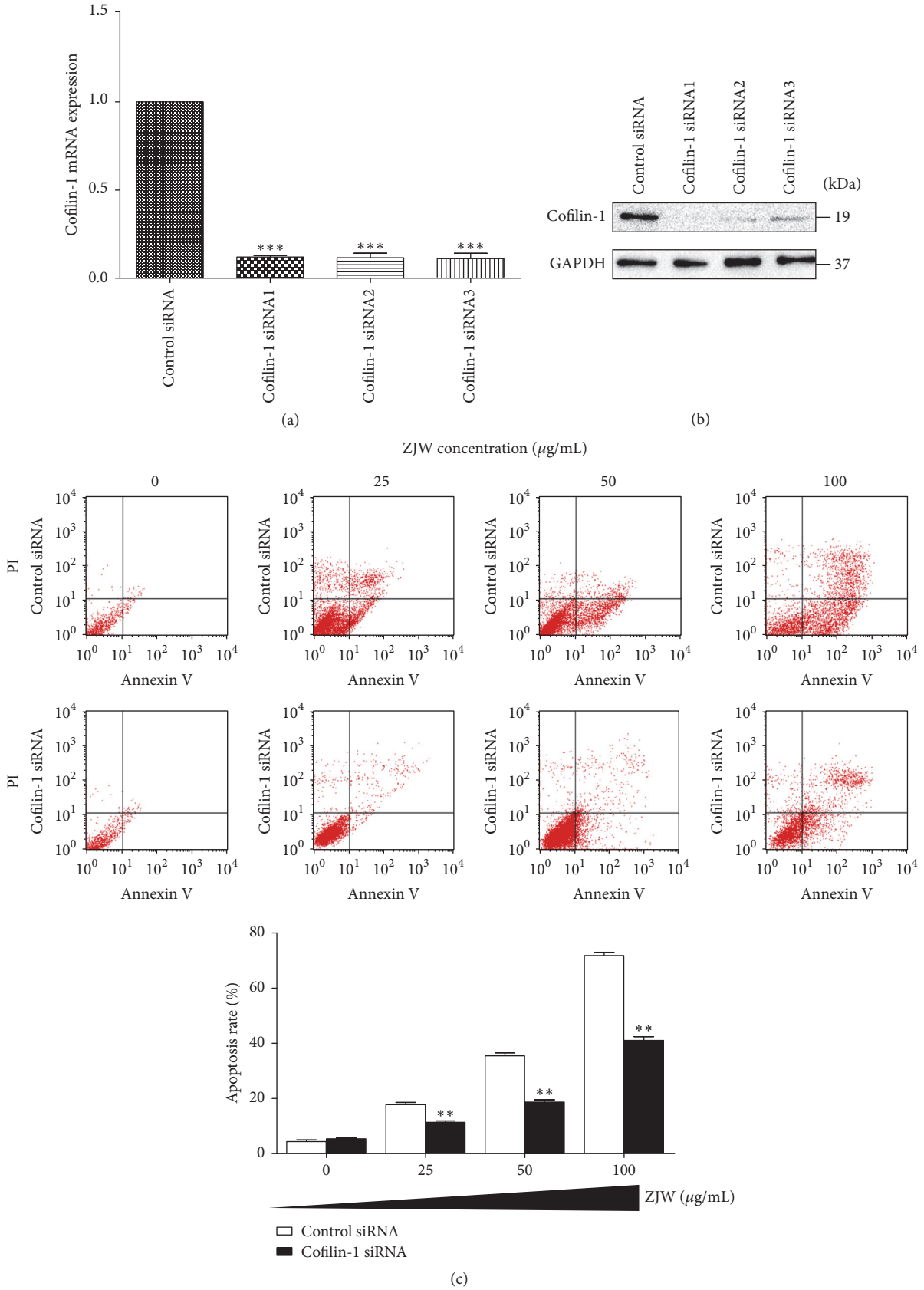

Figure 6: Continued.

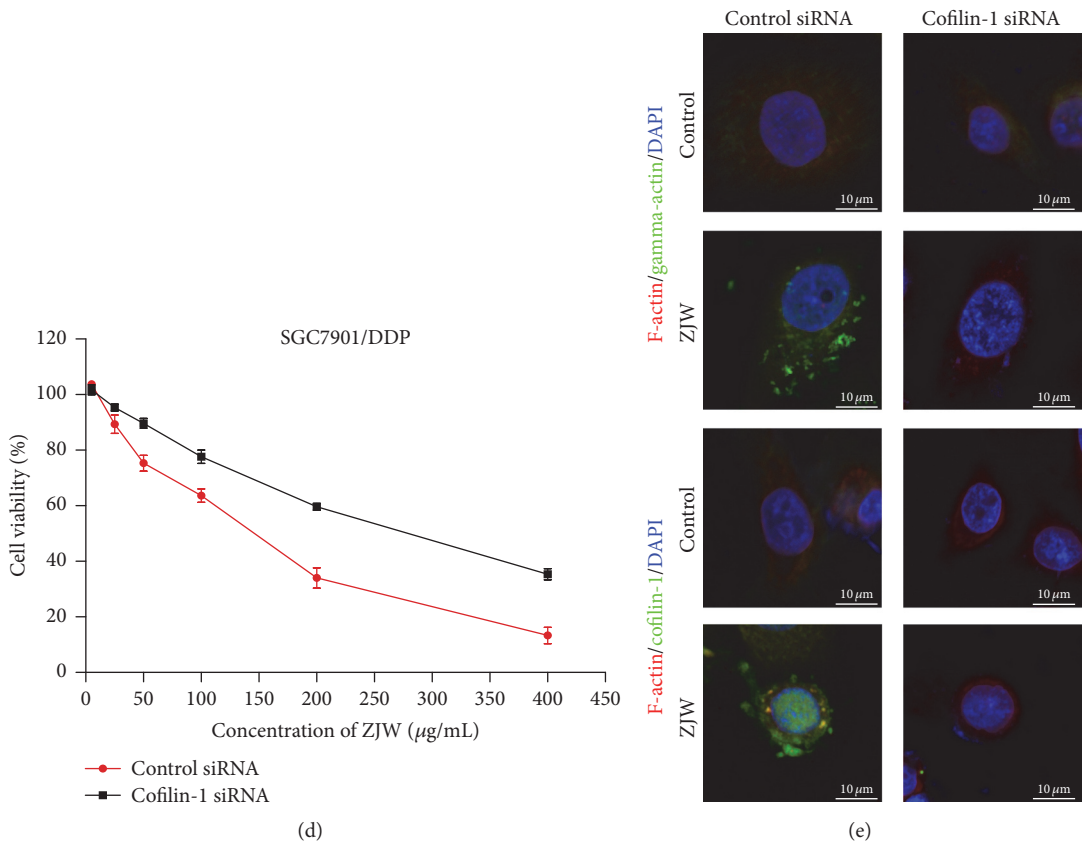

Figure 6: Cofilin-1 knockdown decreases the apoptosis induced by ZJW in SGC7901/DDP cells. (a) The effects of siRNAs specific for the cofilin-1 gene on cisplatin (DDP)-resistant SGC7901/DDP cells were determined using quantitative real-time PCR. $^{***}p < 0.001$, versus control groups treated control siRNA. (b) The effects of siRNAs specific for the cofilin-1 gene were determined by western blotting. (c) Flow cytometry demonstrated the dose-dependent effects of ZJW treatment on the apoptosis rate of SGC7901/DDP cells 24 h after siRNA-mediated cofilin-1 knockdown. $^{**}p < 0.01$, versus control groups treated with cofilin-1 siRNA and ZJW (0 μg/mL). (d) The viabilities of SGC7901/DDP cells subjected to cofilin-1 knockdown and exposed to 0, 25, 50, 100, 200, or 400 μg/mL ZJW for 24 h were determined using a CCK-8 assay. (e) SGC7901/DDP cells with cofilin-1 knockdown exposed to 50 μg/mL ZJW for 24 h, depolymerisation of F-actin, and translocation of G-actin and cofilin-1 from the cytoplasm to the mitochondria were detected using immunofluorescence assay.

detected in cofilin-1 siRNA-treated SGC7901/DDP cells using immunofluorescence. As shown in Figure 6(e), in response to ZJW treatment, control siRNA-treated SGC7901/DDP cells exhibited mitochondrial translocation, accumulation of cofilin-1 and G-actin, and lower levels of F-actin, whereas cofilin-1 siRNA-treated SGC7901/DDP cells exhibited a reduced expression of cofilin-1 and G-actin and an increased level of F-actin.

