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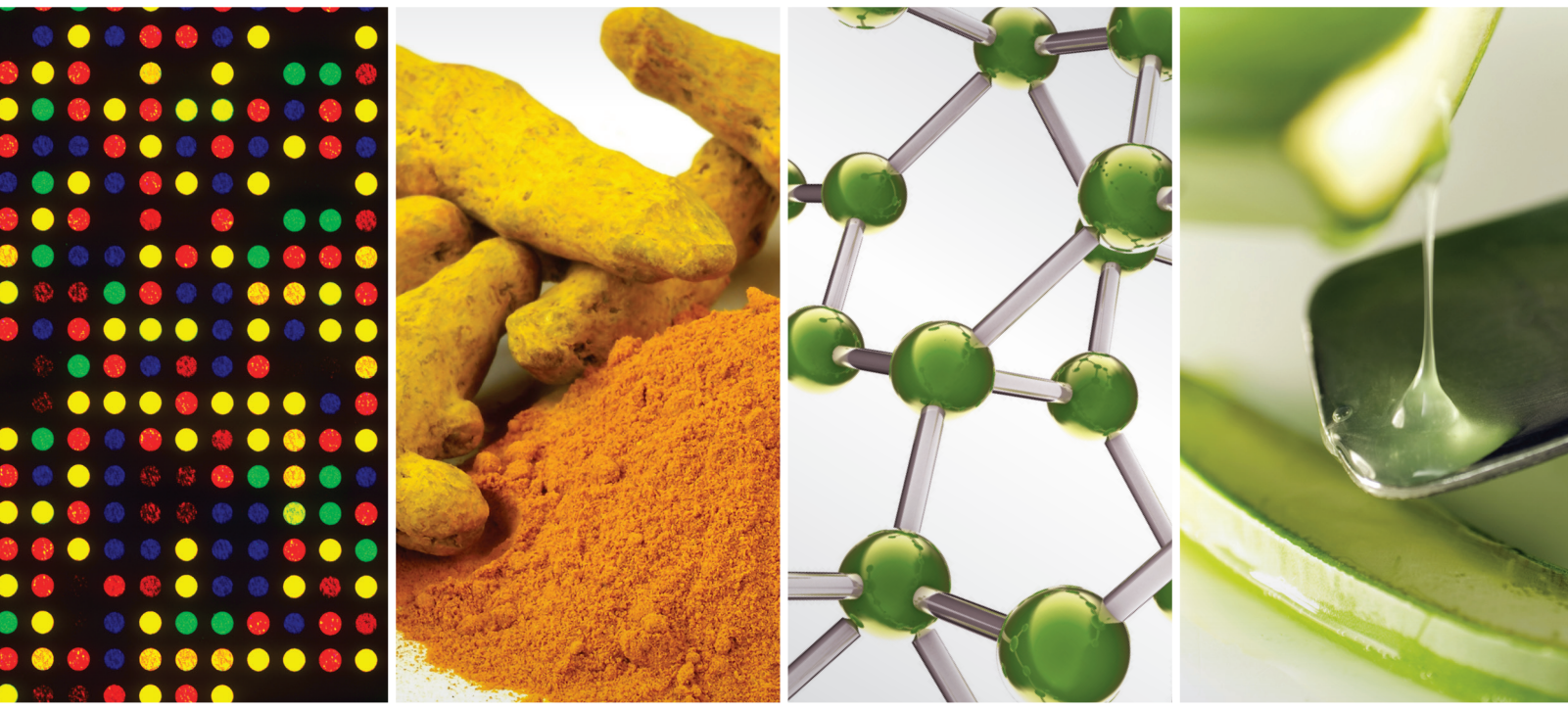
Eastern Medicine: From Nutritional Supplements to Cancer Research

Guest Editors: Dominic P. Lu, Yemeng Chen, Lixian Xu, and Leo M. Lee



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Editorial Board

Eastern Medicine: From Nutritional Supplements to Cancer Research, Dominic P. Lu, Yemeng Chen, Lixian Xu, and Leo M. Lee
Volume 2014, Article ID 817126, 2 pages

Role of JNK Activation and Mitochondrial Bax Translocation in Allicin-Induced Apoptosis in Human Ovarian Cancer SKOV3 Cells, Ling Xu, Jin Yu, Dongxia Zhai, Danying Zhang, Wei Shen, Lingling Bai, Zailong Cai, and Chaoqin Yu
Volume 2014, Article ID 378684, 6 pages

Cryptotanshinone Reverses Reproductive and Metabolic Disturbances in PCOS Model Rats via Regulating the Expression of CYP17 and AR, Jin Yu, Dongxia Zhai, Li Hao, Danying Zhang, Lingling Bai, Zailong Cai, and Chaoqin Yu
Volume 2014, Article ID 670743, 10 pages

Inhibitory Effects of Gymnema (*Gymnema sylvestre*) Leaves on Tumour Promotion in Two-Stage Mouse Skin Carcinogenesis, Ken Yasukawa, Sakiko Okuda, and Yasuhito Nobushi
Volume 2014, Article ID 328684, 5 pages

Impact of Chinese Herbal Medicine on American Society and Health Care System: Perspective and Concern, Winston I. Lu and Dominic P. Lu
Volume 2014, Article ID 251891, 6 pages

Anti-Inflammatory Effects of 81 Chinese Herb Extracts and Their Correlation with the Characteristics of Traditional Chinese Medicine, Chang-Liang Chen and Dan-Dan Zhang
Volume 2014, Article ID 985176, 8 pages

Capilliposide Isolated from *Lysimachia capillipes* Hemsl. Induces ROS Generation, Cell Cycle Arrest, and Apoptosis in Human Nonsmall Cell Lung Cancer Cell Lines, Zheng-hua Fei, Kan Wu, Yun-liang Chen, Bing Wang, Shi-rong Zhang, and Sheng-lin Ma
Volume 2014, Article ID 497456, 11 pages

Hindawi Publishing Corporation
Evidence-Based Complementary and Alternative Medicine
Volume 2014, Article ID 817126, 2 pages
http://dx.doi.org/10.1155/2014/817126

Editorial

Eastern Medicine: From Nutritional Supplements to Cancer Research

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Throughout human history, complementary and alternative medicine in the form of folk medicine has emerged and flourished in every civilization, tribe, and continent. Some forms evolved to become traditional medicine and some disappeared to be forgotten, while others have been labeled untraditional medicine and are now regarded as complementary and alternative medicine. In the past half century, an increasing number of patients and health care providers in the West have become dissatisfied with aspects of traditional Western medicine and have turned their attention to these branches of untraditional medicine. The term integrative or integrated medicine was born recently as the popularity grew in incorporating complementary medicine to reinforce gaps to better fulfill the purpose of traditional medicine.

Complementary and alternative medicine includes many branches such as herbal medicine which increasingly appears in the form of nutritional supplements to elude increasing governmental regulation as demand for these products grow. In an analysis by the International Trade Center that spanned 2010–2013, it was estimated that global medicinal plant production was $50 billion and is growing at a rate of almost 16% annually. The increasing use of various forms of traditional herbal medicine in combatting modern illnesses, particularly the dangerous side effects of pharmaceutical drugs, has proven to be valuable. However, the absence of proper warning labels concerning drug-herb interaction causes an alarming number of emergency clinic cases due to the unwanted consequences of some of these interactions.

Acupuncture is one of these aforementioned branches that has made major inroads into Western medicine. It, along with the increased interest and research in herbal medicine, is likely the most researched branch of alternative medicine in the West. Acupuncture has been recognized for its healing value by the National Institutes of Health in 1997. The subsequent creation of the National Institute of Complementary and Alternative Medicine within the NIH in the United States and the founding of European Congress of Integrative Medicine has promoted research into these various overlooked disciplines. Understanding the value and discovering the merits of each discipline using modern Western scientific methodology is integral in trying to incorporate desirable aspects into traditional medicine. This special issue reviewed and accepted merited articles ranging in topics from the current dilemma of Eastern medicine in the West to the problem of government oversight in the field of herbs that are frequently and misguidedly marketed as nutritional supplements. An article included in this special issue titled "*Impact of Chinese herbal medicine on American society and health care system: perspective and concern*" reflected these issues and concerns. Also included are articles highlighting research into ginkgo biloba and cancer-related herbal research.

Noting all these development, we turn our attention to a systematic way of doing traditional Chinese medicine research. All articles published in this special issue underscore the positive trend of returning to natural approaches for our health care and emphasize better treatment for all types of human sickness.

There were three research articles, in this special issue, on the mechanisms of immune system and apoptosis for the therapeutic studies of antitumor activities. Both in vitro herbal drugs possess an enormous potential for the cure of certain types of cancer diseases.

It is important to have well-designed pharmaceutical studies to help explain the millennia old theory of Chinese herbology and the mechanism, pharmacodynamics, pharmacokinetics, and pharmacognostics that elucidate the efficacy of Chinese herbs and unlock its century-old mysteries. The traditional clinical application of traditional Chinese medicine has been based on the characteristics of taste, flavor, channel entering, and actions of the herbs. This special issue includes an article entitled "*Anti-inflammatory effects of 81 Chinese herb extracts and their correlation with the characteristics of traditional Chinese medicine*" which suggests that herbs with pungent flavors be considered the drugs of choice due to their effective anti-inflammatory agents which can be evaluated by their effects on nitrogen oxide (NO) production and cell growth in LPS/IFNγ-costimulated murine macrophage RAW264.7 cells. This discovery could be used as one of the criteria to select different Chinese herbs for anti-inflammatory purposes. Also included in this special issue is an intensive study on the effect of Cryptotanshinone, extracted from the Chinese herb Dan Shen (Salvia miltiorrhiza Bung) on reversing the reproductive and metabolic disturbances in polycystic ovary syndrome (PCOS) in rats. This study and its analysis into the possible regulatory mechanism would validate the clinical efficacy of this particular herb for the treatment of PCOS patients.

The guest editors of this special issue hope that, through the articles accepted and published, we can bridge the gap between Western and Eastern medicines and bring them closer in order to further understand the human body and to promote the advancement of health care. Western cancer treatments such as radiation and chemotherapy have adverse effect. Eastern medicine could help mitigate those side effects by minimizing the symptoms and reducing dosage requirements when used in conjunction with current Western treatment and therapies. Immunological, enzymatic molecular biology-related research could benefit from studies of effective Eastern medicinal treatments. Studies of the ways in which Western mainstream medicine and technology can be integrated with traditional Eastern medicine are the focus of this special issue.

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Research Article

Role of JNK Activation and Mitochondrial Bax Translocation in Allicin-Induced Apoptosis in Human Ovarian Cancer SKOV3 Cells

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Background. Allicin, the major component of freshly crushed garlic, is one of the most biologically active compounds of garlic; it has been reported to induce apoptosis in cancer cells; however, the mechanism by which allicin exerts its apoptotic effects is not fully understood. The aim of the present study was to further elucidate the apoptotic pathways induced by allicin in the human ovarian cancer cell line SKOV3. *Methods*. Cell proliferation and apoptosis were measured by cell-counting assay and flow cytometry analysis. Activation of the signaling pathway was screened by human phospho-kinase array analysis, and the activated pathway and its related proteins were further confirmed by western blot analysis. *Results*. Allicin induced SKOV3 cell apoptosis and JNK phosphorylation in a time- and dose-dependent manner, but these were significantly blocked by SP600125 (an inhibitor of JNK). The findings suggest that JNK phosphorylation is related to the action of allicin on SKOV3 cells. Furthermore, JNK activation induced Bcl-2 family activation, triggered mitochondria-mediated signaling pathways, and led to the translocation of a considerable amount of Bax and cytochrome *c* release. *Conclusions*. JNK activation and mitochondrial Bax translocation are involved in allicin-induced apoptosis in SKOV3 cells. Our data input new insights to the literature of allicin-induced apoptosis.

1. Introduction

Ovarian cancer is a leading cause of cancer-related death in western countries, and its incidence in Asia is increasing. At present, cytoreductive surgery and chemotherapy were considered to be the conventional treatments of ovarian cancer. However, the success rate of surgery is only 35~50% and the multiple drug resistance (MDR) caused by chemotherapy results in the 5-year-survival rate of advanced ovarian cancer patients in only 20~30%. Therefore, although ovarian cancer is a well-studied cancer, progress in its prevention or cure is still needed.

Garlic (*Allium sativum*) has long been used as food and remedy in oriental countries. Researches have shown that garlic possesses a variety of biological activities, including antiatherosclerotic, antihypertensive, antimicrobial, anticancer, immunomodulatory, radioprotective, and potential antiaging effects [1, 2]. Allicin, the major component of freshly crushed garlic, is one of the most biologically active compounds of garlic [3] and is formed from alliin via the action of alliinase [4, 5]. Allicin has obvious inhibitory effects on different kinds of tumor cells such as gastric cancer, colon cancer, liver cancer, and lung cancer and has been put into clinical treatment as an aid cancer drug.

Activation of apoptosis signaling pathways may be responsible for treatment of malignant diseases. Two apoptotic pathways that converge on caspase-3, with one involving caspase-8 and the other involving the mitochondrial release

of cytochrome *c* as well as the activation of caspase-9, have been described [6, 7]. Mitochondrial apoptosis signaling is initiated by changes in mitochondrial membrane integrity. Soluble apoptosis signaling molecules, such as cytochrome *c*, localized in the mitochondrial intermembrane space are released into the cytosol upon apoptosis induction [8]. Released cytochrome *c* associates with Apaf-1 [9, 10] and activates procaspase-9 in a multimeric complex, the apoptosome [11–13]. Active caspase-9 in turn processes the downstream effector caspases 3, 6, and 7 [14]. Thus, the release of cytochrome *c* into the cytosol represents a pivotal step of apoptosis signaling and analysis of mitochondrial cytochrome *c* release might therefore identify apoptosis in mitochondrial signaling [15]. Studies have demonstrated that some Bcl-2 family members (e.g., Bax, Bcl-xL, Mcl-1, Bcl-2, and Bid) located in the mitochondrial membrane can alter the permeability of the membrane and trigger the activation of caspases [16], thereby, leading to apoptotic cell death. Allicin has been reported to induce apoptosis in human epithelial carcinoma cells through the mitochondrial release of apoptosis induce factor (AIF) and protein kinase A was found to play an important role in caspase-independent apoptotic pathways [17].

The proapoptotic effects of allicin against cancer cells were provided by in vitro studies [18]; however, the mechanism by which allicin exerts its apoptotic effects especially on ovarian cancer is not fully understood. The present study offers new evidence showing that activation of JNK and mitochondrial translocation of Bax are involved in allicin-induced apoptosis in human ovarian cancer SKOV3 cells.

2. Materials and Methods

2.1. Materials. Allicin was purchased from Shanghai Harvest Pharmaceutical Co., Ltd. (Shanghai, China). The purity of allicin used in the experiments was ≥90%, as determined by HPLC. Mouse anti-Hsp60 monoclonal antibody and anti-Bax monoclonal antibody 2D2 were purchased from Santa Cruz Biotechnology, Inc (USA). Antibodies against cytochrome *c* and the JNK inhibitor SP600125 were obtained from Beyotime Institute of Biotechnology, whereas antibodies against β-actin, phospho-JNK, and JNK were purchased from Cell Signaling Technology. Human Phospho-Kinase Array (catalog number ARY003) was obtained from R&D Systems Co. Ltd. (USA). RPMI-1640 medium and fetal bovine serum were purchased from GIBCO (USA).