3.7. ZJW Induces the Dephosphorylation of p-Cofilin-1 by PP1 and PP2A. Expressions of the phosphatases PP1 and PP2A and slingshot (SSH), which participate in intracellular phosphorylation reactions, were evaluated in ZJW-treated SGC7901/DDP cells using western blotting. We observed an increased expression of PP1 and PP2A in these cells, whereas the expression of SSH was not significantly altered (Figure 7(a)).

To verify whether the activation of PP2A and PP1 represented a key step in the ZJW-induced dephosphorylation of p-cofilin-1, the PP1 and PP2A inhibitor calyculin A was used to inhibit the activities of these phosphatases. A co-IP assay with an anti-cofilin-1 antibody demonstrated the strong phosphatase inhibitory capacity of calyculin A; specifically, calyculin A treatment reversed the increases in PP1 and PP2A and decreased p-cofilin-1 expression induced by ZJW (Figure 7(b)).

SGC7901/DDP cells treated with ZJW and calyculin A were subjected to apoptosis evaluation. Either ZJW or calyculin A alone could induce apoptosis (Figure 7(c)). However, the combination of ZJW and calyculin A yielded a significantly lower apoptosis rate than that observed with ZJW alone (Figure 7(d)). These results suggested that calyculin A inhibited the apoptosis-inducing effect of ZJW on SGC7901/DDP cells. Finally, proteins associated with mitochondrial apoptosis were detected. As shown in Figure 7(e), calyculin A inhibited the activation of PARP, caspase-3, and caspase-9 and the release of cytochrome C. These results indicate that the activation of PP2A and PP1 plays an

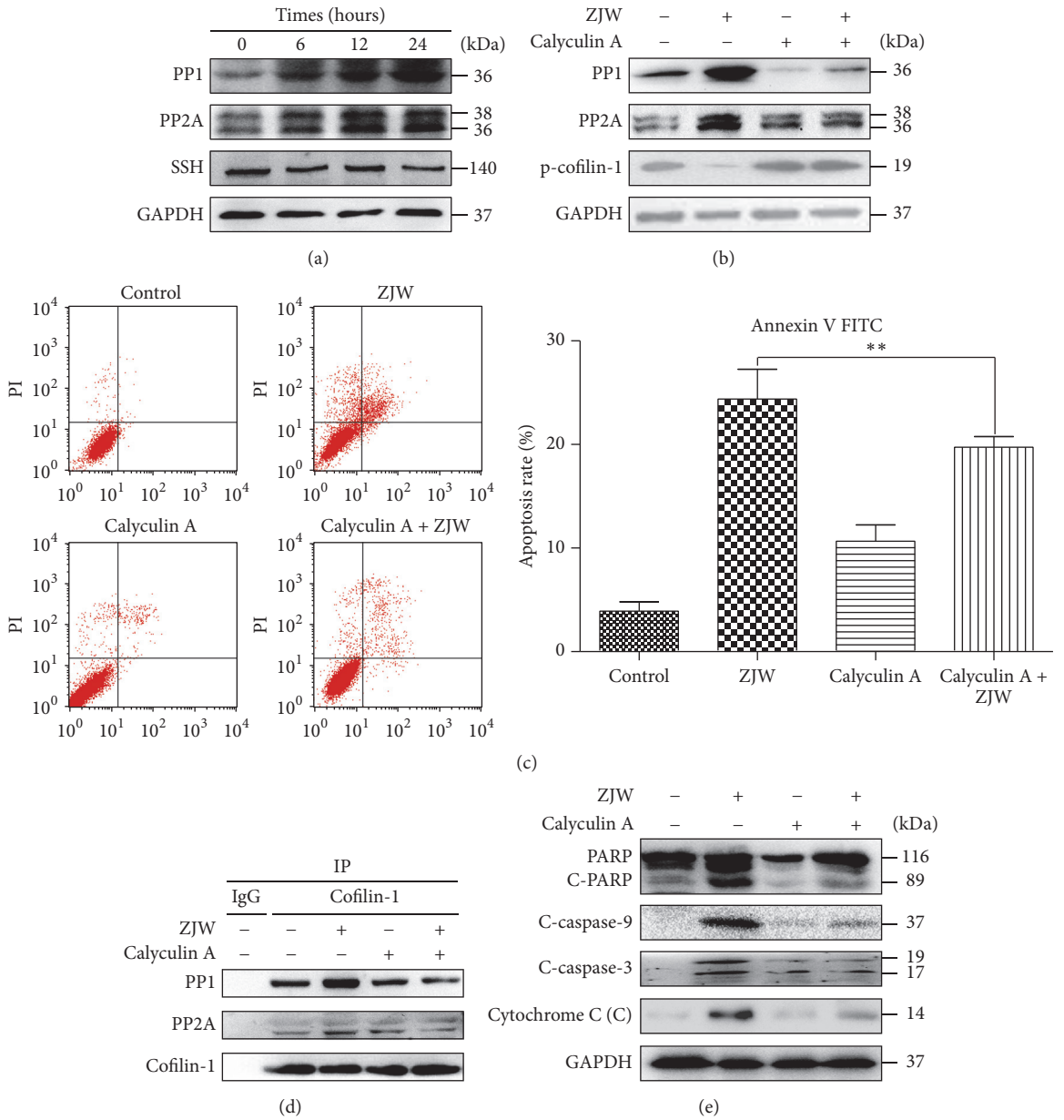

FIGURE 7: ZJW induces the dephosphorylation of p-cofilin-1 by PP1 and PP2A. (a) SGC7901/DDP cells were treated with ZJW (50 μg/mL) for 0, 6, 12, and 24 h as indicated. Whole cell proteins were prepared and subjected to western blotting to detect PP1, PP2A, and SSH. (b) Cells were treated with ZJW (50 μg/mL) and calyculin A (2 ng/mL) for 24 h. Whole cell protein was subjected to western blotting to detect PP1, PP2A, and p-cofilin-1. (c) Cells were treated with ZJW (50 μg/mL) and calyculin A (2 ng/mL) for 24 h and subjected to an apoptosis evaluation using flow cytometry with an Annexin V-FITC/propidium iodide apoptosis detection kit. $^{**}P < 0.01$, groups treated with ZJW and calyculin A versus groups treated only ZJW. (d) A co-IP assay with an anti-cofilin-1 antibody was used to detect the activation of PP1 and PP2A following treatment with ZJW (50 μg/mL) and calyculin A (2 ng/mL) for 24 h. (e) Western blotting detected PARP, cleaved caspase-3 and caspase-9, and cytochrome C in SGC7901/DDP cells following ZJW (50 μg/mL) and calyculin A (2 ng/mL) treatment for 24 h.

important role in ZJW-mediated dephosphorylation of p-cofilin-1.

4. Discussion

In this study, we found a significantly higher expression of p-cofilin-1 in DDP-resistant cell lines, wherein a lower concentration of DDP could not induce the dephosphorylation of p-cofilin-1. Moreover, we further determined that ZJW could induce the dephosphorylation of p-cofilin-1 and enhance the DDP sensitivity of BGC823/DDP and SGC7901/DDP cells, inducing apoptosis through mitochondrial signalling pathways, where ZJW promoting the dephosphorylation of p-cofilin-1 in vitro was via the activation of PP1 and PP2A. These results provided further evidence that ZJW could induce apoptosis in DDP-resistant gastric cancer cells.

DDP, a chemotherapy drug commonly used to treat gastric cancer [20–22] acts by inducing mitochondrial apoptosis in cancer cells; however, drug-resistant gastric cancer cells are not sensitive to this agent, and thus, considerable attention has been placed on this issue [23, 24]. Studies have indicated that TCM combination with chemotherapy for cancer can enhance the efficacy of and diminish the side effects and complications caused by chemotherapy. Rhizoma Coptidis is the rhizome of *Coptis chinensis* Franch, which belongs to the Ranunculaceae family being a kind of perennial herb and grows primarily in china. Rhizoma Coptidis contains many kinds of alkaloids, such as berberine, coptisine, methyl coptisine, and palmatine and can cure acute conjunctivitis, acute bacillary dysentery, acute gastroenteritis, hematemesis, furuncle, and other disease. Rhizoma coptidis and its major compounds alkaloid berberine have been reported to reverse drug resistance in cancer [25–27]. Fructus evodiae is of Rutaceae family; its fruit is the herb for ZJW. Evodiamine, an alkaloid extract from Fructus evodiae, has been reported to induce apoptosis and inhibit proliferation in tumors [28–30]. Although the anticancer activity produced by Rhizoma Coptidis and Fructus evodiae and their alkaloids singly used had been verified, the formula ZJW showed much better effect [12]. Therefore, we determined the effect of ZJW on drug resistance in DDP-resistant gastric cancer cells. The results also verified the effect of reversing drug-resistance of ZJW in DDP-resistant gastric cancer cells.

Recent studies demonstrated that high-expression levels of cofilin-1 in many cancers correlated with invasion and metastasis, chemotherapy resistance, and poor prognosis [4, 6, 31–33]. The mammalian cofilin-encoding gene encodes two members of the actin-binding protein family: cofilin-1, which is expressed in various nonmuscle tissues, and cofilin-2, which is mainly expressed in muscle tissue [34]. The main function of cofilin-1 is the decomposition of actin microfilaments; this leads to an increase in actin monomers, which is dependent on the rate of dissociation of the ends of actin microfilaments, and promotes the circulation of actin microfilaments, thus influencing actin cytoskeletal reorganisation and regulating cytoskeletal remodelling. The importance of this process is underscored by the involvement of the actin filament cytoskeleton in many important physiological processes, such as cell growth, differentiation, metastasis, membrane reorganisation, and cell dynamics [35]. Cofilin-1 protein exists in two states, activated (cofilin-1) and inactivated (p-cofilin-1), and these states play different roles in the cell [36]. The dephosphorylation of p-cofilin-1 can induce degradation of F-actin to G-actin and promote the translocation of actin and cofilin-1 complexes to the mitochondria, triggering mitochondrial apoptosis [7, 8]. The phosphoric acid lipases, PP1, PP2A, and SSH, can activate cofilin-1, allowing cofilin-1 to bind F-actin and promote the depolymerisation of actin filaments. Previous studies demonstrated that changes in cofilin-1 or p-cofilin-1 patterns played an important role in multidrug resistance in tumour cells [4, 5, 33]. In this study, high expression of p-cofilin-1 was found in DDP-resistant gastric cells. At the same concentration, DDP could induce p-cofilin-1 dephosphorylation in DDP-sensitive gastric cancer cells but not in DDP-resistant gastric cancer cells. The results suggested p-cofilin-1 as an effective therapeutic target of gastric cancer. Our results thus verified the ability of ZJW to induce the activation of PP1 and PP2A, which further promote the dephosphorylation of p-cofilin-1. Certainly, degradation of F-actin to G-actin, the translocation of actin and cofilin-1 complexes to the mitochondria, and mitochondrial apoptosis were observed in DDP-resistant gastric cancer cells.

5. Conclusion

In accordance with our previous studies, we conclude that ZJW can be used as an inhibitor of chemoresistance in gastric cancer, which may partly be due to dephosphorylation of p-cofilin-1 via the activation of PP1 and PP2A. We believe that our study thus demonstrates a naturally derived drug resistance inhibitor in human cancers. In addition, this combination of herbs might yield better results than modern medicine in the context of cancer treatment. ZJW-based treatments should be explored as potential therapeutic strategies for human drug-resistant cancers. Certainly, the cause of high expression of p-cofilin-1 and the mechanism of activation of PP1 and PP2A of ZJW in DDP-resistant gastric cells deserve further investigation.

Competing Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contributions

Qing-Feng Tang and Jian Sun contributed equally to this work.

Acknowledgments

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References

[1] A. Jemal, F. Bray, M. M. Center, J. Ferlay, E. Ward, and D. Forman, "Global cancer statistics," *CA—A Cancer Journal for Clinicians*, vol. 61, no. 2, pp. 69–90, 2011.

[2] X. Paoletti, K. Oba, T. Burzykowski et al., "Benefit of adjuvant chemotherapy for resectable gastric cancer: a meta-analysis," *The Journal of the American Medical Association*, vol. 303, no. 17, pp. 1729–1737, 2010.

[3] A. D. Wagner, W. Grothe, J. Haerting, G. Kleber, A. Grothey, and W. E. Fleig, "Chemotherapy in advanced gastric cancer: a systematic review and meta-analysis based on aggregate data," *Journal of Clinical Oncology*, vol. 24, no. 18, pp. 2903–2909, 2006.

[4] M. Li, J. Yin, N. Mao, and L. Pan, "Upregulation of phosphorylated cofilin 1 correlates with taxol resistance in human ovarian

cancer in vitro and in vivo," *Oncology Reports*, vol. 29, no. 1, pp. 58–66, 2013.

[5] H. Zhang, Y. Wang, F. Xing et al., "Overexpression of LIMK1 promotes migration ability of multidrug-resistant osteosarcoma cells," *Oncology Research*, vol. 19, no. 10-11, pp. 501–509, 2012.

[6] M. Becker, M. A. De Bastiani, C. B. Müller, M. M. Markoski, M. A. A. Castro, and F. Klamt, "High cofilin-1 levels correlate with cisplatin resistance in lung adenocarcinomas," *Tumor Biology*, vol. 35, no. 2, pp. 1233–1238, 2014.

[7] B. T. Chua, C. Volbracht, K. O. Tan, R. Li, V. C. Yu, and P. Li, "Mitochondrial translocation of cofilin is an early step in apoptosis induction," *Nature Cell Biology*, vol. 5, no. 12, pp. 1083–1089, 2003.

[8] Q. Tang, Q. Ji, Y. Tang et al., "Mitochondrial translocation of cofilin-1 promotes apoptosis of gastric cancer BGC-823 cells induced by ursolic acid," *Tumor Biology*, vol. 35, no. 3, pp. 2451–2459, 2014.

[9] L. Xu, Y. Qi, L. Lv et al., "In vitro anti-proliferative effects of Zuojinwan on eight kinds of human cancer cell lines," *Cytotechnology*, vol. 66, no. 1, pp. 37–50, 2014.

[10] J. Du, Y. Sun, X.-F. Wang, Y.-Y. Lu, Q.-M. Zhou, and S.-B. Su, "Establishment of an experimental breast cancer ZHENG model and curative effect evaluation of zuo-jin wan," *Evidence-Based Complementary and Alternative Medicine*, vol. 2013, Article ID 324732, 6 pages, 2013.

[11] D.-C. Chao, L.-J. Lin, S.-T. Kao et al., "Inhibitory effects of Zuo-Jin-Wan and its alkaloidal ingredients on activator protein 1, nuclear factor-κB, and cellular transformation in HepG2 cells," *Fitoterapia*, vol. 82, no. 4, pp. 696–703, 2011.