2.2. Cell Culture and Treatment. The human ovarian cell line SKOV3 was obtained from the China Center for Type Culture Collection (Wuhan, China). The cells were routinely cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum in a humidified atmosphere with 5% CO_2 incubation at 37°C. Treatments were performed with different amounts of allicin, ranging from 0 to 100 μg/mL. Unless otherwise specified, the concentration of allicin selected for all the experiments was 25 μg/mL; an equal amount of phosphate buffered saline or dimethyl sulfoxide was added to cells as control.

2.3. Cell Proliferation and Apoptosis Assay. SKOV3 cells (2×10^4) were seeded in each well of 96-well plates and incubated at various concentrations of allicin for different periods. After treatment, the proliferative potential of the cells was analyzed using Cell Counting Kit-8 (Dojindo, Kumamoto, Japan) according to the manufacturer's protocol. For apoptosis assay, the SKOV3 cells were grown to approximately 75% confluence in 6-well plates and then treated with or without allicin (25 μg/mL, 48 h) and/or JNK inhibitors (20 μM, 30 min). After treatment, the cells were collected, washed, and resuspended in 200 μL of binding buffer at 2×10^5 cells/mL. The samples were subsequently incubated with 2.5 μL of Annexin V-FITC and 5 μL of propidium iodide in the dark for 15 min at room temperature and then analyzed by flow cytometry (Miltenyi, Germany).

2.4. Phospho-Kinase Proteome Profiling and Western Blot Analysis. The cells were seeded at a density of 1×10^7 cells per 60 cm^2 dish, cultured for 24 h, treated using indicated concentrations of allicin for 48 h, and processed using Human Phospho-Kinase Array Kit (Proteome Profiler; R&D Systems, Minneapolis, USA) following the manufacturer's instructions. Phospho-kinase array data were developed on X-ray films following exposure to chemiluminescent reagents. The results were confirmed by western blot analysis, as previously described [19].

2.5. Detection of Bax Translocation and Cytochrome c Release in Mitochondria. Crude mitochondrial and cytosolic extracts were prepared from SKOV3 cells with indicated treatments, as described by Parone et al. [20]. Bax and cytochrome *c* in the cytosol and mitochondria were detected by western blot analysis.

2.6. Statistical Analysis. Data were obtained from three independent experiments and expressed as mean ± SD. Differences were analyzed using Student's *t*-test or one-way ANOVA, as appropriate. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Allicin Inhibits SKOV3 Cell Proliferation and Induces Apoptosis. The antiproliferative effect of allicin on SKOV3 cells was examined by exposing the cells to different concentrations of allicin for 24, 48, and 72 h. Cell growth was inhibited in a dose- and time-dependent manner (Figure 1). In the presence of 25 μg/mL of allicin, SKOV3 cells exhibited approximately 60% inhibition of proliferation after treatment for 48 h. As such, this concentration and the treatment time were used in the following experiments. Flow cytometry analysis showed that allicin induced apoptosis significantly, which was also significantly blocked by pretreatment with

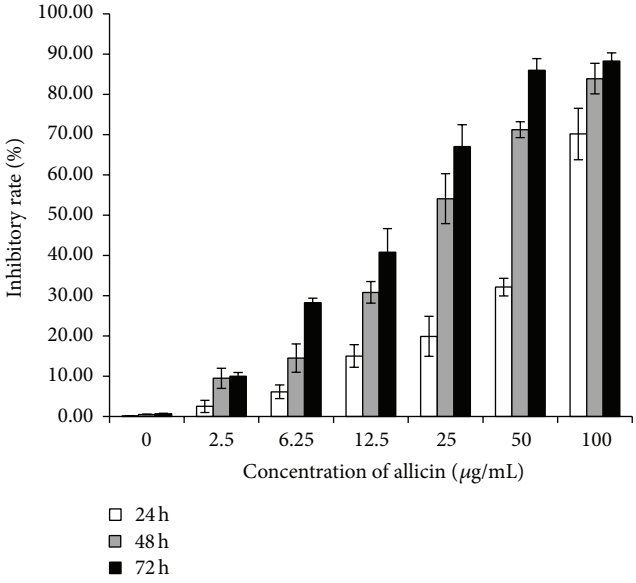

Figure 1: Inhibitory effect of allicin on SKOV3 cell proliferation. SKOV3 cells were treated with various doses of allicin for 24, 48, and 72 h. Cell proliferation was determined using cell-counting assay and expressed as the percentage of the absorbance value obtained without allicin.

SP600125 (Figure 2); however, SP600125 alone could not inhibit apoptosis.

3.2. Activation of the Signaling Pathway by Allicin in SKOV3 Cells. Human phospho-kinase array assays were performed to discover which signaling pathways are involved in allicin-induced SKOV3 cell apoptosis. The AKT and JNK pathways were activated (see supplementary data in Supplementary Material available online at http://dx.doi.org/10.1155/2014/378684). As activation of the JNK pathway is a novel finding in this setting, we focused on it in the following experiments. Phospho-JNK increased in a dose-dependent manner (Figure 3(a)), and peak phosphorylation was detected at 15 min—when the cells were treated with 25 μg/mL of allicin (Figure 3(b)). Furthermore, SP600125 could partially inhibit JNK phosphorylation as activated by allicin (Figure 3(c)), revealing that allicin-induced apoptosis is related to the JNK MAPK signaling pathway in SKOV3 cells.

3.3. JNK Activation by Allicin Results in Bax Translocation and Cytochrome c Release in Mitochondria. The Bax (2D2) and cytochrome *c* levels in the mitochondrial and cytosolic fractions were examined to further elucidate whether the JNK pathway is involved in downstream molecular events of apoptosis. As shown in Figure 4(a), the mitochondrial Bax level decreased in a time-dependent manner but simultaneously increased in the cytosolic fraction. The opposite was observed for the cytochrome *c* level. Interestingly, SP600125 markedly blocked cytochrome *c* release from mitochondria in SKOV3 cells exposed to allicin (Figure 4(b)). Allicin-induced JNK clearly leads directly to an increase in cytochrome *c* content. These biochemical changes confirm that allicin-induced apoptosis is mediated by JNK activation.

4. Discussion

Apoptosis, programmed cell death process, is an important way to remove aging, damage, and mutation of cells. Along with the in-depth study of apoptosis and its mechanisms, researchers come to realize that inducing tumor cell apoptosis is an effective way for the treatment of the tumor [21]. Therefore, exploring new therapy of regulating the cellular mechanisms and inducing apoptosis to treat tumors is becoming one of the hotspot researches in the field of oncology.

Apoptosis is a tightly regulated process controlled by several signaling pathways, such as the caspase and mitochondrial pathways [22]. The Bcl-2 family of proteins, either proapoptotic (Bax) or antiapoptotic (Bcl-2) proteins, plays an important role in apoptosis that leads to the release of cytochrome *c* from mitochondria [23]. Similarly, mitochondria are known to play a central role in mediating "intrinsic death signals" and could therefore serve as a novel target for chemotherapy. Cytochrome *c* is a mitochondrial protein whose release into the cytosol is regulated by Bcl-2 family members [24]. Once it is released into the cytosol, cytochrome *c* interacts with procaspase-9, after which it switches on caspase-3 or caspase-7, leading to apoptosis [25].

Recent research has shown that MAPK proteins are important mediators of apoptosis induced by stressful stimuli [26]. JNK and p38 MAPK are collectively termed "stress-activated protein kinases" because they are activated by various stress-related stimuli and chemotherapy drugs [27]. The JNK signaling pathway has been reported to affect members of the Bcl-2 family. For example, JNK not only can inactivate antiapoptotic Bcl-2 proteins but also can activate the mitochondrial translocation of Bax [28].

In the present study, allicin activated the AKT, P53, and JNK (c-Jun) pathway in SKOV3 cells by human phospho-kinase array analysis; however, the signals were developed weakly, for the control signal was extremely strong (supplementary data). Since the JNK pathway was novel finding in this setting, we focused on the JNK pathway in this study and the JNK activation pattern was further confirmed by western blot. JNK activation subsequently induced mitochondrial Bax translocation and the release of cytochrome *c* from mitochondria into the cytosol. SP600125 could markedly block these actions. In addition, the expression of Bcl-xL slightly decreased following treatment with allicin (data not shown). These results indicate that caspase-independent pathways are involved in allicin-induced apoptosis. In conclusion, our data provide new evidence that allicin can activate the JNK pathway, which leads to mitochondrial Bax translocation and mitochondrial release of cytochrome *c*, thus inducing SKOV3 cell apoptosis.

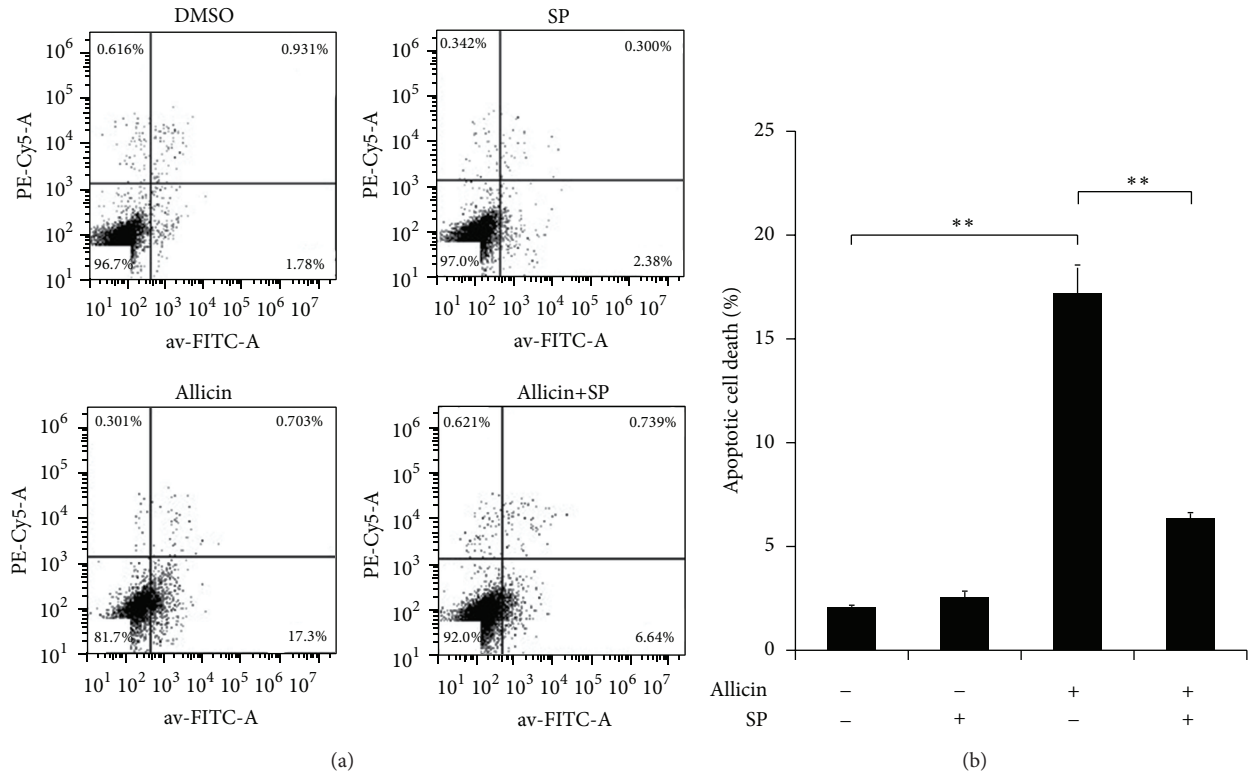

Figure 2: Flow cytometry analysis of allicin and/or SP600125 in SKOV3 cell apoptosis. SKOV3 cells were pretreated with 20 μM SP600125 for 30 min before incubation with 25 μg/mL of allicin, and apoptotic cells were measured by cytometry after 48 h. Data (mean ± SD) are representative of three experiments. (a) is a representative figure and (b) is a statistical graph. Asterisks indicate statistically significant difference ($^{**}P < 0.01$).

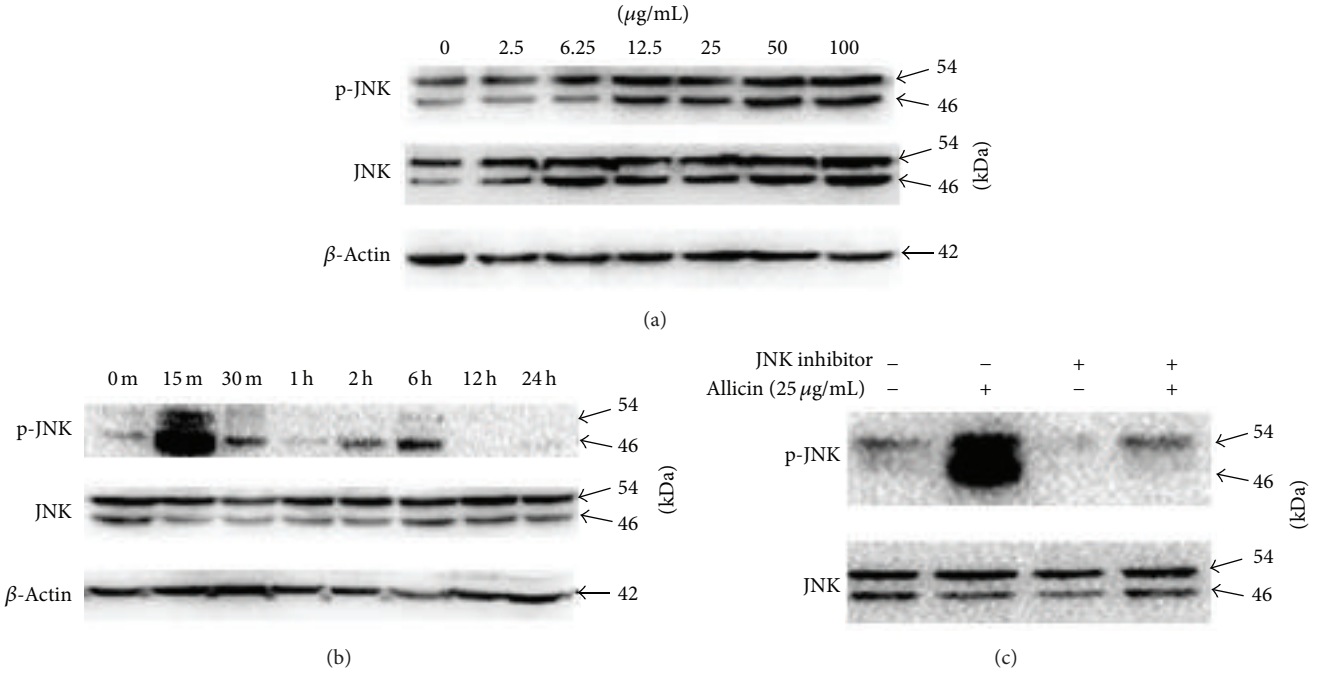

Figure 3: Effect of allicin and/or SP600125 on the phosphorylation of JNK in SKOV3 cells. (a) Treatment with various concentrations of allicin for 15 min. (b) Treatment with 25 μg/mL of allicin at indicated times. (c) Pretreatment with 20 μM SP600125 for 30 min before incubation with 25 μg/mL of allicin for 15 min; JNK phosphorylation was measured by western blot analysis after 48 h.