[12] X.-N. Wang, L.-N. Xu, J.-Y. Peng, K.-X. Liu, L.-H. Zhang, and Y.-K. Zhang, "In vivo inhibition of S180 tumors by the synergistic effect of the Chinese medicinal herbs *Coptis chinensis* and *Evodia rutaecarpa*," *Planta Medica*, vol. 75, no. 11, pp. 1215–1220, 2009.

[13] H. Sui, X. Liu, B.-H. Jin et al., "Zuo Jin Wan, a traditional chinese herbal formula, reverses P-gp-mediated MDR in vitro and in vivo," *Evidence-Based Complementary and Alternative Medicine*, vol. 2013, Article ID 957078, 13 pages, 2013.

[14] H. Sui, S.-F. Pan, Y. Feng et al., "Zuo Jin Wan reverses P-gp-mediated drug-resistance by inhibiting activation of the PI3K/Akt/NF-κB pathway," *BMC Complementary and Alternative Medicine*, vol. 14, article 279, 2014.

[15] Q.-F. Tang, Q. Ji, Y.-Y. Qiu et al., "Synergistic effect of Zuo Jin Wan on DDP-induced apoptosis in human gastric cancer SGC-7901/DDP cells," *Evidence-Based Complementary and Alternative Medicine*, vol. 2014, Article ID 724764, 10 pages, 2014.

[16] I. Rasmussen, L. H. Pedersen, L. Byg, K. Suzuki, H. Sumimoto, and F. Vilhardt, "Effects of F/G-actin ratio and actin turnover rate on NADPH oxidase activity in microglia," *BMC Immunology*, vol. 11, article 44, 2010.

[17] D. P. Turner, O. Moussa, M. Sauane, P. B. Fisher, and D. K. Watson, "Prostate-derived ETS factor is a mediator of metastatic potential through the inhibition of migration and invasion in breast cancer," *Cancer Research*, vol. 67, no. 4, pp. 1618–1625, 2007.

[18] C. Wang, G.-L. Zhou, S. Vedantam, P. Li, and J. Field, "Mitochondrial shuttling of CAP1 promotes actin- and cofilin-dependent apoptosis," *Journal of Cell Science*, vol. 121, no. 17, pp. 2913–2920, 2008.

[19] R. Li, X. Wang, X.-H. Zhang, H.-H. Chen, and Y.-D. Liu, "Ursolic acid promotes apoptosis of SGC-7901 gastric cancer cells through ROCK/PTEN mediated mitochondrial translocation of cofilin-1," *Asian Pacific Journal of Cancer Prevention*, vol. 15, no. 22, pp. 9593–9597, 2014.

[20] D. Huang, H. Duan, H. Huang et al., "Cisplatin resistance in gastric cancer cells is associated with HER2 upregulation-induced epithelial-mesenchymal transition," *Scientific Reports*, vol. 6, Article ID 20502, 2016.

[21] M. Zhuang, Q. Shi, X. Zhang et al., "Involvement of miR-143 in cisplatin resistance of gastric cancer cells via targeting IGF1R and BCL2," *Tumor Biology*, vol. 36, no. 4, pp. 2737–2745, 2015.

[22] X. Zhi, J. Tao, G. Xiang et al., "APRIL induces cisplatin resistance in gastric cancer cells via activation of the NF-λB pathway," *Cellular Physiology and Biochemistry*, vol. 35, no. 2, pp. 571–585, 2015.

[23] H. J. Cho, K. E. Baek, S.-M. Park et al., "RhoGDI2 confers gastric cancer cells resistance against cisplatin-induced apoptosis by upregulation of Bcl-2 expression," *Cancer Letters*, vol. 311, no. 1, pp. 48–56, 2011.

[24] V. V. Subhash, S. H. Tan, W. L. Tan et al., "GTSE1 expression represses apoptotic signaling and confers cisplatin resistance in gastric cancer cells," *BMC Cancer*, vol. 15, article 550, 2015.

[25] Y. D. Min, M. C. Yang, K. H. Lee, K. R. Kim, S. U. Choi, and K. R. Lee, "Protoberberine alkaloids and their reversal activity of P-gp expressed multidrug resistance (MDR) from the rhizome of *Coptis japonica* Makino," *Archives of Pharmacal Research*, vol. 29, no. 9, pp. 757–761, 2006.

[26] C. He, R. Rong, J. Liu, J. Wan, K. Zhou, and J. X. Kang, "Effects of Coptis extract combined with chemotherapeutic agents on ROS production, multidrug resistance, and cell growth in A549 human lung cancer cells," *Chinese Medicine*, vol. 7, no. 1, article 11, 2012.

[27] L. Fu, W. Chen, W. Guo et al., "Berberine targets AP-2/hTERT, NF-κB/COX-2, HIF-1α/VEGF and cytochrome-c/caspase signaling to suppress human cancer cell growth," *PLoS ONE*, vol. 8, no. 7, Article ID e69240, 2013.

[28] J. Yang, L.-J. Wu, S.-I. Tashino, S. Onodera, and T. Ikejima, "Critical roles of reactive oxygen species in mitochondrial permeability transition in mediating evodiamine-induced human melanoma A375-S2 cell apoptosis," *Free Radical Research*, vol. 41, no. 10, pp. 1099–1108, 2007.

[29] W.-T. Wei, H. Chen, Z.-H. Wang et al., "Enhanced antitumor efficacy of gemcitabine by evodiamine on pancreatic cancer via regulating PI3K/Akt pathway," *International Journal of Biological Sciences*, vol. 8, no. 1, pp. 1–14, 2011.

[30] L. I. Yang, X. Liu, D. Wu et al., "Growth inhibition and induction of apoptosis in SGC7901 human gastric cancer cells by evodiamine," *Molecular Medicine Reports*, vol. 9, no. 4, pp. 1147–1152, 2014.

[31] W. Wang, R. Eddy, and J. Condeelis, "The cofilin pathway in breast cancer invasion and metastasis," *Nature Reviews Cancer*, vol. 7, no. 6, pp. 429–440, 2007.

[32] H. Yamaguchi and J. Condeelis, "Regulation of the actin cytoskeleton in cancer cell migration and invasion," *Biochimica et Biophysica Acta - Molecular Cell Research*, vol. 1773, no. 5, pp. 642–652, 2007.

[33] C. B. Müller, M. A. De Bastiani, M. Becker et al., "Potential crosstalk between cofilin-1 and EGFR pathways in cisplatin resistance of non-small-cell lung cancer," *Oncotarget*, vol. 6, no. 6, pp. 3531–3539, 2015.

[34] V. DesMarais, M. Ghosh, R. Eddy, and J. Condeelis, "Cofilin takes the lead," *Journal of Cell Science*, vol. 118, no. 1, pp. 19–26, 2005.

[35] J. R. Bamburg and B. W. Bernstein, "Roles of ADF/cofilin in actin polymerization and beyond," *F1000 Biology Reports*, vol. 2, article 62, 2010.

[36] M. Van Troys, L. Huyck, S. Leyman, S. Dhaese, J. Vandekerkhove, and C. Ampe, "Ins and outs of ADF/cofilin activity and regulation," *European Journal of Cell Biology*, vol. 87, no. 8-9, pp. 649–667, 2008.

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Research Article

Efficacy of Compound Kushen Injection in Combination with Induction Chemotherapy for Treating Adult Patients Newly Diagnosed with Acute Leukemia

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We assessed the clinical effectiveness and safety of CKI (compound Kushen injection) plus standard induction chemotherapy for treating adult acute leukemia (AL). We randomly assigned 332 patients with newly diagnosed AL to control ($n = 165$, receiving DA (daunorubicin and cytarabine) or hyper-CVAD (fractionated cyclophosphamide, doxorubicin, vincristine, and dexamethasone)) or treatment ($n = 167$, receiving CKI and DA or hyper-CVAD) groups. Posttreatment, treatment group CD3+, CD4+, CD4+/CD8+, natural killer (NK) cell, and immunoglobulin (IgG, IgA, and IgM) levels were significantly higher than those of the control group ($p < 0.05$), and CD8+ levels were lower in the treatment group than in the control group ($p < 0.05$). Treatment group interleukin- (IL-) 4 and IL-10 levels were significantly higher compared to the control posttreatment (both $p < 0.05$) as were complete remission, overall response, and quality of life (QoL) improvement rates ($p < 0.05$). The control group had more incidences of grade 3/4 hematologic and nonhematologic toxicity ($p < 0.05$). Responses to induction chemotherapy, QoL improvement, and adverse events incidence between control group patients with acute myeloid leukemia and acute lymphocytic leukemia were not significantly different. CKI plus standard induction chemotherapy is effective and safe for treating AL, possibly by increasing immunologic function.