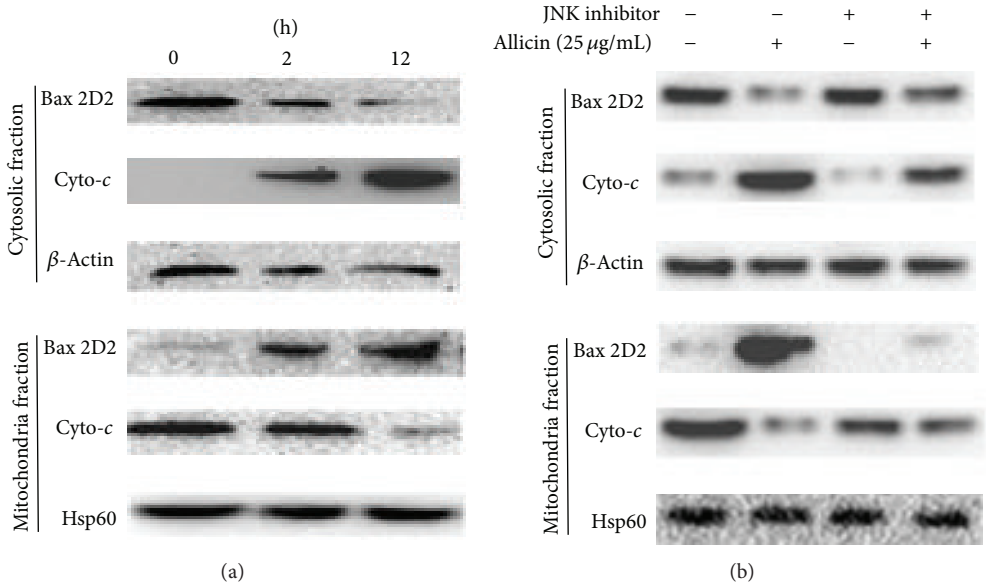

Figure 4: Western blot analysis showing cytochrome *c* and Bax levels in response to allicin. (a) SKOV3 cells were treated with 25 μg/mL of allicin for 12 h. Subsequently, cytosolic and mitochondrial fractions were prepared and western blot analysis was carried out (20 μg of protein) as described in Materials and Methods. (b) Pretreatment with or without the JNK inhibitor SP600125 for 30 min, followed by treatment with allicin for 12 h to analyze Bax and cytochrome *c*. Data are representative of three independent experiments showing a similar pattern of expression. β-Actin and Hps60 were used as internal control.

Conflict of Interests

The authors declared no potential conflict of interests with respect to the authorship and/or publication of this paper.

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References

[1] H. Amagase, B. L. Petesch, H. Matsuura, S. Kasuga, and Y. Itakura, "Intake of garlic and its bioactive components," *Journal of Nutrition*, vol. 131, no. 3, pp. 955S–962S, 2001.

[2] H. Amagase, "Clarifying the real bioactive constituents of garlic," *Journal of Nutrition*, vol. 136, no. 3, pp. S716–S725, 2006.

[3] M. Ali, M. Thomson, and M. Afzal, "Garlic and onions: their effect on eicosanoid metabolism and its clinical relevance," *Prostaglandins Leukotrienes and Essential Fatty Acids*, vol. 62, no. 2, pp. 55–73, 2000.

[4] K. Hirsch, M. Danilenko, J. Giat et al., "Effect of purified allicin, the major ingredient of freshly crushed garlic, on cancer cell proliferation," *Nutrition and Cancer*, vol. 38, no. 2, pp. 245–254, 2000.

[5] G. Sigounas, J. Hooker, A. Anagnostou, and M. Steiner, "S-allylmercaptocysteine inhibits cell proliferation and reduces the viability of erythroleukemia, breast, and prostate cancer cell lines," *Nutrition and Cancer*, vol. 27, no. 2, pp. 186–191, 1997.

[6] H. Steller, "Mechanisms and genes of cellular suicide," *Science*, vol. 267, no. 5203, pp. 1445–1449, 1995.

[7] X. Sun, M. MacFarlane, J. Zhuang, B. B. Wolf, D. R. Green, and G. M. Cohen, "Distinct caspase cascades are initiated in receptor-mediated and chemical-induced apoptosis," *Journal of Biological Chemistry*, vol. 274, no. 8, pp. 5053–5060, 1999.

[8] X. Liu, C. N. Kim, J. Yang, R. Jemmerson, and X. Wang, "Induction of apoptotic program in cell-free extracts: requirement for dATP and cytochrome c," *Cell*, vol. 86, no. 1, pp. 147–157, 1996.

[9] X. Jiang and X. Wang, "Cytochrome c promotes caspase-9 activation by inducing nucleotide binding to Apaf-1," *Journal of Biological Chemistry*, vol. 275, no. 40, pp. 31199–31203, 2000.

[10] P. Li, D. Nijhawan, I. Budihardjo et al., "Cytochrome c and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade," *Cell*, vol. 91, no. 4, pp. 479–489, 1997.

[11] K. Cain, D. G. Brown, C. Langlais, and G. M. Cohen, "Caspase activation involves the formation of the aposome, a large (~ 700 kDa) caspase-activating complex," *The Journal of Biological Chemistry*, vol. 274, no. 32, pp. 22686–22692, 1999.

[12] H. Zou, Y. Li, X. Liu, and X. Wang, "An APAf-1·cytochrome C multimeric complex is a functional apoptosome that activates procaspase-9," *Journal of Biological Chemistry*, vol. 274, no. 17, pp. 11549–11556, 1999.

[13] A. Saleh, S. M. Srinivasula, S. Acharya, R. Fishel, and E. S. Alnemri, "Cytochrome c and dATP-mediated oligomerization of Apaf-1 is a prerequisite for procaspase-9 activation," *Journal of Biological Chemistry*, vol. 274, no. 25, pp. 17941–17945, 1999.

[14] E. A. Slee, M. T. Harte, R. M. Kluck et al., "Ordering the cytochrome c-initiated caspase cascade: hierarchical activation of caspases-2,-3,-6,-7,-8, and -10 in a caspase-9-dependent

manner," *Journal of Cell Biology*, vol. 144, no. 2, pp. 281–292, 1999.

[15] K. Stahnke, A. Mohr, J. Liu, L. H. Meyer, L. Karawajew, and K.-M. Debatin, "Identification of deficient mitochondrial signaling in apoptosis resistant leukemia cells by flow cytometric analysis of intracellular cytochrome c, caspase-3 and apoptosis," *Apoptosis*, vol. 9, no. 4, pp. 457–465, 2004.

[16] J. M. Adams and S. Cory, "The Bcl-2 protein family: arbiters of cell survival," *Science*, vol. 281, no. 5381, pp. 1322–1326, 1998.

[17] S. Park, S. Cho, H. Kwon, K. Lee, D. Rhee, and S. Pyo, "Caspase-independent cell death by allicin in human epithelial carcinoma cells: involvement of PKA," *Cancer Letters*, vol. 224, no. 1, pp. 123–132, 2005.

[18] S. Oommen, R. J. Anto, G. Srinivas, and D. Karunagaran, "Allicin (from garlic) induces caspase-mediated apoptosis in cancer cells," *European Journal of Pharmacology*, vol. 485, no. 1–3, pp. 97–103, 2004.

[19] S. Chen, J. Zheng, Q. Hao et al., "p53-insensitive PUMA down-regulation is essential in the early phase of liver regeneration after partial hepatectomy in mice," *Journal of Hepatology*, vol. 52, no. 6, pp. 864–871, 2010.

[20] P. A. Parone, D. I. James, S. D. Cruz et al., "Inhibiting the mitochondrial fission machinery does not prevent Bax/Bak-dependent apoptosis," *Molecular and Cellular Biology*, vol. 26, no. 20, pp. 7397–7408, 2006.

[21] K. Lei and R. J. Davis, "JNK phosphorylation of Bim-related members of the Bcl2 family induces Bax-dependent apoptosis," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 100, no. 5, pp. 2432–2437, 2003.

[22] G. S. Salvesen and V. M. Dixit, "Caspases: intracellular signaling by proteolysis," *Cell*, vol. 91, no. 4, pp. 443–446, 1997.

[23] A. Gross, J. M. McDonnell, and S. J. Korsmeyer, "BCL-2 family members and the mitochondria in apoptosis," *Genes and Development*, vol. 13, no. 15, pp. 1899–1911, 1999.

[24] C. N. Chen, H. H. Huang, C. L. Wu et al., "Isocostunolide, a sesquiterpene lactone, induces mitochondrial membrane depolarization and caspase-dependent apoptosis in human melanoma cells," *Cancer Letters*, vol. 246, no. 1-2, pp. 237–252, 2007.

[25] Z. Wang, S. Wang, Y. Dai, and S. Grant, "Bryostatin 1 increases 1-β-D-arabinofuranosylcytosine-induced cytochrome c release and apoptosis in human leukemia cells ectopically expressing Bcl-xL," *The Journal of Pharmacology and Experimental Therapeutics*, vol. 301, no. 2, pp. 568–577, 2002.

[26] G. L. Johnson and R. Lapadat, "Mitogen-activated protein kinase pathways mediated by ERK, JNK, and p38 protein kinases," *Science*, vol. 298, no. 5600, pp. 1911–1912, 2002.

[27] H. H. Lin, J. H. Chen, C. C. Huang, and C. J. Wang, "Apoptotic effect of 3,4-dihydroxybenzoic acid on human gastric carcinoma cells involving JNK/p38 MAPK signaling activation," *International Journal of Cancer*, vol. 120, no. 11, pp. 2306–2316, 2007.

[28] H. J. Lee, C. J. Wang, H. C. Kuo, F. P. Chou, L. F. Jean, and T. H. Tseng, "Induction apoptosis of luteolin in human hepatoma HepG2 cells involving mitochondria translocation of Bax/Bak and activation of JNK," *Toxicology and Applied Pharmacology*, vol. 203, no. 2, pp. 124–131, 2005.

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Research Article

Cryptotanshinone Reverses Reproductive and Metabolic Disturbances in PCOS Model Rats via Regulating the Expression of CYP17 and AR

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Objective. To explore the effect of Cryptotanshinone on reversing the reproductive and metabolic disturbances in polycystic ovary syndrome (PCOS) model rats and the possible regulatory mechanisms. *Methods*. PCOS model rats were induced by subcutaneous injection of dehydroepiandrosterone (DHEA) and verified by histological screening of vaginal exfoliated cells. After Cryptotanshinone intervention, the rats' body weight and ovary morphological were observed; the serum biochemical assessments were analyzed by radioimmunoassay (RIA) and key genes and proteins related with anabolism of androgen and insulin were detected by Real-Time PCR and Immunohistochemical (IHC). *Results*. The estrous cyclicity of PCOS model rats was significantly recovered by Cryptotanshinone. The body weight, ovarian coefficient, and ovarian morphology had been improved and the serum biochemical indicators including testosterone (T), androstenedione (A2), luteinizing hormone (LH), LH/follicle stimulating hormone (FSH), sexual binding globulin (SHBG), low density cholesterol (LDL-C), fasting insulin (FINS) were reversed after Cryptotanshinone intervention. Specifically, the levels of Cytochrome P450, 17-a hydroxylase/17,20 lyase (CYP17), and androgen receptor (AR) were downregulated significantly. *Conclusions*. Our data suggest that Cryptotanshinone could rebalance reproductive and metabolic disturbances in PCOS model rats and could be a potential therapeutic agent for the treatment of PCOS.

1. Introduction

Polycystic ovary syndrome (PCOS), a complex genetic condition, is a highly prevalent heterogeneous syndrome of clinical and/or biochemical hyperandrogenism, oligoanovulation, and polycystic ovaries (PCO), excluding other endocrinopathies according to the 2003 Rotterdam criteria [1, 2]. Women with PCOS are at increased risk of reproductive abnormalities, and two-thirds of them also have metabolic dysfunction and, thereby, have an increased risk of developing type 2 diabetes mellitus (T2DM) and cardiovascular disease (CVD) [3]. So the therapy of reversing the high level of androgen and improving the insulin sensitivity is significant for PCOS patients.

Cryptotanshinone, traditionally known as tanshinone, was originally isolated from the dried roots of *Salvia miltiorrhiza* Bunge [4]. In traditional Chinese medicine, Cryptotanshinone has been widely prescribed for several pathologies, including acne, cardiovascular, and some endocrine metabolic diseases such as diabetes [5]. Our study made efforts to explore the effects and mechanisms whereby Cryptotanshinone ameliorates androgen excess and insulin resistance in a PCOS rat model induced by DHEA.

2. Materials and Methods

2.1. Animals. SPF grade female Wistar rats (age 21 d; body wt 35–40 g; $n = 50$) were purchased from Shanghai Slack laboratory animal co., LTD (license: SCXK(HU)2007-0003) and raised in Second Military Medical University Animal Center, 25°C constant temperature (Humidity 50%), 12 h light : 12 h dark cyclical alternates. All procedures described here were reviewed and approved by the Ethical Committee of Second Military Medical University.

2.2. Drugs. Cryptotanshinone (purity 99%) was purchased from Shanghai Ziyi biological technology co., LTD. (batch number: E - 0024); DHEA (purity ≥ 99%) was purchased from Sigma (batch number: D4000); Sesame oil (for injection) was purchased from Sigma (batch number: S3547).

2.3. Rat Model. The PCOS rat model, according to Anderson et al. [6, 7], was induced by subcutaneous injection of DHEA and verified by histological screening of vaginal exfoliated cells. Specifically, on the age of 23 d, 40 rats (as the DHEA-ed group) were injected subcutaneously with DHEA 6 mg/kg/day (dissolved into 0.2 mL sesame oil), while the other 10 (as the oil-ed group) were injected subcutaneously with sesame oil 0.2 mL/day. Both of the two groups were administrated for 20 days; then the test of vaginal exfoliated cytology was taken for 10 continuously days and the successful PCOS model rats were selected. The oil-ed group rats have the normal estrous cycle.

2.4. Drug Intervention. At age 53 d, 24 successful PCOS model rats chosen from the DHEA-ed group, randomly, were divided into two groups: one was Model group ($n = 12$) and the other was Drug group ($n = 12$). The 10 normal rats (the oil-ed group ones) were referred to as Control group ($n = 10$); both Model group rats and Control group rats were arranged to orally receive the following vehicle: normal saline (dose: 0.01 mL/body wt(g)/day), while Drug group rats orally received Cryptotanshinone (dissolved in normal saline, dose: 0.027 mg/body wt(g)/day) [8]. All rats of the three groups were treated for 4 weeks between 9:00 and 10:00 A.M everyday.

2.5. RIA Analysis. Biochemical assessments of rat serum weredetected by RIA analysis. The abdominal aortic blood (about 4 mL) was obtained from the experimental rats anaesthetized by 3.5% chloral hydrate (350 mg/kg body mass). After being left for 2 h at room temperature, the whole blood was centrifuged for 15 min (2500 g) and the serum was separated and stored in −20°C refrigerator before test. At last, the serum levels of T, A2, estradiol (E2), LH, FSH, SHBG, triglycerides (TG), total cholesterol (TC), high density cholesterol (HLD-C), LDL-C, fasting plasma glucose (FPG), and FINS were detected by RIA kits. This part of the test experiment was coperformed with Shanghai Audi kang Biotechnology co., LTD. China.

Table 1: The primer sequences of target genes.

Target gene	Primer sequences
Rat β-actin	F5′-CACAATGCTGGGACACAAAC-3′
	R5′-TGGCGTGAGCAGTTTATCAG-3′
Rat 3β-hsd	F5′-GTGTGCCAGCCTTCATCTAC-3′
	R5′-GGAGCAGTGGTGATGTATGG-3′
Rat cyp17	F5′-ATCAGGCCGGTGGCTCCCAT-3′
	R5′-TCGGGGACCAGCTCCGAAGG-3′
Rat cyp19	F5′-CCATCTGGTCTCCTGCTAG-3′
	R5′-CCACTTACCCTCAACACACA-3′
Rat ar	F5′-CGTCGCTCCTGGGAGGTCCA-3′
	R5′-CTGCTGCCAGAGCAGCCCAG-3′
Rat igf-1	F5′-ACATCTCTTCTACCTGGCACTCT-3′
	R5′-AAGCAACACTCATCCACAATG-3′
Rat gdf-9	F5′-AGCTCAAATGGGACAAACTGGAT-3′
	R5′-GGGACAGTCCCCTTTACACTACCT-3′

2.6. Light Microscope Analysis. Bilateral ovarian tissues of rats were surgically detached and the wet weight values were obtained from electronic scales. One side of the ovarian tissues was immediately stored in liquid nitrogen; the other side was quickly fixed in 10% Formalin for 24 h and then dehydrated in increasing concentrations of ethanol, followed by immersion in xylene and embedding in paraffin wax. The paraffin-embedded ovary sections (4 μm) were stained with hematoxylin and eosin and analyzed under a conventional light microscope. In addition, 3 fields of each ovary section were selected randomly and the number of follicles (including follicles at all levels) was counted. Two investigators, blinded to the sections' origin, independently analyzed the sections under a conventional microscope, took the available pictures, and calculated the number of follicles.

2.7. Real-Time PCR Analysis. Six ovarian tissues from each group preserved in liquid nitrogen were randomly selected for Real-Time PCR analysis. Total RNA was isolated from ovarian tissues using Trizol reagent and following the manufacturer's instructions. Total RNA (3 μg) was reverse transcribed with Reverse Transcription kit (Tatar, USA) as described by the manufacturer. The resulting cDNA was diluted 10-fold in sterile water and aliquots were subjected to Real-Time PCR. PCR primer pairs for the analysis were designed and synthetized by Invitrogen biological technology co., LTD, Shanghai, China (Table 1). Finally, the 3β-hsd, cyp17, cyp19, ar, igf-1, and gdf-9 mRNA levels were, respectively, analyzed by quantitative PCR instrument (ROTORGENE6000, Carbett.). The relative expression of each target gene compared to β-actin was calculated using the $2^{\Delta\Delta Ct}$ method.

2.8. IHC Analysis. Immunohistochemistry method was performed using a two-step EnVision/HRP technique (Dako Cytomation, Denmark) according to the manufacturer's instruction as the description of protocol referenced to the publication [9]. The proteins included 3β-hydroxy steroid

Table 2: Antibodies used in this research.

Antibody	Host species	Source
3β-HSD	Rabbit	Santa Cruz
CYP17	Goat	Santa Cruz
CYP19	Rabbit	Santa Cruz
AR	Rabbit	Santa Cruz
IGF-1	Rabbit	Santa Cruz
GDF-9	Goat	Santa Cruz
Goat anti-rabbit IgG, Rabbit anti-goat IgG		Santa Cruz

Table 3: The success rate of PCOS rat model induced by DHEA.

Method	Total number	Disorder number	Success rate
Subcutaneous injection of sesame oil	10	0	0.00%
Subcutaneous injection of DHEA	40	25	62.50%

Table 4: The recovery rate of Cryptotanshinone intervention.

Group	Total number	Recovery number	Recovery rate
Model group	12	1	8.33%
Drug group	12	7	58.33%*

Drug group versus Model group: $^*P < 0.05$.

dehydrogenase (3β-HSD), CYP17, Cytochrome P450 aromatase (CYP19), AR, insulin-like growth factors (IGF-1), and growth differentiation factor-9 (GDF-9). Table 2 lists the antibodies used in this research. Two investigators assessed degree of immunostaining by blinded examination (Table 2).

2.9. Statistical Analysis. Statistical evaluations were analyzed using the Statistical Package for the Social Sciences (SPSS version 16.0). Values are expressed as means ± SD. Independent t-tests assessed differences and the one-way ANOVA was used to analyze the differences among groups. $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. Estrous Cyclicity. According to the predominant cell type in vaginal smears obtained daily (10 consecutive days) determined by microscopic analysis, the estrous cyclicity of rats can be observed. After being injected with DHEA for 20 days, the estrous cyclicity of 25 rats was in disorder in DHEA-ed group (40 rats in all), and the success rate of PCOS rat model induced by DHEA was 62.50%. In addition, the disordered estrous cycle of 7 rats in Drug group (12 rats in all) was reversed after Cryptotanshinone intervention for 28 days and the recovery rate was 58.33% which was statistically significant comparing with the natural recovery rate of Model group 8.33% (Tables 3 and 4).

3.2. Body Weight and Ovaries Quotiety. From the age of 53 d, the rats of each group were weighed as a frequency of once

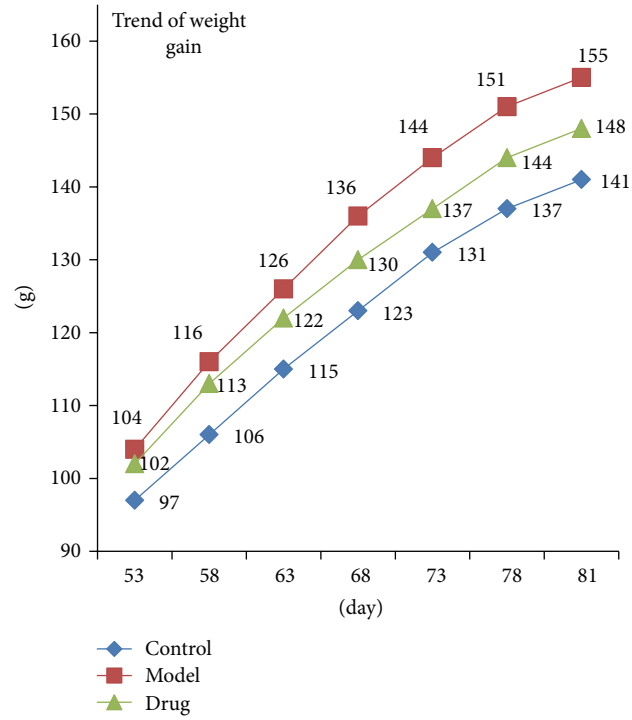


Figure 1: Trends of the weight gain in three groups. The blue, red, and green lines represent the Control group, Model group, and Drug group, respectively. As is shown above, a gain in weight value of Drug group had been slowed down compared to the Model group rats.

in five daysuntil the end of the drug intervention; the average weight value was calculated and the trend of the weight gains was observed (Figure 1).

At the age of 91 d, all rats were fasting but water for 12 h and then weighed by electronic scale and compared between groups. The average body weight value of Model group rats (169.22 ± 13.25 g) was significantly higher than that of both the Control group rats (152.36 ± 11.84 g) and the Drug group rats (158.57 ± 12.47 g). In addition, Ovaries Quotiety = ovarian weight/body weight (mg/100 g). According to the results of statistical data, the average Ovaries Quotiety of Model group rats [(28.08 ± 3.48 mg/100 g)] was significantly increased compared with the Control group ones [(22.40 ± 3.15 mg/100 g)]; while compared with the Model group, the average Ovaries Quotiety of the Drug group was statistically decreased [(23.90 ± 3.61 mg/100 g)] (Figure 2).

3.3. Biochemical Assessments. After detection by RIA, there were significant differences in serum level of T, A2, E2, LH, LH/FSH, SHBG, TC, LDH-C, HDL-C, and FINS between Control group and Model group ($P < 0.05$). To compare Model group with Drug group, there were obvious differences in serum concentration of T, A2, LH, LH/FSH, SHBG, LDH-C, and FINS, while the serum levels of E2, TC, and HDL-C were nonsignificant between the two groups. What is more, there were no statistical differences in serum level of FSH, TG, and FPG among the three groups (Table 5).

Table 5: Serum biochemical assessments by radioimmunoassay.

Radioimmunoassay index Group	Number	T (nmol/L)	A2 (nmol/L)	E2 (pmol/L)	LH (IU/L)	FSH (IU/L)	LH/FSH (IU/L)	SHBG (nmol/L)	TC (nmol/L)	TG (nmol/L)	LDH-C (μmol/L)	HDL-C (μmol/L)	FPG (mIU/L)	FINS (mIU/L)
Control group	10	0.76 ± 0.07	9.24 ± 2.34	88.76 ± 5.23	28.21 ± 3.82	12.42 ± 1.92	2.27 ± 0.13	40.52 ± 3.76	7.22 ± 1.37	1.00 ± 0.22	32.04 ± 7.56	81.68 ± 15.17	5.36 ± 0.09	5.57 ± 1.01
Model group	12	1.03 ± 0.14*	16.02 ± 6.31*	126.73 ± 17.08*	32.82 ± 1.22*	10.36 ± 2.25	3.17 ± 0.31*	28.52 ± 5.37*	8.63 ± 2.50*	1.89 ± 0.34	55.95 ± 9.85*	140.50 ± 19.91*	6.19 ± 0.17	13.79 ± 3.74*
Drug group	12	0.90 ± 0.11$^{\triangle}$	12.31 ± 2.38$^{\triangle}$	121.46 ± 9.56	29.04 ± 5.11$^{\triangle}$	10.57 ± 2.41	2.75 ± 0.18$^{\triangle}$	35.64 ± 4.82$^{\triangle}$	7.68 ± 1.00	2.05 ± 0.44	49.89 ± 7.18$^{\triangle}$	162.32 ± 30.51	6.25 ± 0.21	9.95 ± 1.68$^{\triangle}$

Model group versus Control group: $^{*}P < 0.05$; Drug group versus Model group: $^{\triangle}P < 0.05$.

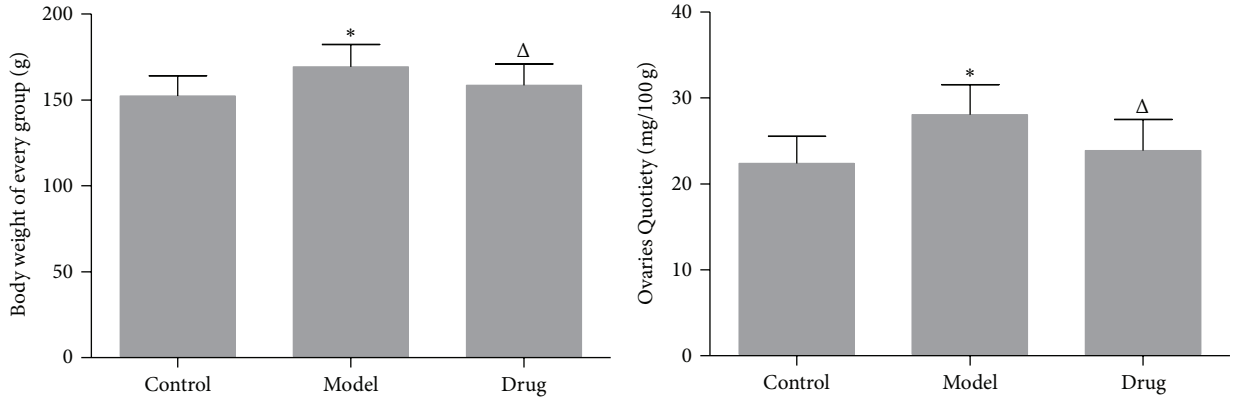


FIGURE 2: The comparison of body weight value and Ovaries Quotiety among the three groups; Model group ($n = 12$) versus Control group ($n = 10$): $^{*}P < 0.05$; Drug group ($n = 12$) versus Model group ($n = 12$): $^{\Delta}P < 0.05$.

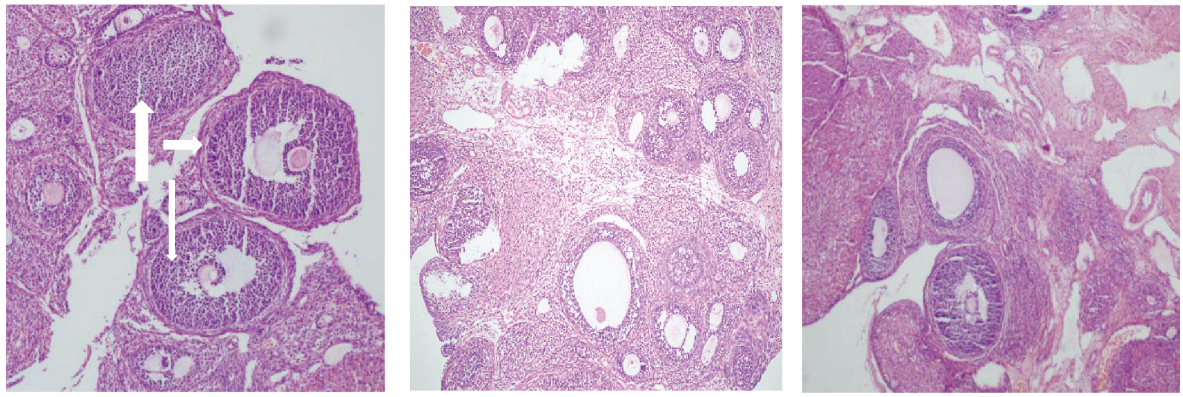


FIGURE 3: Pathological morphology of ovarian tissues analyzed by light microscope. As is shown above, there were significant differences in color, the numbers of follicles (short thick arrows) and corpora lutea (long thick arrow), and the numbers of granulosa cell layer (long thin arrows) not only between the Model group and the Control group but also between the Drug group and the Model group.

3.4. Pathological Morphology. Light microscope analysis showed no structural abnormalities in Control group rats: the ovarian tissue was pink, follicles (the number was 5 ± 2/field) and corpora lutea were in varying stages of development, and granulosa cell layers were normal (the number of granulosa cell layer was 6–8). Differences in Model group rats, however, were significant: the color of the ovarian tissue was lighter as an overall observation; the number of cystic follicles (a large fluid-filled cyst, the number was 15 ± 4/field) increased and the granulosa cell layers were abnormal (the number. of granulosa cell layer was 2–4 or even none); besides, the number of corpora lutea dropped sharply. After Cryptotanshinone intervention, the ovarian pathological morphology of Drug group was greatly reversed, the color of the ovarian tissue and the number of granulosa cell layer had a certain degree of recovery, and the number of cystic follicles (the number was 8 ± 3/field) decreased while the number of corpus luteums increased (Figure 3).