1. Introduction

Acute leukemia (AL), a malignant neoplasm, is characterized by clonal blood cell proliferation within the bone marrow. Historically, AL diagnosis is linked with poor prognosis, particularly in older adults. Improved disease treatment and management have led to increased overall survival trends [1]. The Surveillance Epidemiology and End Results reported 24% and 65% 2002–2008 relative five-year survival rates in adults with acute myeloid leukemia (AML) or acute lymphoblastic leukemia (ALL), respectively [2]. The nature of adult AL necessitates emergent, aggressive inpatient chemotherapy. However, standard chemotherapy is not always tolerated by patients; the adverse events during chemotherapy, such as myelosuppression, gastrointestinal reaction, infection, and cardiotoxicity, often lead to interruption of chemotherapy [3]; consequently, there is treatment failure. Therefore, such patients urgently need effective, low-toxicity therapy.

Therapy integrating traditional Chinese and Western medicine has been the most distinctive method for treating malignant tumors in China [4–7]. As a representative of traditional Chinese medicine (TCM), compound Kushen injection (CKI) is extracted from the Kushen (*Radix Sophorae Flavescentis*) and Baituling (*Rhizoma smilacis Glabrae*) herbs; its primary components are oxymatrine and matrine [8, 9]. Additional File 1 (in Supplementary Material available online at http://dx.doi.org/10.1155/2016/3121402) contains the CKI

fingerprint. The State Food and Drug Administration of China approved CKI for treating cancer in 1992 [10]. Since then, CKI has been extensively used in the Chinese clinical setting. There are many clinical reports demonstrating its anticancer effect and these reports include using CKI to treat gastric cancer, liver cancer, lung cancer, breast cancer, ovarian cancer, colorectal cancer, and other cancer types [11–14]. It is reported that CKI attenuates chemotherapy and radiotherapy side effects by improving quality of life (QoL), regulating immune function, and synergizing chemotherapy and radiotherapy therapeutic effects [15, 16]. In addition, it is reported that there is a positive effect of CKI on bone cancer pain: compared with radiotherapy or bisphosphonates, CKI showed significant effects on the improvement of pain relief in patients with bone cancer pain and the increase in Karnofsky Performance Status (KPS). The patients treated with CKI achieved statistically significant reductions in the incidence of leukopenia and nausea. No severe adverse events were found and no treatment was stopped because of adverse events of CKI in the treatment groups [17]. However, the scientific literature contains scarce empirical data involving CKI in ALs, and the underlying complex mechanisms of its anticancer effect are not fully understood. In the study, our primary aim was to determine whether CKI with induction chemotherapy has a similar or better effect in improving the objective response rate (ORR) in adult AL. The secondary aims were to compare immunologic function, quality of life (QoL), clinical symptoms, and adverse drug reactions (ADRs) and to explore the reasons therein.

2. Materials and Methods

2.1. Study Groups. We selected 332 previously untreated patients (aged 18–78 years) with AL who were treated at our hospital between January 2011 and January 2016. According to the World Health Organization criteria, 175 and 157 patients were diagnosed with AML and ALL, respectively. The patients were randomly assigned to a treatment (n = 167) or control (n = 165) group. No patient had central nervous system diseases, other malignancies, or uncontrolled infections. They also had no inflammatory conditions, pathological antecedents, or endocrine dysfunctions. We excluded patients with known confusion or who were deemed too ill to participate. The Wuhan University Ethics Committee approved this study and it met international standards for patient confidentiality. All patients signed informed consent forms according to the Declaration of Helsinki.

2.2. Treatment Methods. Both groups received induction chemotherapy, in which patients with confirmed AML received the DA regimen, and patients with ALL received the hyper-CVAD regimen. The treatment group received CKI in addition to the DA or hyper-CVAD regimens.

The DA regimen consisted of 60 mg/m^2 daunorubicin on days 1–3 and 200 mg/m^2 cytarabine on days 1–7. Hyper-CVAD treatment consisted of 300 mg/m^2 fractionated cyclophosphamide twice daily on days 1–3, 50 g/m^2 doxorubicin on day 4, 2 mg vincristine on days 4 and 11, and 40 mg dexamethasone on days 1–4 and 11–14. In the treatment group, according to the instructions, 20 mL CKI (Shanxi Zhendong Pharmacy Limited Company, Chinese medicine permit number Z14021231) plus 200 mL saline was administered by intravenous drip, at 40–60 drips each min, once daily for 14 days. After the end of the first induction course, a bone marrow biopsy was used to assess the treatment response. Patients with complete remission (CR) received continued consolidation therapy or autologous or allogeneic stem cell transplantation, and patients who did not achieve CR in 2 cycles had their induction programs adjusted.

2.3. Observation Indices. The present analysis involved only 1-2 cycle of induction chemotherapy. Treatment response, QoL, and toxicity (hematologic and nonhematologic (including gastrointestinal reaction, pneumonia, hepatotoxicity, neurological dysfunction, renal dysfunction, and skin reactions)) were evaluated. One day before treatment and 1 week after the end of the treatment course in both groups, the peripheral blood T-lymphocyte subgroup (CD3+, CD4+, and CD8+), natural killer (NK) cell, and immunoglobulin (IgG, IgA, and IgM) levels were detected with flow cytometry, the CD4+/CD8+ ratios were calculated, and cytokines (interleukin- (IL-) 4 and IL-10) were detected by enzyme-linked immunosorbent assay (ELISA).

2.4. Statistical Analysis. We used SPSS version 20.0 for the statistical analyses. All data are presented as the mean ± standard deviation ($x \pm s$). The groups were compared using independent sample t-tests. The differences in characteristics between the two groups were examined using the χ^2 test. $p < 0.05$ was considered significant.

3. Results

3.1. Baseline Characteristics. The clinicopathological information of the treatment and control groups did not significantly differ ($p > 0.05$, Table 1).

3.2. Changes in Immune Function Indices. The peripheral blood CD3+, CD4+, CD8+, CD4+/CD8+, NK cell, and immunoglobulin levels in the treatment and control groups did not differ significantly 1 day before treatment ($p > 0.05$). After the first course of induction chemotherapy, in the control group, CD3+, CD4+, CD4+/CD8+, NK cell, and immunoglobulin (IgA, IgM, and IgG) levels decreased significantly ($p < 0.05$) and CD8+ was increased significantly ($p < 0.05$); in the treatment group, levels of CD3+, IgA, and IgM decreased significantly ($p < 0.05$), CD4+ was increased significantly ($p < 0.05$), and CD8+, CD4+/CD8+, NK cell, and IgG levels did not change significantly ($p > 0.05$). This suggests that immunity function has been decreased in AL patients with induction chemotherapy. The serum CD3+, CD4+, CD4+/CD8+, NK cells, and immunoglobulin levels were significantly lower in the control group than in the treatment group ($p < 0.05$, Table 2), which implies that CKI improves immunologic function in patients with AL.

Table 1: Comparison of main status between treatment and control groups (n).