3.5. Genes and Proteins Expression. The levels of key genes and proteins related to anabolism of androgen and insulin were detected by Real-Time PCR and IHC. The expression of cyp17, cyp19, ar, and igf-1 in Model group was significantly higher than that of the Control group; after Cryptotanshinone intervention, the expression of cyp17 and ar was reversed; however, the level of cyp19 and igf-1 was nonsignificant. The level of 3β-hsd and gdf-9 had no statistical differences according to the analysis of Real-Time PCR. Besides, as IHC analyzed, except the fact that the protein CYP17 and AR had been downregulated significantly, it seemed that Cryptotanshinone had little effect on reversing the expression of protein CYP19 and IGF-1 increased in Model group, while the protein 3β-HSD and GDF-9 had almost no significant differences among groups, all of which were consistent with the results of Real-Time PCR (Figures 4, 5, and 6).

4. Discussion

Cryptotanshinone, a kind of fat-soluble 2 terpenoids material [10], has been isolated from the herb of *Salvia miltiorrhiza* and identified as the major chemical constituent [11].

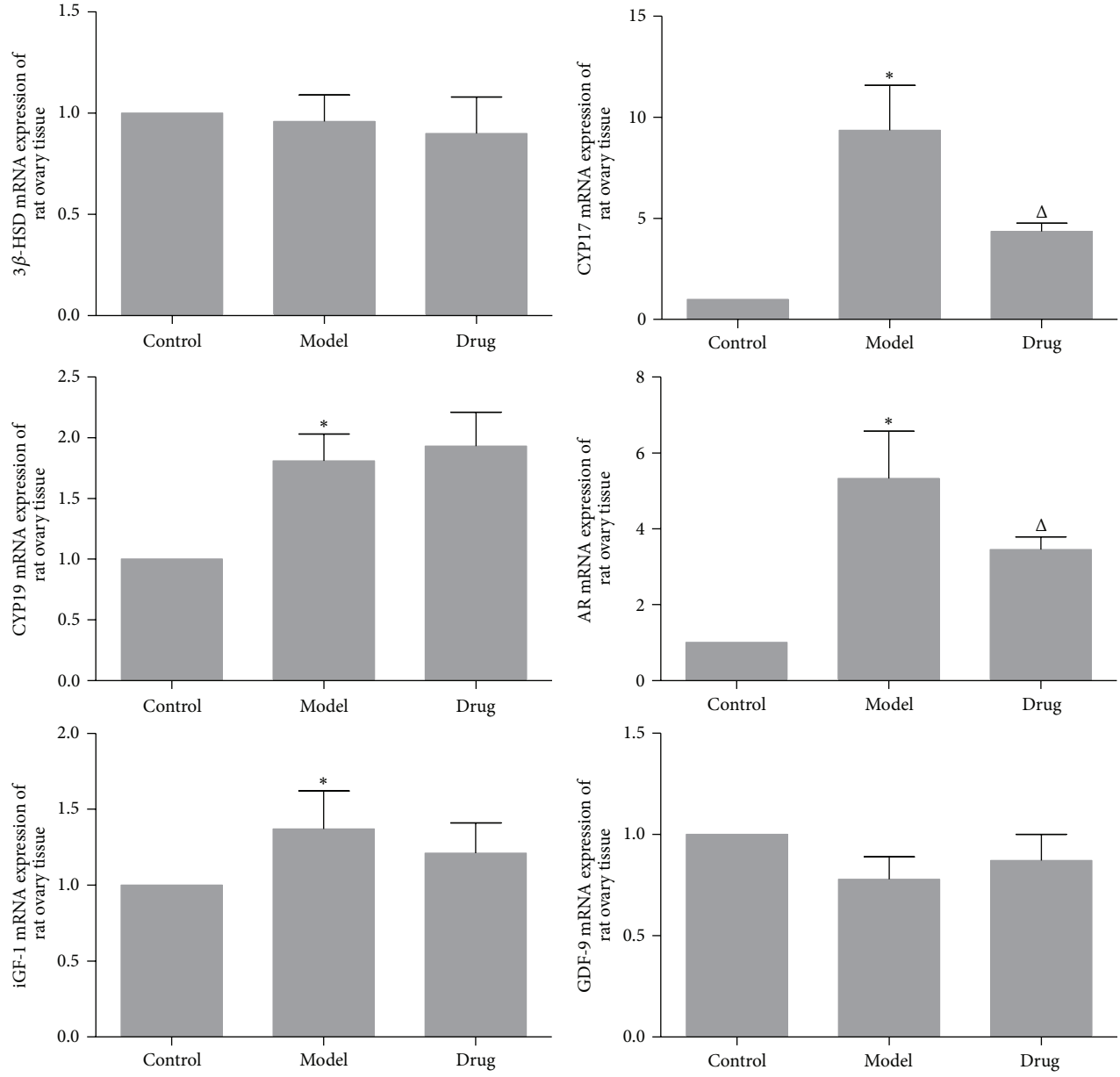


Figure 4: The genes expression of ovarian tissues analyzed by Real-Time PCR. As is shown above, there were no obvious differences between 3β-hsd mRNA and gdf-9 mRNA among the three groups. The expression of cyp17 mRNA (9.35 ± 2.23), cyp19 mRNA (1.81 ± 0.22), ar mRNA (5.33 ± 1.24), and igf-1 mRNA (1.37 ± 0.25) in Model group was increased significantly than in the Control group (1.00 ± 0.00). After drug intervention, the expression of cyp17 mRNA (4.36 ± 0.39) and ar mRNA (3.45 ± 0.34) was statistically decreased, while cyp19 mRNA and igf-1 mRNA had no statistical differences (cyp19 mRNA: 1.93 ± 0.28; igf-1 mRNA: 1.21 ± 0.20). Model group ($n = 6$) versus Control group ($n = 6$): $^{*}P < 0.05$; Drug group ($n = 6$) versus Model group ($n = 6$): $^{\Delta}P < 0.05$.

Its molecular formula is $C_{19}H_{20}O_3$, the Mol. wt. is 296.35, and the chemical structural is as in Figure 7.

Modern pharmacological studies have shown that Cryptotanshinone has the effects of antibacterial, anti-inflammatory, antiobesity, and antidiabetic activity [12, 13] and has been prescribed for several diseases, including coronary heart disease (CHD), angina pectoris, and myocardial damage. Recently, some scholars have focused their interests on researching the relationship between Cryptotanshinone and some endocrine metabolic diseases. For instance, Yang et al. [8] reported that Cryptotanshinone could reverse the disturbances in prenatally androgenized rats; Qi et al. [14] discovered that Cryptotanshinone could improve insulin resistance of pig ovarian theca cell cultured in vitro.

In our experiment, we successfully induced the PCOS rat model by subcutaneous injection DHEA, a kind of exogenous androgen. The rat model, in general, recapitulated the reproductive and metabolic features of human PCOS, including the irregular estrous cycles, increased body weight,

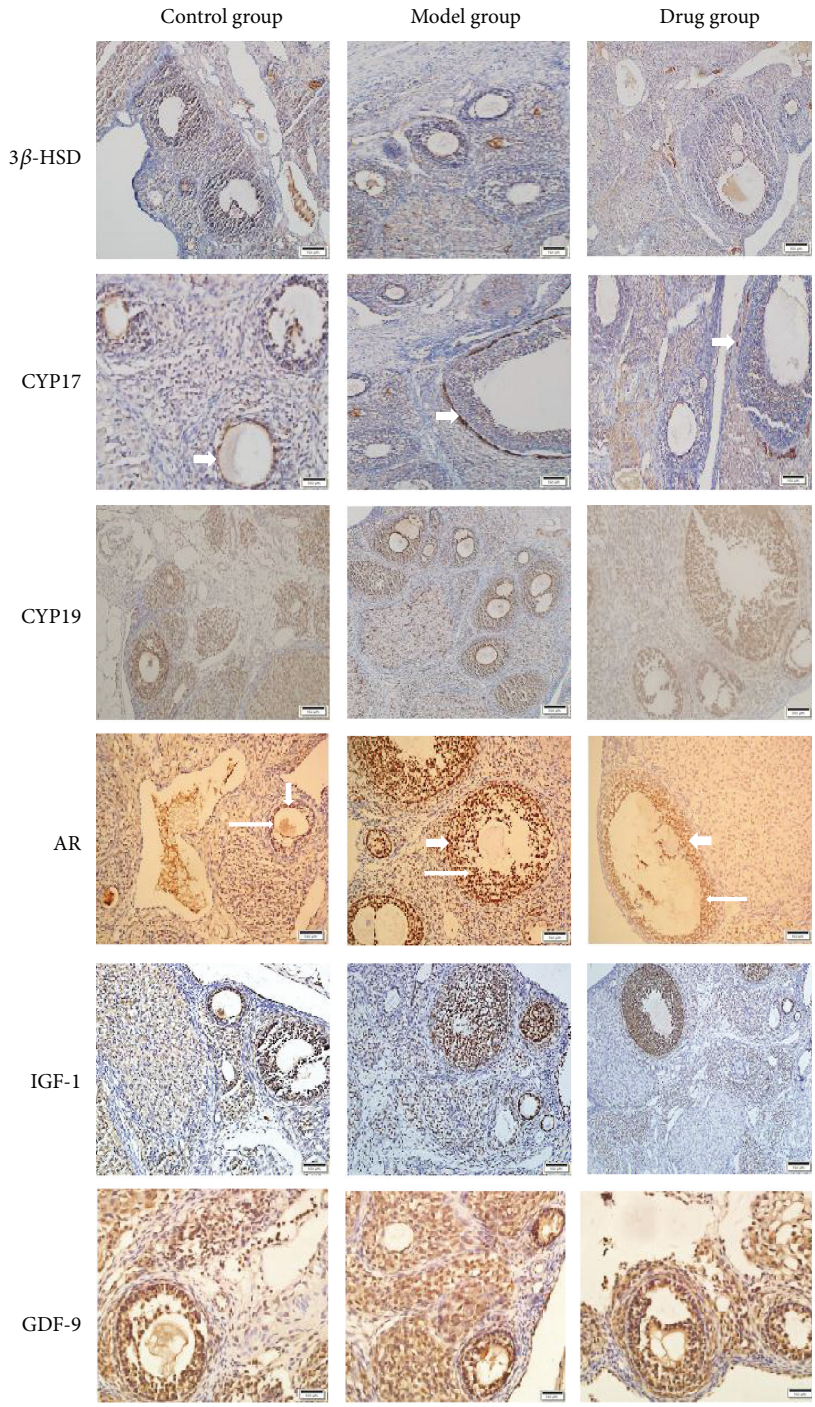


Figure 5: Immunohistochemical staining of 3β-HSD, CYP17, CYP19, AR, IGF-1, and GDF-9 in three groups. As is shown above, CYP17 was primarily localized in theca cells (short thick arrows), AR was primarily expressed both in theca cells (short thick arrows) and in granulosa cells (long thin arrows), while the expressions of other proteins were not obvious.

and ovarian coefficient and the abnormal serum biochemical level of T, A2, E2, LH, LH/FSH, SHBG, TC, LDH-C, HDL-C, and FINS. After Cryptotanshinone intervention, the estrous cyclicity of model rats was obviously restored. Besides, the body weight, ovarian coefficient, and ovarian morphology of PCOS model rats had been improved. Even more noteworthy is that the serum biochemical indicators including T, A2, LH, LH/FSH, SHBG, LDH-C, and FINS had been reversed significantly.

Ovary is one of the sources of androgen and insulin anabolic in the human body. In PCOS ovarian tissue, theca cells and granule cells abnormally express mRNA for key genes involved in anabolic of androgen and insulin, including 3β-hsd, cyp17, cyp19, ar, igf-1, and gdp-9. Our data showed

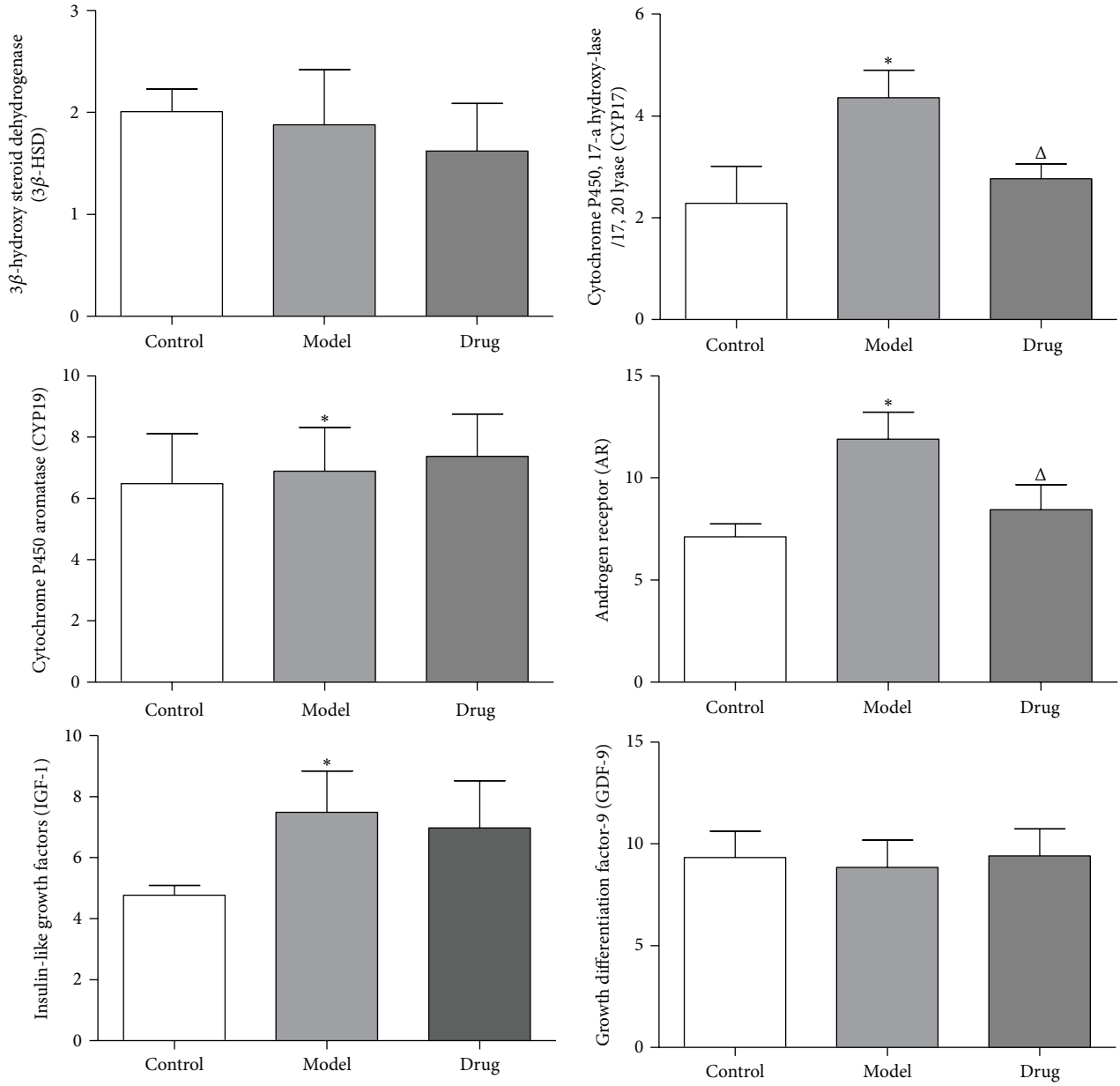
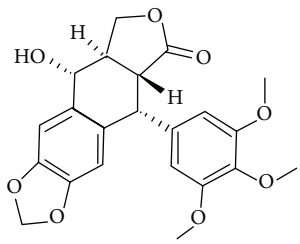


Figure 6: The protein expression of ovarian tissues analyzed by IHC. As is shown in the histogram above, except 3β-HSD and GDF-9, the expression of CYP17, CYP19, AR, and IGF-1 significantly increased in Model group than that of Control group, while in Drug group only CYP17 and AR were decreased according to statistics. Model group ($n = 6$) versus Control group ($n = 6$): $^{*}P < 0.05$; Drug group versus Model group ($n = 6$): $^{\Delta}P < 0.05$.