Variable	Treatment group (n = 167)	Control group (n = 165)	χ^2	p
Age (years)				
≤60	78	74	0.115	0.734
>60	89	91		
Sex				
Men	87	88	0.015	0.821
Women	80	77		
AML				
M1	3	4		
M2	11	10		
M4	39	40	0.306	0.989
M5	29	28		
M6	6	5		
ALL				
L1	44	44		
L2	26	25	0.013	0.993
L3	9	9		
KPS				
≥90	17	21		
70–90	116	105	1.299	0.522
≤70	34	39		

Notes: AML: acute myeloid leukemia, ALL: acute lymphocytic leukemia, and KPS: Karnofsky Performance Status.

3.3. Cytokine Changes. Before treatment, the treatment and control groups did not significantly differ in the peripheral blood counts for IL-4 and IL-10 ($p > 0.05$). After the end of treatment, IL-4 and IL-10 levels were increased significantly in the treatment group and were significantly higher than those of the control group (both $p < 0.05$); however, those of the control group were decreased significantly compared to pretreatment levels ($p < 0.05$, Table 2).

3.4. Response to Induction Chemotherapy. Our evaluation was based on the National Comprehensive Cancer Network (NCCN) Guidelines (Version 1. 2014) for evaluating response criteria for blood and bone marrow in AL: CR (no circulating blasts or extramedullary disease, trilineage hematopoiesis (TLH) < 5% blasts, absolute neutrophil count (ANC) > 1000/μL, platelets > 1000,000/μL, and no recurrence for 4 weeks); CRi (CR with incomplete blood count recovery: recovery of platelets but <1000,000 or ANC = 1000/μL); ORR (ORR = CR + CRi). We assessed disease status via bone marrow biopsies performed 14 days (±2 days) after induction chemotherapy. After the first cycle of induction chemotherapy, the treatment group ORR rate was significantly higher than that of the control (AML: 92% versus 73.6%, $p < 0.05$; ALL: 89.9% versus 69.2%, $p < 0.05$) (Table 3), which showed that CKI plus chemotherapy significantly improved the ORR as compared with induction chemotherapy alone.

3.5. Evaluation of QoL. QoL evaluation was according to the Karnofsky Performance Status (KPS) and was classified as improvement (improved KPS of ≥10 points posttreatment); stabilization (improved or decreased KPS of <10 points posttreatment); deterioration (decreased KPS of ≥10 points posttreatment). Before treatment, the two groups patients did not significantly differ in KPS ($p > 0.05$, Table 1). After completing the first cycle of induction therapy, the treatment group KPS improvement rate was significantly higher than that of the control (AML: 55.7% versus 28.7%, $p < 0.05$; ALL: 59.5% versus 24.4%, $p < 0.05$), and KPS deterioration rate was significantly lower in the treatment group than in the control (AML: 9.1% versus 28.7%, $p < 0.05$; ALL: 11.4% versus 38.5%, $p < 0.05$) (Table 4), indicating that CKI plus chemotherapy can improve patient QoL.

3.6. Toxicity Reactions. According to the National Cancer Institute (NCI) Common Terminology Criteria for Adverse Events (CTCAE) v 3.0 [18], drug toxicity and adverse reactions were classified into four grades (1–4). Grade 1/2 adverse reactions were observed in many patients and were not considered in this study.

Whether in patients with AML or ALL, there was more hematologic toxicity in the control group ($p < 0.05$). Thrombocytopenia and anemia were observed in 9 patients (5.4%) in the treatment group as compared with 38 patients (23%) in the control group, and the patients received blood platelet or red blood cell transfusions ($p < 0.05$). Similarly, nonhematologic toxicity occurred significantly more often in the control group. In total, 2 patients (1.2%) in the treatment group compared with 15 patients (9.1%) in the control group developed grades 3 and 4 pneumonia ($p < 0.05$); 5 control group patients died of pneumonia during treatment; no treatment group patient died. The incidences of adverse events between patients with AML and ALL in the same treatment groups were not significantly different (Table 5).

The results show that chemotherapy-related complications are reduced following CKI plus chemotherapy as compared with chemotherapy alone.

4. Discussion

Traditional Chinese Medicine (TCM) holds that, in treating tumors, eliminating pathogenic factors and strengthening genuine Qi are of equal importance: eliminating pathogenic factors is killing tumor cells by using radiotherapy or chemotherapy and strengthening genuine Qi protects immunologic functions of the organism by using drugs, increasing immunity of the organism [19, 20]. Hematopoietic stem cells that have lost the normal differentiation capacity into mature blood cells give rise to leukemia [21, 22]. In AL treatment, induction chemotherapy to achieve CR is considered basic therapy [23]. However, chemotherapy cannot avoid injuring the immune system, which mainly manifests in the fact that chemotherapy drugs directly or indirectly kill the immunologic effector cells, leading to decreased immune function [24, 25]. Antitumor immune responses are T-cell-mediated specific responses. T-cells can be divided into CD4+

Table 2: Comparison of immune function indices and cytokines in the treatment and control groups before and after treatment ($x \pm s$).

Variable	Treatment group ($n = 167$)		Control group ($n = 165$)	
	Before treatment	After treatment	Before treatment	After treatment
CD3+	56.43 ± 4.22	55.45 ± 4.22^{*a}	55.38 ± 5.33	34.29 ± 4.93^{b}
CD4+	31.55 ± 5.33	35.35 ± 4.24^{*a}	33.19 ± 5.39	17.13 ± 5.81^{b}
CD8+	13.32 ± 2.33	13.12 ± 2.23*	14.31 ± 3.02	15.23 ± 3.03^{b}
CD4+/CD8+	1.69 ± 0.33	1.73 ± 0.22*	1.68 ± 0.42	1.08 ± 0.33^{b}
NK cells	15.44 ± 3.35	15.41 ± 4.02*	15.12 ± 3.72	10.12 ± 3.11^{b}
IgG	12.5 ± 1.3	12.3 ± 1.2*	12.4 ± 1.3	7.2 ± 1.5^{b}
IgA	2.4 ± 0.3	2.3 ± 0.3^{*a}	2.4 ± 0.3	1.2 ± 0.5^{b}
IgM	1.71 ± 0.30	1.58 ± 0.4^{*a}	1.81 ± 0.47	0.45 ± 0.53^{b}
IL-4	20.0 ± 2.4	22.2 ± 2.3^{*a}	22.5 ± 3.2	11.8 ± 2.2^{b}
IL-10	17.10 ± 2.2	19.4 ± 4.2^{*a}	18.90 ± 2.1	9.80 ± 3.2^{b}

Notes: compared with the control group after treatment, $^{*}p < 0.05$; compared with the treatment group before treatment, $^{a}p < 0.05$; compared with the control group before treatment, $^{b}p < 0.05$.

Table 3: Response to induction chemotherapy in the treatment and control groups.

Variable	AML (%)		ALL (%)	
	Treatment group ($n = 88$)	Control group ($n = 87$)	Treatment group ($n = 79$)	Control group ($n = 78$)
CR (%)	72 (81.8)	58 (66.7)	64 (81)	51 (65.4)
CRi (%)	9 (10.2)	6 (6.9)	7 (8.9)	3 (3.8)
ORR (%)	81 (92)^{a}	64 (73.6)	71 (89.9)^{a}	54 (69.2)

Notes: AML: acute myeloid leukemia, ALL: acute lymphocytic leukemia, CR: complete remissions, CRi: CR with incomplete blood count recovery, and ORR: overall response rate. Compared with the control group, $^{a}p < 0.05$.

Table 4: KPS index changes of two groups before and after treatment.

Variable	AML (%)		ALL (%)	
	Treatment group ($n = 88$)	Control group ($n = 87$)	Treatment group ($n = 79$)	Control group ($n = 78$)
Improvement (%)	49 (55.7)^{a}	25 (28.7)	47 (59.5)^{a}	19 (24.4)
Stabilization (%)	31 (35.2)	37 (42.5)	23 (29.1)	29 (37.2)
Deterioration (%)	8 (9.1)^{a}	25 (28.7)	9 (11.4)^{a}	30 (38.5)

Notes: AML: acute myeloid leukemia, ALL: acute lymphocytic leukemia. Compared with the control group, $^{a}p < 0.05$.