C19H20O3 296.35

Figure 7

that the levels of cyp17 and ar were obviously downregulated by Cryptotanshinone; accordingly, the protein levels of these two genes were also downregulated as detected by IHC.

The possible regulatory mechanisms of Cryptotanshinone on reversing the reproductive and metabolic disturbances in PCOS model rats might be as follows: large amount of exogenous DHEA prompts the pituitary gland sensitive to gonadotrophin-releasing hormone (GnRH) and increases the excessive secretion of LH; the increased GnRH/LH can

enhance the activity of cytochrome P450 and 17-α hydroxylase/17,20 lyase (coded by gene cyp17), one of the key enzymes in theca cells, and result in the synthetic of A2 and T increasing [15]. The excessive androgen in ovarian, on one hand, inhibits the development of follicle and makes it difficult to form a dominant follicle and at last leads to the state of polycystic ovary sample (PCO) [16]; On the other hand, small follicles in the ovaries can secrete E2, the equivalent of the early follicles, and A2 can be converted into estrone (E1) by cytochrome P450 aromatase (coded by gene cyp19) outside the tissues. Sustained release of E1 and a certain level of E2 effect on the hypothalamus and pituitary [17] make a positive feedback on LH secretion, further increase the amplitude and frequency of LH secretion, and make the ratio of LH/FSH inversed. Cryptotanshinone can directly and/or indirectly reduce the levels of A2 and T probably by inhibiting the expression CYP17 and reducing the biosynthesis of androgen. In addition, our test also found that the level of AR was downregulated by Cryptotanshinone, thereby reducing the role of androgen by limiting the expression of AR.

Modern scientific researches widely believe that PCOS is a kind of metabolic disorder; most of PCOS patients suffer from insulin resistance and lipid metabolism dysfunction. Hyperinsulinemia of PCOS could directly impact the hypothalamus or pituitary gland and raise the level of LH and therefore indirectly enhance the secretion of androgen mediated by LH [18]. At the same time, hyperinsulinemia could suppress the synthesis of SHBG in liver and cause the increasing of free testosterone (FT) [19]. At present, it is believed that the level of SHBG in peripheral blood can reflect the degree of insulin resistance [18]. In our research, we found the serum biochemical indices of SHBG and FINS significantly regulated after Cryptotanshinone intervention. Besides, the level of igf-1 had a downward trend in Cryptotanshinone group, and although it had no statistical significance, we cannot exclude the situation that Cryptotanshinone enhanced insulin sensitivity and improved insulin resistance by inhibiting the expression of IGF-1. In addition, some reports showed that androgen can strengthen lipoprotein lipase (LPL) catabolism [20] and insulin has an upregulated effect on LDL-C receptor activity [21]. Interestingly, the serum biochemical level of LDL-C was also descended after Cryptotanshinone intervention in our research.

5. Conclusions

Cryptotanshinone can reverse the reproductive and metabolic disturbances in PCOS model rats via downregulating the expression of CYP17 and AR, and it may indirectly increase the insulin sensitivity. Therefore, it could be a potential therapeutic agent for the treatment of PCOS patients.

Conflict of Interests

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

Authors' Contribution

Dongxia Zhai was responsible for the surgery to rats in the experimental process. Danying Zhang was responsible for the statistics. Lingling Bai was responsible for analyzing the photos under microscope.

Acknowledgments

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References

[1] B. C. J. M. Fauser, "Revised 2003 consensus on diagnostic criteria and long-term health risks related to polycystic ovary syndrome," *Fertility and Sterility*, vol. 81, no. 1, pp. 19–25, 2004.

[2] S. M. Sirmans and K. A. Pate, "Epidemiology, diagnosis, and management of polycystic ovary syndrome," *Clinical Epidemiology*, vol. 6, pp. 1–13, 2014.

[3] M. O. Goodarzi, D. A. Dumesic, G. Chazenbalk, and R. Azziz, "Polycystic ovary syndrome: etiology, pathogenesis and diagnosis," *Nature Reviews Endocrinology*, vol. 7, no. 4, pp. 219–231, 2011.

[4] X.-Y. Ji, B. K.-H. Tan, and Y.-Z. Zhu, "Salvia miltiorrhiza and ischemic diseases," *Acta Pharmacologica Sinica*, vol. 21, no. 12, pp. 1089–1094, 2000.

[5] X. Wang, S. L. Morris-Natschke, and K.-H. Lee, "New developments in the chemistry and biology of the bioactive constituents of Tanshen," *Medicinal Research Reviews*, vol. 27, no. 1, pp. 133–148, 2007.

[6] E. Anderson, G. Y. Lee, and K. Obrein, "Polycystic ovarian condition in the dehydroepiandrosterone-treated rat model: hyperandrogenism and the resumption of meiosis are major initial events associated with cystogenesis of antral follicles," *The Anatomical Record*, vol. 249, no. 1, pp. 44–53, 1997.

[7] M. T. Lee, E. Anderson, and G. Y. Lee, "Changes in ovarian morphology and senlm hormones in the rat after treatment tll dehydrcepiandrosterone," *The Anatomical Record*, vol. 231, no. 2, pp. 185–192, 1991.

[8] X. Yang, Y. Zhang, X. Wu et al., "Cryptotanshinone reverses reproductive and metabolic disturbances in prenatally androgenized rats via regulation of ovarian signaling mechanisms and androgen synthesis," *American Journal of Physiology, Regulatory Integrative and Comparative Physiology*, vol. 300, no. 4, pp. R869–R875, 2011.

[9] M. He, C. Zuo, J. Wang et al., "Prognostic significance of the aggregative perivascular growth pattern of tumor cells in primary central nervous system diffuse large B-cell lymphoma," *Neuro-Oncology*, vol. 15, no. 6, pp. 727–734, 2013.

[10] J. M. Hur, J. S. Shim, H. J. Jung, and H. J. Kwon, "Cryptotanshinone but not tanshinone IIA inhibits angiogenesis in vitro," *Experimental and Molecular Medicine*, vol. 37, no. 2, pp. 133–137, 2005.

[11] L. Zhou, Z. Zuo, and M. S. S. Chow, "Danshen: an overview of its chemistry, pharmacology, pharmacokinetics, and clinical use," *Journal of Clinical Pharmacology*, vol. 45, no. 12, pp. 1345–1359, 2005.

[12] D.-Z. Jin, L.-L. Yin, X.-Q. Ji, and X.-Z. Zhu, "Cryptotanshinone inhibits cyclooxygenase-2 enzyme activity but not its expression," *European Journal of Pharmacology*, vol. 549, no. 1-3, pp. 166–172, 2006.

[13] J. K. Eun, S.-N. Jung, H. S. Kun et al., "Antidiabetes and antiobesity effect of cryptotanshinone via activation of AMP-activated protein kinase," *Molecular Pharmacology*, vol. 72, no. 1, pp. 62–72, 2007.

[14] B. Qi, J. X. Song, L. Yang et al., "Studies on effect of cryptotanshinone on insulin resistant ovarial granular cells by DNA microarray," *Technology Review*, vol. 27, pp. 39–40, 2009.

[15] The Practice Committee of the American Society for Reproductive Medicine, "The evaluation and treatment of androgen excess," *Fertility and Sterility*, vol. 86, no. 5, pp. S241–S247, 2006.

[16] H.-Y. Kuang, H. Shao, L.-H. Hou, and X.-K. Wu, "Effects of ginseng total saponins on nerve growth factor expression in rat with estradiol valerate-induced polycystic ovaries," *Journal of Chinese Integrative Medicine*, vol. 6, no. 7, pp. 723–728, 2008.

[17] J. Le, X. Xie, Z. Q. Lin et al., *Gynaecology*, vol. 5, Press of Renmin Weisheng, Beijing, China, 7th edition, 2009.

[18] J. E. Nestler, L. P. Powers, D. W. Matt et al., "A direct effect of hyperinsulinemia on serum sex hormone-binding globulin levels in obese women with the polycystic ovary syndrome," *Journal of Clinical Endocrinology and Metabolism*, vol. 72, no. 1, pp. 83–89, 1991.

[19] L.-N. Jia and X.-J. Wang, "Clinical observation on treatment of 43 women polycystic ovary syndrome based on syndrome differentiation," *Journal of Chinese Integrative Medicine*, vol. 4, no. 6, pp. 585–588, 2006.

[20] R. Azziz, R. S. Rittmaster, L. M. Fox, E. L. Bradley Jr., H. D. Potter, and L. R. Boots, "Role of the ovary in the adrenal androgen excess of hyperandrogenic women," *Fertility and Sterility*, vol. 69, no. 5, pp. 851–859, 1998.

[21] G. M. Reaven, Y.-D. I. Chen, J. Jeppesen, P. Maheux, and R. M. Krauss, "Insulin resistance and hyperinsulinemia in individuals with small, dense, low density lipoprotein particles," *The Journal of Clinical Investigation*, vol. 92, no. 1, pp. 141–146, 1993.

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Research Article

Inhibitory Effects of Gymnema (*Gymnema sylvestre*) Leaves on Tumour Promotion in Two-Stage Mouse Skin Carcinogenesis

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Ethanol extracts of gymnema (*Gymnema sylvestre*) leaves exhibited marked antitumour-promoting activity in an *in vivo* two-stage carcinogenesis test in mice using 7,12-dimethylbenz[*a*]anthracene as an initiator and 12-*O*-tetradecanoylphorbol-13-acetate (TPA) as a promoter. From the active fraction of the ethanol extract of the gymnema leaves, three triterpenoids were isolated and identified. These compounds were evaluated for their inhibitory effects on TPA-induced inflammation (1 μg/ear) in mice. The tested compounds showed marked anti-inflammatory effects, with a 50% inhibitory dose of 50–555 nmol/ear.

1. Introduction

The chemoprevention of cancer is an urgent priority in the field of public health. A method of prevention that acts at the promotion stage of carcinogenesis is most desirable, as such a method could be applied even after exposure to tumour-promotion agents, which in many cases is unavoidable in daily life. Many tumour promoters have inflammatory activity [1]. A correlation between the inhibitory effects against 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced ear inflammation and inhibition of TPA-induced tumour promotion in a two-stage carcinogenesis experiment was observed in mice [2]. To screen for new inhibitors as chemopreventive agents, we intentionally selected natural compounds.

In the course of our studies on bioactive components from natural medicines, we found that the ethanol extracts of gymnema (*Gymnema sylvestre*; Asclepiadaceae) leaves showed inhibitory effects on TPA-induced inflammatory ear oedema in mice. Gymnema is a perennial herbaceous plant native to southern and central India and Sri Lanka. Gymnema has been used externally for diabetes [3, 4]. In chemical studies of gymnema leaves, the isolation and structural determination of flavonoids [5, 6], triterpenes [7, 8], and triterpene saponins [9–22] have been reported. With regard to biological activities, antiarthritic action [23] and inhibition of lipid absorption [24, 25] have been reported.

In the present study, ethanol extracts of gymnema leaves were found to inhibit TPA-induced tumour promotion during two-stage carcinogenesis in mouse skin. Three triterpenoids were isolated from ethanol extracts of gymnema leaves for inhibitory activity against TPA-induced inflammatory ear oedema in mice. The 50% inhibitory doses of these compounds for TPA-induced inflammatory ear oedema were 55–555 nmol/ear. Of the total assayed triterpenoids, 28-acetyl-21-tigloylgymnemagenin (**3**) showed similar activity as hydrocortisone, a steroidal anti-inflammatory drug, and gymnemagenin (**2**) and gymnemic acid III (**4**) showed greater suppression than indomethacin, a nonsteroidal anti-inflammatory drug.

2. Materials and Methods

2.1. General Experimental Procedures. ^{1}H- and ^{13}C-NMR spectra were measured with a JEOL LA-600 (^{1}H, 600 MHz; ^{13}C, 150 MHz) spectrometer, and chemical shifts are presented as values relative to tetramethylsilane as an internal standard. Mass spectra were measured with a JEOL JMS-GC mate spectrometer at an ionization voltage of 70 eV. HPLC

was performed on a C_{18} silica column (Cosmosil Cholester 10 id × 250 mm, Kyoto, Japan).

2.2. Chemicals. TPA was purchased from Chemicals for Cancer Research, Inc. (Eden Prairie, MN). 7,12-Dimethylbenz[*a*]anthracene, indomethacin, and hydrocortisone were obtained from Sigma Chemical Co. (St. Louis, MO). Acetone, chloroform, and methanol were obtained from Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan).

2.3. Materials. Ethanol extracts of gymnema (*Gymnema sylvestre* R. Br.) leaves were obtained from Tokiwa Phytochemical Institute in April 2008. Voucher specimens "SM0802" were deposited at the Laboratory of Self Medication, School of Pharmacy, Nihon University.

2.4. Extraction and Isolation. Ethanol extracts (50 g) of gymnema leaves were partitioned within EtOAc-H_2O (1 : 1) to yield an EtOAc extract (9.83 g). EtOAc extracts were partitioned within *n*-hexane-MeOH-H_2O (19 : 19 : 2), which afforded *n*-hexane (685 mg) and MeOH-H_2O (9.10 g) extracts, respectively. The H_2O solution was partitioned within *n*-BuOH-H_2O (1 : 1), yielding an *n*-BuOH extract (19.5 g) and an H_2O extract (20.2 g), respectively.

MeOH-H_2O extracts (9 g) were subjected to column chromatography (CC) on Silica gel 60 (E.Merck, Germany) using $CHCl_3$-MeOH (100 : 0 → 0 : 100) to obtain five fractions: fraction 1 (81.6 mg), fraction 2 (758 mg), fraction 3 (1.14 g), fraction 4 (4.09 g), and fraction 5 (2.84 g). Fraction 3 (1.0 g) was further separated on ODS 75 C_{-18} (Nacalai Tesque, Kyoto, Japan) using MeOH : H_2O (20 : 80 → 100 : 0) to obtain nine fractions: 3-1 (77.4 mg), 3-2 (15.9 mg), 3-3 (58.8 mg), 3-4 (2.57 mg), 3-5 (24.1 mg), 3-6 (7.48 mg), 3-7 (18.1 mg), 3-8 (31.8 mg), and 3-9 (699 mg). Fraction 3-3 (50.0 mg) was purified by reversed-phase preparative HPLC to isolate **1** (5.3 mg). Fractions 3-5 (20.0 mg) and 3-7 (13.0 mg) were treated by the same method to isolate **2** (5.5 mg) and **3** (7.6 mg), respectively. Fraction 4 (4.0 g) was separated on ODS 75 C_{-18} using MeOH-H_2O (50 : 50 → 100 : 0) to obtain three fractions: 4-1 (215 mg), 4-2 (2.67 g), and 4-3 (1.09 g). Fraction 4-2 (2.5 g) was separated by Silica gel 60 using $CHCl_3$-MeOH : H_2O (90 : 10 : 1 → 0 : 100 : 0) to obtain four fractions: 4-2-1 (388 mg), 4-2-2 (255 mg), 4-2-3 (641 mg), and 4-2-4 (1.03 g). Fraction 4-2-1 was then purified by HPLC to isolate **4** (4.8 mg).

2.5. Identification. Identification of compounds **1**, **2**, and **4** was performed by spectral comparison with literature data (Figure 2). Compound **1** was identified as phenethyl *β*-D-glucoside [26]. Compounds **2** and **4** were identified as gymnemagenin (**2**) and gymnemic acid III (**4**) [14]. Compound **3**, a pale yellow amorphous powder, $[\alpha]_D$ + 37.8° (*c* = 1.0, MeOH), possessed the molecular formula $C_{37}H_{58}O_8$, HR-FAB-MS (positive mode): *m/z* 631.42124 $[M + H]^+$ (calcd. 631.42097), suggesting that **3** was composed of **2**, acetic acid, and tiglic acid. By comparison of **3** with **2** on ^{13}C-NMR spectra, two acylation shifts were observed at the C-21 (position) [+1.9 ppm (C-21)] and the C-28 (position) [+4.1 ppm (C-28)] [17]. In addition, the chemical shifts of **3** accorded with that of the aglycone moiety of gymnemic acid I in ^{13}C-NMR spectral data [9, 13, 17]. These results suggest that **3** is 28-acetyl-21-tigloylgymnemagenin (**3**) (^{13}C-NMR δ (in pridine-d_5, 150 MHz): 171.2 (Ac-1), 168.4 (Tig-1), 141.7 (C-13), 137.1 (Tig-3), 129.9 (Tig-2), 124.3 (C-12), 79.2 (C-3), 73.7 (C-21), 72.0 (C-22), 68.3 (C-16), 67.9 (C-23), 62.7 (C-28), 48.9 (C-5), 47.6 (C-9), 46.2 (C-17), 46.2 (C-19), 43.3 (C-4), 43.1 (C-14), 42.9 (C-18), 40.6 (C-8), 39.4 (C-1), 37.4 (C-10), 37.0 (C-20), 36.7 (C-15), 33.0 (C-7), 29.7 (C-29), 28.0 (C-27), 27.8 (C-2), 24.4 (C-11), 21.1 (Ac-2), 20.1 (C-30), 18.8 (C-6), 17.5 (C-26), 16.5 (C-25), 14.5 (Tig-4), 13.4 (C-24), 12.8 (Tig-5)). Full details of the identification, as well as the spectral data, are available on request from the corresponding author.

2.6. Animals. Experiments were performed in accordance with the Guidelines of the Institutional Animal Care and Use Committee of the College of Pharmacy, Nihon University, Chiba, Japan. Female ICR mice (age: 7 weeks) were purchased from Japan SLC Inc. (Shizuoka, Japan) and were housed in an air-conditioned specific pathogen-free room (24 ± 2°C) lit from 08:00 to 20:00. Food and water were available *ad libitum*.

2.7. TPA-Induced Inflammation Assay in Mice. TPA (1 μg) dissolved in acetone (20 μL) was applied to the right ear of ICR mice by means of a micropipette. A volume of 10 μL was delivered to both the inner and outer surfaces of the ear. The sample (0.02–1.0 mg/ear) or vehicle, methanol-chloroform-water (2 : 1 : 1; 20 μL) or methanol-chloroform (1 : 1; 20 μL), as a control, was applied topically about 30 min before TPA treatment. For ear thickness determination, a pocket thickness gauge (Mitsutoyo Co. Ltd., Tokyo, Japan) with a range of 0–9 mm (graduated at 0.01-mm intervals and modified so that the contact surface area was increased, thus reducing tension) was applied to the tip of the ear.

Ear thickness was determined before TPA treatment (*a*).

Oedema was measured at 6 h after TPA treatment (*b*: TPA with vehicle; *b*′: TPA with sample). The following values were then calculated:

Oedema A: oedema induced by TPA with vehicle (*b* – *a*).

Oedema B: oedema induced by TPA plus sample (*b*′ – *a*).

Inhibitory ratio (%) = [(oedema A – oedema B)/oedema A] × 100.

Each value was the mean of individual determinations from four mice.

2.8. Two-Stage Carcinogenesis Experiment. The backs of mice (age: 7 weeks) were shaved with electric clippers. Initiation was accomplished by a single topical application of 50 μg of DMBA. Promotion with 1 μg TPA, applied twice weekly, was started 1 week after initiation. Ethanol extracts of gymnema leaves (1.0 mg/mouse) or vehicle, acetone-dimethylsulfoxide-water (8 : 1 : 1; 100 μL), was applied topically 30 min before each TPA treatment. DMBA and TPA were dissolved in

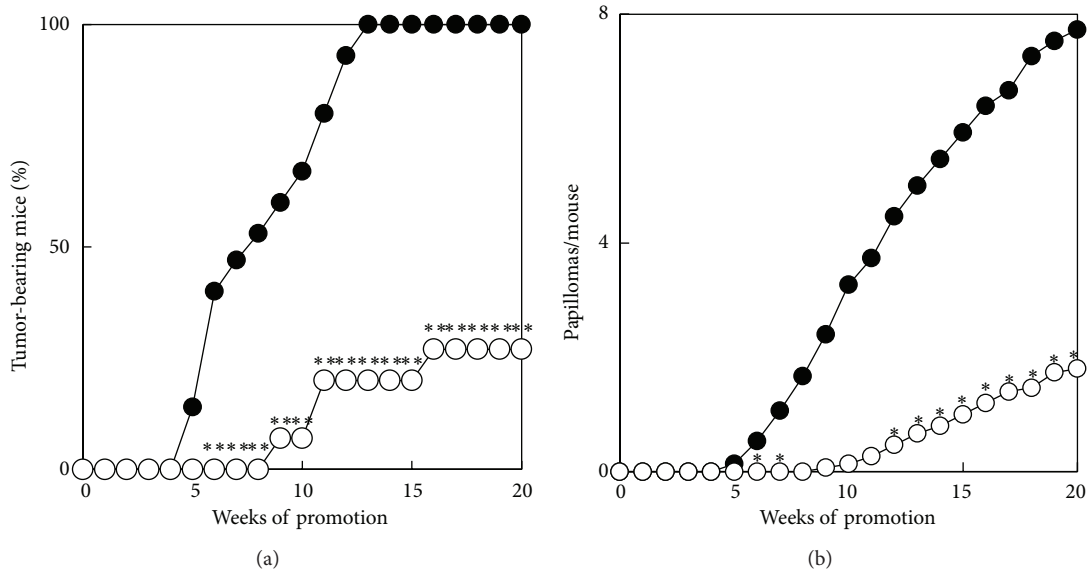

Figure 1: Inhibitory effects of ethanol extracts of gymnema leaves on tumour promotion of skin papillomas by TPA in DMBA-initiated mice. From 1 week after initiation with a single topical application of 50 μg of DMBA, 1 μg of TPA was applied twice weekly. Topical application of ethanol extract (1 mg) and vehicle was performed 30 min before each TPA treatment. Data are expressed as the percentage of mice bearing papillomas (a) and as the average number of papillomas per mouse (b). ●, +TPA with vehicle alone; ○, +TPA with ethanol extract of gymnema leaves. The treated group was determined to be statistically different from the control group by Mann-Whitney U exact test (a) and by Student's t-test (b). $^{*}P < 0.05$ and $^{**}P < 0.01$.

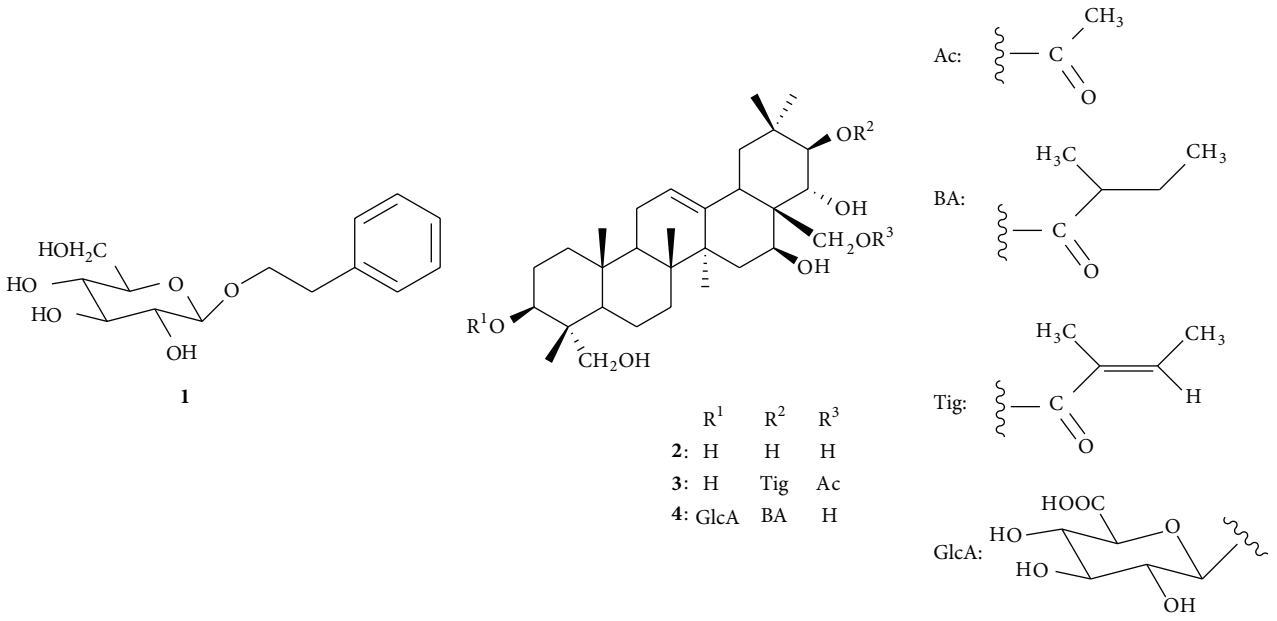

Figure 2: Chemical structures of components from gymnema leaves.

acetone and applied to the shaved area in a volume of 100 μL using a micropipette. The back of each animal was shaved once a week to remove hair. The number and diameter of skin tumours were measured every week, and the experiment was continued for 20 weeks. Experimental and control groups each consisted of 15 mice.

2.9. Statistical Analysis. The 50% inhibitory dose (ID_{50}) values and their 95% confidence intervals (95% CI) were obtained by nonlinear regression using the GraphPad program 5.0 (Intuitive Software for Science, San Diego, CA). Differences between experimental groups were compared by Student's t-test and Mann-Whitney U exact test.

Table 1: Inhibitory effects of gymnema leaves on TPA-induced inflammatory ear oedema.

Sample	I.R.
EtOH extract (1.0 mg/ear)	82**
n-Hexane extract of EtOH extract (1.0 mg/ear)	75**
MeOH-H_2O extract of EtOH extract (1.0 mg/ear)	89**
n-BuOH extract of EtOH extract (1.0 mg/ear)	43**
H_2O extract of EtOH extract (1.0 mg/ear)	9
Fraction 1 from MeOH-H_2O extract of EtOH extract (0.5 mg/ear)	29*
Fraction 2 from MeOH-H_2O extract of EtOH extract (0.5 mg/ear)	45**
Fraction 3 from MeOH-H_2O extract of EtOH extract (0.5 mg/ear)	62**
Fraction 4 from MeOH-H_2O extract of EtOH extract (0.5 mg/ear)	89**
Fraction 5 from MeOH-H_2O extract of EtOH extract (0.5 mg/ear)	41**

I.R.: inhibitory ratio. $^{*}P < 0.05$ and $^{**}P < 0.01$.

3. Results and Discussion

As can be seen in Table 1, extracts from gymnema leaves inhibited TPA-induced inflammation in mice. The inhibitory effects of the ethanol extract of gymnema leaves in a two-stage carcinogenesis test on mouse skin using DMBA as an initiator and TPA as a tumour promoter were then investigated. Figure 1(a) illustrates the time course of skin tumour formation in the groups treated with DMBA plus TPA, with or without the ethanol extract of gymnema leaves. The first tumour appeared at week 5 in the group treated with DMBA plus TPA and all 15 mice had tumours at week 13. In the group treated with DMBA plus TPA and ethanol extract of gymnema leaves, the first tumour appeared at week 9. The percentage of tumour-bearing mice treated with DMBA plus TPA and ethanol extract of gymnema leaves was 27% at week 20. Figure 1(b) shows the average number of tumours per mouse. The group treated with DMBA plus TPA produced 7.7 tumours per mouse at week 20; the group treated with DMBA plus TPA and ethanol extract of gymnema leaves had 1.8 tumours per mouse. Treatment with ethanol extract of gymnema leaves caused a 77% reduction in the average number of tumours per mouse at week 20.

Active components were then isolated from the ethanol extract of gymnema leaves. The isolated compounds showed inhibitory activity against TPA-induced ear inflammatory oedema. As can be seen in Table 2, the ID_{50} values of **2**–**4** against TPA-induced inflammation were 49.7–555 nmol/ear, respectively.

This is the first report to find that ethanol extracts of gymnema leaves inhibit tumour promotion by TPA following initiation with DMBA in ICR mouse skin. Furthermore, the active constituents were identified as three triterpenes from ethanol extracts of gymnema leaves. This is the first report of phenethyl glucoside in the genus *Gymnema* and the first report of 28-acetyl-21-tigyolgymnemagenin in nature.

Table 2: Inhibitory effects of components from gymnema leaves on TPA-induced inflammatory ear oedema.

Component	ID50 (nmol/ear)	95% CI (nmol/ear)
Gymnemagenin (**2**)	555	417–739
28-Acetyl-21-tigloylgymnemagenin (**3**)	49.7	38.3–64.7
Gymnemic acid III (**4**)	212	160–281
Indomethacin[a]	908	755–1092
Hydrocortisone[a]	69.1	64.3–75.4

ID_{50}: 50% inhibitory dose. 95% CI: 95% confidence intervals. [a]Reference compound.

These results demonstrate the efficacy to two triterpenes and triterpene glycosides in the components of gymnema leaves. In our study, we reported that numerous triterpenes and their glycosides are effective for preventing cancer [27, 28]. Therefore, we inferred that triterpenes and their glycosides in gymnema leaves are the active components.

Conflict of Interests

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

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References

[1] H. Fujiki, M. Mori, M. Nakayasu, M. Terada, and T. Sugimura, "A possible naturally occurring tumor promoter, teleocidin B from *Streptomyces*," *Biochemical and Biophysical Research Communications*, vol. 90, no. 3, pp. 976–983, 1979.