Table 5: Grade 3/4 drug toxicity and adverse events occurring during the induction period in the treatment and control groups.

Variable	AML (%)		ALL (%)	
	Treatment group ($n = 88$)	Control group ($n = 87$)	Treatment group ($n = 79$)	Control group ($n = 78$)
Hematologic toxicity	6 (6.8)^{a}	21 (24.1)	3 (3.8)^{a}	17 (21.8)
Gastrointestinal reaction	2 (2.3)^{a}	10 (11.5)	1 (1.3)^{a}	8 (10.3)
Pneumonia	1 (1.1)^{a}	9 (10.3)	1 (1.3)	6 (7.7)
Cardiotoxicity	0 (0)	4 (4.6)	1 (1.3)	5 (6.4)
Hepatotoxicity	3 (3.4)^{a}	11 (12.6)	2 (2.5)^{a}	9 (11.5)
Neurological dysfunction	1 (1.1)^{a}	7 (8)	1 (1.3)^{a}	8 (10.3)
Renal dysfunction	3 (3.4)^{a}	16 (18.4)	1 (1.3)^{a}	11 (14.1)
Skin reactions	1 (1.1)^{a}	7 (8)	0 (0)^{a}	5 (6.4)

Notes: AML: acute myeloid leukemia, ALL: acute lymphocytic leukemia. Compared with the control group, $^{a}p < 0.05$.

helper/induced T-cells and CD8+ suppressor/killer T-cells at a CD4+/CD8+ ratio of 1.5–2.0. When the immunologic function in patients with cancer is impaired, CD4+ cells will decrease, CD8+ cells will increase, and the CD4+/CD8+ ratio decreases or even inverts [26]. CD3+ cells reflect the total CD4 and CD8 levels. When cellular immunologic function decreases, the NK cells ratio will also decrease, as they are unable to effectively kill tumor cells. Besides, immunoglobulins (Ig) also play an important role in adaptive immunity [27]. Therefore, flow cytometry determination of

the changes in peripheral blood T-cell subgroups, NK cells, and immunoglobulins (IgG, IgA, and IgM) can illustrate the changes in immunologic function.

In our study, T-cell subgroups, NK cells, and immunoglobulins decreased after induction chemotherapy in the control group as compared with pretreatment levels, indicating that chemotherapy has an inhibitory action on immunologic function; however, among patients in the treatment group, the T-cell subgroups, NK cells, and immunoglobulin levels were significantly higher than those of the control group, suggesting that CKI protects the immune functions of patients who undergo chemotherapy, which is similar to previous research results. In addition, after induction chemotherapy, the treatment group QoL improvement rate was higher than that in the control group. Therefore, we conclude that CKI aids antitumor therapy, resists the toxicity of chemotherapy drugs, and improves the QoL by increasing the immune system function in patients with AL who receive chemotherapy. Also, our results showed that the treatment group CR and ORR were higher than those in the control after 1-2 cycles of induction chemotherapy, whereas the long-term effect regarding treatment response and survival needs further investigation. During induction chemotherapy, no any special adverse events were observed and no treatment was stopped because of adverse events of CKI in the treatment group, and the incidence of grades 3 and 4 chemotherapy-related toxicity was quite low in the treatment group than in the control, which means that CKI is safe for patients with AL and its primary active components oxymatrine and matrine have no direct interactions with used chemotherapies prescription drugs.

Traditional Chinese Medicine (TCM) is mostly prescribed in combination to obtain synergistic effects and reduce possible adverse reactions [28, 29]. Hence, the compatibility of Chinese medicinal herbs is an important theory in the combination of TCM. According to the record of Chinese herbal medicine toxicology database, Sophorae flavescentis root and seeds are poisonous; the symptom of poisoning is given priority with the nervous system, which performed for salivation, breathing and pulse acceleration, gait instability, serious eclampsia, or death from respiratory depression [30]. So reducing the toxicity of Sophorae flavescentis radix is helpful to improve the safety of clinical medication. Glycyrrhizae radix is prescribed in many Chinese traditional formulas for its medical potential effects in anti-inflammation [31], immunoregulation [32], and antiallergy nature [33]. Long-term clinical practice has also confirmed that Glycyrrhizae radix has certain detoxification effect for a variety of poisoning from drugs, animals, or the body's metabolic products [34]. In addition, after compatibility of Sophorae flavescent radix and Glycyrrhizae radix, the mortality of mice was reduced, and there were changes in the content of four kinds of indicator elements, brain tissue, and liver tissue pathology [35, 36]. Future studies may combine them together to determine the interaction of Sophorae flavescentis radix and Glycyrrhizae radix.

We acknowledge that there are some limitations to the current study. First, patients of all ages were enrolled, which resulted in a heterogeneous distribution of adjuvant treatment procedures. Additionally, the duration of follow-up was too short for CKI. We adjusted the treatment programs for patients who did not achieve CR within 2 cycles, and patients with CR received consolidation therapy or autologous or allogeneic stem cell transplantation and were no longer evaluated in the study. We may state that induction chemotherapy is only one important part of leukemia treatment, and follow-up treatment is directly related to survival, for which there may also be many uncertain factors closely based on mutations related to the patients themselves.

5. Conclusions

In summary, CKI combined with proven induction chemotherapy regimens for AL appears to have better short-term efficacy and lower toxicity, which may depend on increasing the patient's immunologic function and improving the QoL. In Chinese clinics, we commonly administer CKI to synergize the therapeutic effects of chemotherapy or radiotherapy. Whether CKI can prolong the survival of patients with AL requires further investigation.

Competing Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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References

[1] D. Pulte, A. Gondos, and H. Brenner, "Expected long-term survival of patients diagnosed with acute myeloblastic leukemia during 2006–2010," *Annals of Oncology*, vol. 21, no. 2, pp. 335–341, 2010.

[2] N. Howlader, A. M. Noone, M. Krapcho et al., *SEER Cancer Statistics Review, 1975–2009*, National Cancer Institute, Bethesda, Md, USA, 2012.

[3] J. M. Vagace and G. Gervasini, "Chemotherapy toxicity in patients with acute leukemia," in *Acute Leukemia—The Scientist's Perspective and Challenge*, M. Antica, Ed., chapter 17, InTech, Rijeka, Croatia, 2011.

[4] "Challenges in research and development of traditional chinese medicines," *Chinese Herbal Medicines*, no. 1, pp. 1–28, 2009.

[5] Q. Ji, Y. Luo, W. Wang, X. Liu, Q. Li, and S. Su, "Research advances in traditional Chinese medicine syndromes in cancer patients," *Journal of Integrative Medicine*, vol. 14, no. 1, pp. 12–21, 2016.

[6] C.-L. Qian, F. Yan, Y.-Z. Song, D. Li, K.-Z. Dong, and Y.-M. Zhu, "Is the traditional Chinese medicine helpful for patients with hematologic malignant diseases? A meta-analysis of randomized controlled trials," *World Journal of Meta-Analysis*, vol. 3, no. 3, pp. 163–180, 2015.

[7] Z. Liu, S. Chen, J. Cai et al., "Traditional Chinese Medicine syndrome-related herbal prescriptions in treatment of malignant tumors," *Journal of Traditional Chinese Medicine*, vol. 33, no. 1, pp. 19–26, 2013.

[8] J. Tian, W.-H. Wang, H.-M. Cao, and Z.-M. Wang, "Determination of matrine, sophoridine and oxymatrine in compound Kushen injection by HPLC," *China Journal of Chinese Materia Medica*, vol. 32, no. 3, pp. 222–224, 2007.

[9] Q. I. Liang, J. Zhang, and Z. Zhang, "Determination of four alkaloids in compound Kushen Injection by high performance liquid chromatography with ionic liquid as mobile phase additive," *Chinese Journal of Chromatography*, vol. 31, no. 3, pp. 249–253, 2013.

[10] Z. Z. Qian, Y. Dan, Y. Z. Liu, and Y. Peng, "Pharmacopoeia of the People's Republic of China (2010 edition): a milestone in development of China's healthcare," *Chinese Herbal Medicines*, vol. 2, no. 2, pp. 157–160, 2010.

[11] M. Sun, H. Cao, L. Sun et al., "Antitumor activities of kushen: literature review," *Evidence-Based Complementary and Alternative Medicine*, vol. 2012, Article ID 373219, 11 pages, 2012.

[12] X. Ma, R.-S. Li, J. Wang et al., "The therapeutic efficacy and safety of compound kushen injection combined with transarterial chemoembolization in unresectable hepatocellular carcinoma: an update systematic review and meta-analysis," *Frontiers in Pharmacology*, vol. 7, article 70, 2016.

[13] W. Xu, H. Lin, Y. Zhang et al., "Compound Kushen Injection suppresses human breast cancer stem-like cells by down-regulating the canonical Wnt/β-catenin pathway," *Journal of Experimental and Clinical Cancer Research*, vol. 30, no. 1, article 103, 2011.

[14] L. Yin, W. L. Zheng, Q. Z. Sun, Y. F. Peng, and M. A. Wen-Li, "Meta-analysis of efficiency in treatment of gastric cancer by compound Kushen injection combined with chemotherapy," *Chinese Journal of Experimental Traditional Medical Formulae*, vol. 19, no. 14, pp. 325–330, 2013.

[15] Z. Y. Wang, G. S. Li, and H. X. Huang, "Clinical observation on treatment of 75 mid-late stage cancer patients with Yanshu injection," *Chinese Journal of Integrated Traditional & Western Medicine*, vol. 26, no. 8, pp. 681–684, 2006.

[16] J. Chen, Q. Mei, Y.-C. Xu, J. Du, Y. Wei, and Z.-M. Xu, "Effects of Matrine injection on T-lymphocyte subsets of patients with malignant tumor after gamma knife radiosurgery," *Journal of Chinese Integrative Medicine*, vol. 4, no. 1, pp. 78–79, 2006.

[17] B. Yanju, L. Yang, B. Hua et al., "A systematic review and meta-analysis on the use of traditional Chinese medicine compound kushen injection for bone cancer pain," *Supportive Care in Cancer*, vol. 22, no. 3, pp. 825–836, 2014.

[18] L. Guerretta, "Common Terminology Criteria for Adverse Events v3.0 (CTCAE)," 2003.

[19] R. Guangxin, L. I. Hegen, L. Zhao, L. Liu, L. Shen, and L. Zhou, "Effect of method of strengthening genuine qi on tumor microenvironment of advanced non-small cell lung cancer," *Journal of Liaoning University of Traditional Chinese Medicine*, no. 6, pp. 157–159, 2013.

[20] M. Xu, G. Zhang, L. Pan et al., "Effects of Chinese herbs of strengthening and invigorating genuine Qi on multidrug resistant lung cancer cells through regulation of mitochondria related molecules," *Chinese Journal of Clinical Oncology*, vol. 37, no. 13, pp. 725–728, 2011.

[21] M. R. Corces-Zimmerman and R. Majeti, "Pre-leukemic evolution of hematopoietic stem cells: the importance of early mutations in leukemogenesis," *Leukemia*, vol. 28, no. 12, pp. 2276–2282, 2014.

[22] S. Kanji, V. J. Pompili, and H. Das, "Plasticity and maintenance of hematopoietic stem cells during development," *Recent Patents on Biotechnology*, vol. 5, no. 1, pp. 40–53, 2011.

[23] A. Nazha and F. Ravandi, "Acute myeloid leukemia in the elderly: do we know who should be treated and how?" *Leukemia & Lymphoma*, vol. 55, no. 5, pp. 979–987, 2014.

[24] C. Rébé and F. Ghiringhelli, "Cytotoxic effects of chemotherapy on cancer and immune cells: how can it be modulated to generate novel therapeutic strategies?" *Future Oncology*, vol. 11, no. 19, pp. 2645–2654, 2015.

[25] R. G. van der Most, A. Currie, B. W. S. Robinson, and R. A. Lake, "Cranking the immunologic engine with chemotherapy: using context to drive tumor antigen cross-presentation towards useful antitumor immunity," *Cancer Research*, vol. 66, no. 2, pp. 601–604, 2006.

[26] P. Olioso, R. Giancola, M. Di Riti, A. Contento, P. Accorsi, and A. Iacone, "Immunotherapy with cytokine induced killer cells in solid and hematopoietic tumours: a pilot clinical trial," *Hematological Oncology*, vol. 27, no. 3, pp. 130–139, 2009.

[27] S. Alanko, T.-T. Pelliniemi, and T. T. Salmi, "Recovery of blood B-lymphocytes and serum immunoglobulins after chemotherapy for childhood acute lymphoblastic leukemia," *Cancer*, vol. 69, no. 6, pp. 1481–1486, 1992.

[28] X. Liu, H. Li, L. Wu et al., "Simultaneous quantification of chrysophanol and physcion in rat plasma by ultra fast liquid chromatography-tandem mass spectrometry and application of the technique to comparative pharmacokinetic studies of *Radix et Rhei Rhizoma* extract alone and Dahuang Fuzi Decoction," *Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences*, vol. 980, pp. 88–93, 2015.

[29] Y. Jin, C. Qu, Y. Tang et al., "Herb pairs containing Angelicae Sinensis Radix (Danggui): a review of bio-active constituents and compatibility effects," *Journal of Ethnopharmacology*, vol. 181, pp. 158–171, 2016.

[30] A. K. Drew, A. Bensoussan, I. M. Whyte, A. H. Dawson, X. Zhu, and S. P. S. Myers, "Chinese herbal medicine toxicology database: monograph on Radix Sophorae Flavescentis, 'Ku Shen,'" *Journal of Toxicology: Clinical Toxicology*, vol. 40, no. 2, pp. 173–176, 2002.

[31] G. P. Zhang, S. H. Xie, X. G. Zhu et al., "Experimental study on effects of radix glycyrrhizae on toxicity and efficacy of radix aconiti carmichaeli in compatibility," *Chinese Journal of Information on Traditional Chinese Medicine*, no. 6, pp. 31–34, 2012.

[32] J. Y. Lee, J.-H. Lee, J. H. Park et al., "Liquiritigenin, a licorice flavonoid, helps mice resist disseminated candidiasis due to *Candida albicans* by Th1 immune response, whereas liquiritin, its glycoside form, does not," *International Immunopharmacology*, vol. 9, no. 5, pp. 632–638, 2009.

[33] Y.-W. Shin, E.-A. Bae, B. Lee et al., "In vitro and in vivo antiallergic effects of *Glycyrrhiza glabra* and its components," *Planta Medica*, vol. 73, no. 3, pp. 257–261, 2007.

[34] S. C. Kim, S. H. Byun, C. H. Yang, C. Y. Kim, J. W. Kim, and S. G. Kim, "Cytoprotective effects of Glycyrrhizae radix extract and its active component liquiritigenin against cadmium-induced toxicity (effects on bad translocation and cytochrome c-mediated PARP cleavage)," *Toxicology*, vol. 197, no. 3, pp. 239–251, 2004.

[35] L. Shi, X. Tang, X. Dang et al., "Investigating herb-herb interactions: the potential attenuated toxicity mechanism of the

combined use of *Glycyrrhizae radix* et rhizoma (Gancao) and Sophorae flavescentis radix (Kushen)," *Journal of Ethnopharmacology*, vol. 165, pp. 243–250, 2015.

[36] X. Wang, X. Huang, N. Wang et al., "Toxicity of the water extraction of radix sophorae flavescentis formulated with radix glycyrrhizae on mice," *Chinese Archives of Traditional Chinese Medicine*, vol. 33, no. 7, pp. 1653–1655, 2015.