[2] K. Yasukawa, M. Takido, M. Takeuchi, and S. Nakagawa, "Effect of chemical constituents from plants on 12-*O*-tetradecanoylphorbol-13-acetate-induced inflammation in mice," *Chemical and Pharmaceutical Bulletin*, vol. 37, no. 4, pp. 1071–1072, 1989.

[3] G. Y. Yeh, D. M. Eisenberg, T. J. Kaptchuk, and R. S. Phillips, "Systematic review of herbs and dietary supplements for glycemic control in diabetes," *Diabetes Care*, vol. 26, no. 4, pp. 1277–1294, 2003.

[4] R. Nahas and M. Moher, "Complementary and alternative medicine for the treatment of type 2 diabetes," *Canadian Family Physician*, vol. 55, no. 6, pp. 591–596, 2009.

[5] B. Mukhopadhyay and R. A. Field, "Convergent synthesis of a trisaccharide as its 2-(trimethylsilyl)ethyl glycoside related to the flavonoid triglycoside from *Gymnema sylvestre*," *Carbohydrate Research*, vol. 341, no. 10, pp. 1697–1701, 2006.

[6] X. Liu, W. Ye, B. Yu, S. Zhao, H. Wu, and C. Che, "Two new flavonol glycosides from *Gymnema sylvestre* and *Euphorbia*

ebracteolata," *Carbohydrate Research*, vol. 339, no. 4, pp. 891–895, 2004.

[7] W.-C. Ye, X. Liu, S.-X. Zhao, and C.-T. Che, "Triterpenes from *Gymnema sylvestre* growing in China," *Biochemical Systematics and Ecology*, vol. 29, no. 11, pp. 1193–1195, 2001.

[8] A. Zarrelli, M. DellaGreca, A. Ladhari, R. Haouala, and L. Previtera, "New triterpenes from *Gymnema sylvestre*," *Helvetica Chimica Acta*, vol. 96, no. 6, pp. 1036–1045, 2013.

[9] K. Yoshikawa, K. Amimoto, S. Arihara, and K. Matsuura, "Structure studies of new antisweet constituents from *Gymnema sylvestre*," *Tetrahedron Letters*, vol. 30, no. 9, pp. 1103–1106, 1989.

[10] K. Yoshikawa, K. Amimoto, S. Arihara, and K. Matsuura, "Gymnemic acid V, VI and VII from gur-ma, the leaves of *Gymnema sylvestre* R. Br," *Chemical and Pharmaceutical Bulletin*, vol. 37, no. 3, pp. 852–854, 1989.

[11] F. Kiuchi, H.-M. Liu, and Y. Tsuda, "Two new gymnemic acid congeners containing a hexulopyranoside moiety," *Chemical and Pharmaceutical Bulletin*, vol. 38, no. 8, pp. 2326–2328, 1990.

[12] K. Yoshikawa, S. Arihara, K. Matsuura, and T. Miyaset, "Dammarane saponins from *Gymnema sylvestre*," *Phytochemistry*, vol. 31, no. 1, pp. 237–241, 1992.

[13] K. Yoshikawa, M. Nakagawa, R. Yamamoto, S. Arihara, and K. Matsuura, "Antisweet natural products. V. Structures of gymnemic acids VIII–XII from *Gymnema sylvestre* R. Br," *Chemical and Pharmaceutical Bulletin*, vol. 40, no. 7, pp. 1779–1782, 1992.

[14] K. Yoshikawa, Y. Kondo, S. Arihara, and K. Matsuura, "Antisweet natural products. IX. Structures of gymnemic acids XV–XVIII from *Gymnema sylvestre* R. Br. V," *Chemical and Pharmaceutical Bulletin*, vol. 41, no. 10, pp. 1730–1732, 1993.

[15] H.-M. Liu, F. Kiuchi, and Y. Tsuda, "Isolation and structure elucidation of gymnemic acids, antisweet principles of *Gymnema sylvestre*," *Chemical and Pharmaceutical Bulletin*, vol. 40, no. 6, pp. 1366–1375, 1992.

[16] N. Murakami, T. Murakami, M. Kadoya, H. Matsuda, J. Yamahara, and M. Yoshikawa, "New hypoglycemic constituents in "gymnemic acid" from *Gymnema sylvestre*," *Chemical and Pharmaceutical Bulletin*, vol. 44, no. 2, pp. 469–471, 1996.

[17] M. Yoshikawa, T. Murakami, M. Kadoya et al., "Medicinal foodstuffs. IX. The inhibitors of glucose absorption from the leaves of *Gymnema sylvestre* R. BR. (Asclepiadaceae): structures of gymnemosides a and b," *Chemical and Pharmaceutical Bulletin*, vol. 45, no. 10, pp. 1671–1676, 1997.

[18] M. Yoshikawa, T. Murakami, and H. Matsuda, "Medicinal foodstuffs. X. Structures of new triterpene glycosides, gymnemosides-c, -d, -e, and -f, from the leaves of *Gymnema sylvestre* R. BR.: influence of gymnema glycosides on glucose uptake in rat small intestinal fragments," *Chemical and Pharmaceutical Bulletin*, vol. 45, no. 12, pp. 2034–2038, 1997.

[19] N. P. Sahu, S. B. Mahato, S. K. Sarkar, and G. Poddar, "Triterpenoid saponins from *Gymnema sylvestre*," *Phytochemistry*, vol. 41, no. 4, pp. 1181–1185, 1996.

[20] Y. Sugihara, H. Nojima, H. Matsuda, T. Murakami, M. Yoshikawa, and I. Kimura, "Antihyperglycemic effects of gymnemic acid IV, a compound derived from *Gymnema sylvestre* leaves in streptozotocin-diabetic mice," *Journal of Asian Natural Products Research*, vol. 2, no. 4, pp. 321–327, 2000.

[21] W.-C. Ye, Q.-W. Zhang, X. Liu, C.-T. Che, and S.-X. Zhao, "Oleanane saponins from *Gymnema sylvestre*," *Phytochemistry*, vol. 53, no. 8, pp. 893–899, 2000.

[22] X.-M. Zhu, P. Xie, Y.-T. Di, S.-L. Peng, L.-S. Ding, and M.-K. Wang, "Two new triterpenoid saponins from *Gymnema sylvestre*," *Journal of Integrative Plant Biology*, vol. 50, no. 5, pp. 589–592, 2008.

[23] J. K. Malik, F. V. Manvi, B. R. Nanjware, D. K. Dwivedi, P. Purohit, and S. Chouhan, "Anti-arthritic activity of leaves of *Gymnema sylvestre* R. Br. Leaves in rats," *Der Pharmcia Letters*, vol. 2, no. 1, pp. 336–341, 2010.

[24] N. Shigematsu, R. Asano, M. Shimosaka, and M. Okazaki, "Effect of long term-administration with *Gymnema sylvestre* R. BR on plasma and liver lipid in rats," *Biological and Pharmaceutical Bulletin*, vol. 24, no. 6, pp. 643–649, 2001.

[25] N. Shigematsu, R. Asano, M. Shimosaka, and M. Okazaki, "Effect of administration with the extract of *Gymnema sylvestre* R. Br leaves on lipid metabolism in rats," *Biological and Pharmaceutical Bulletin*, vol. 24, no. 6, pp. 713–717, 2001.

[26] K. Kurashima, M. Fujii, Y. Ida, and H. Akita, "Simple synthesis of β-D-glycopyranosides using β-glycosidase from almonds," *Chemical and Pharmaceutical Bulletin*, vol. 52, no. 2, pp. 270–275, 2004.

[27] K. Yasukawa, "Cancer chemopreventive agents: natural pentacyclic triterpenoids," in *Pentacyclic Triterpenes as Promising Agents in Cancer*, J. A. R. Salvador, Ed., pp. 127–157, Nova Science Publishers, New York, NY, USA, 2010.

[28] K. Yasukawa, "Cancer chemopreventive agents: tetracyclic triterpenoids," in *Horizons in Cancer Research*, H. S. Watanabe, Ed., vol. 51, pp. 89–114, Nova Science Publishers, New York, NY, USA, 2013.

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Review Article

Impact of Chinese Herbal Medicine on American Society and Health Care System: Perspective and Concern

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Many Americans, not completely satisfied with traditional western medicine, have turned to alternative and complementary medicine which explains the increasing popularity of the herbal products and the Chinese herbal medicine. The lack of government regulations and the increasing advertisements by the manufactures have created an impression to the common public that the natural herbal remedies are inherently safer and cheaper than conventional medicine. The skyrocketing rise of healthcare cost and the adverse reaction and side effects incurred from the prescribed drugs have both reinforced such an impression. Herbs in the USA and in many European countries have been prepared as capsules, tablets, teas, lozenges, juice extracts, tincture, and ointments. Most of the herbs are administered as a single herb in the USA and Europe. However, the traditional Chinese herbal medicine contains multiple active ingredients from various herbs and is prepared as concoctions by simmering them for hours to produce pharma-therapeutic properties useful for the treatment of a particular disease. Those prepared concoctions are taken gingerly with specific treatment purposes. In the USA and some European counties, herbs are distributed and labeled as dietary supplements and are taken by many individuals for a long period of time creating some medical and dental complex problems among them, especially in terms of anesthesia-surgery complications. This paper provides insight into basic differences in how herbs are prepared before administration to the patients in China versus a single unprepared herb sold in the USA and Europe. Also addressed are the interdisciplinary issues with health professionals, the proper regulations for better quality control of imported herbs, and the proper warning on the labels of the herbs.

1. Impact of Chinese Herbal Medicine in the United States

The World Health Organization estimates that 80% of the world population uses herbal medicine [1]. Many Americans, not satisfied with western medicine, have turned to alternative and complementary medicine resulting in increasing popularity of herbal products and traditional Chinese medicine, especially acupuncture and Chinese herbal medicine. Traditional Chinese medicine (TCM) includes 5 branches, namely, acupuncture and Moxibustion, Chinese herbology (commonly known as Chinese herbal medicine), Qigong healing, Tuina therapeutic massage, and Chinese dietary therapy. This paper will focus on the impact of Chinese herbology (Chinese herbal medicine) on the American society and the health care system.

In America and Europe, traditional folk medicine uses many herbs as in Chinese herbal medicine (CHM), but they are often handled and prepared differently with different therapeutic results. There are very few sources of information in the USA about CHM, although many American herbs have the similar properties as their Chinese counterparts. While herbs are considered drugs in China, herbs are consumed in the USA as nutritional or food supplements without necessary warning labels on containers. We provide a background of CHM and information on herbal preparation.

2. Background Information

Generally speaking, traditional Chinese medicine offers an alternative to the potentially toxic effects of western medication, since acupuncture does not inject any drug into the

body and many Chinese herbs are natural products, tend to be milder, and can be combined with western medicines. For example, Chinese herbal medicines improve and augment the effectiveness of western medications in the treatment of liver cirrhosis, arthritis, and cancer, thereby decreasing the dosage and adverse side effects of western chemotherapies [2–4]. Since 1974, when former president Nixon's third exchange delegation to China covered the subject of herbal pharmacology, much information had been shared by eastern and western scientists. Western research into CHM has appealed to both Chinese and western medical establishments [5], but concerns remain. One concern is that in China, few regulations have been applied in herb producing areas or plants and some merchants ignore existing regulations, resulting in quality control problems [6].

Since herbs are widely available in pharmacies throughout the USA, many people assume pharmacists are aware of drug-herb interactions and appropriate warnings, but this is not true. There is also the problem of improper self-administration of herbs by patients who do not inform their physicians, dentists, or pharmacists and many American clinicians lack sufficient knowledge of Chinese herbs to advise patients [7, 8].

3. Current Status of Chinese Herbal Medicine in American Society

Because therapeutic effects of Chinese herbs have been exaggerated or distorted, herbs have often been misused. For example, ma huang has been used in China for several thousand years as an antiasthmatic medication and researchers subsequently extracted from it the alkaloid ephedrine. In the USA, ephedrine has been illegally processed into a stimulant "designer drug" (ecstasy) with ma huang as a major ingredient. Hospitalization and deaths have occurred after mixing ecstasy with liquor, heroin, and sometimes cocaine [5].

To avoid government regulation many American manufacturers and distributors have no cautions or warnings on labels. For example, Ginseng (Panax Chinese or Korean) has a good therapeutic effect with cardiovascular benefits especially for convalescent patients, and inadequate warnings have allowed indiscriminative usage and incidents of Ginseng Abuse Syndrome (GAS). GAS is manifested by diarrhea, skin lesions, CNS stimulation, and interference with homeostasis. Ginseng also potentiates the antiplatelet effect of Coumadin, aspirin, and NSAIDs (although okay with COX-2 sedative) and taking these drugs with ginseng may pose surgical risks. Siberian ginseng may elevate the blood pressure and enhance irritability with long term use, although it is known to enhance t-lymphocyte activity and may improve antibiotic effectiveness [7].

Ginkgo biloba often combined with Herba Ephedrae (mahuang), Pruni Armeniacae, and others for cough and wheezing. This herb alone is known in China to be slightly toxic and not to be taken in large quantity or long term. But no such warning is labeled in the product information to the consumers in the USA. It is known to decrease platelet aggregation with the anticoagulant warfarin which could interfere with homeostasis. *Ginkgo biloba* will also react with narcotic analgesics causing hypotension [7, 8]. Dong quai (angelicae sinensis) [6], used in China for pain and trauma or "female problems," can also interfere with warfarin in high doses. It may potentiate skin cancer or raise blood glucose levels. People who are diabetic, have heart problems, or are taking anticoagulants should not take dong quai. Another popular herb known as licorice root (*Glycyrrhiza glabra*) and its Chinese counterpart (*Glycyrrhiza uralensis*) contain salts of glycyrrhizic acid. This glycoside can intensify platelet aggregation thereby decreasing coumadin function [9]. In China, it is common knowledge that herbs cannot be taken continuously and certain foods must not be taken with certain herbs. From time and time, doctors of traditional Chinese medicine must be consulted for proper herbal dosage adjustment, to meet individual needs and provide necessary warnings. These precautions are not followed in the USA.

4. Chinese Method of Decoction Preparation

One main difference between western herbal medicine and CHM is that in the USA herbs are sold by having the herb ground into powder and encapsulated or by putting together the powder of several herbs into one capsule. But in CHM, herbs are often combined under certain preparation and treatment. CHM, through thousands of years of experimentation and clinical testing, had developed sophisticated and complex methods of herbal preparation. Their therapeutic effects are often achieved through the synergistic and combined actions of different compounds. In addition, certain minerals and animal parts are sometimes used in these concoctions. Organic compounds of high complexity tend to decompose to lower weight compounds upon exposure to heat (during boiling preparations of herbs) and these compounds may react with one another to form other compounds. Molecular properties such as water solubility, molecular affinity between compounds, and the length and type of molecular bonding play a major role in how they react. Under such complex pharmacodynamic processes with various decompositions, reactions, and preparative conditions, it is likely that the end products may have entirely different pharmacologic properties than did the original herbal ingredients. However, under FDA labeling guidelines, original ingredients are listed and this may confuse consumers and pharmacists [2].

Throughout the history of CHM, there have been different ways in which medicinal substances can be combined. Combination classifications are based upon disease pattern symptom complex, etiology, form of application (e.g., external applied or internally ingested), type of usage (gynecology, pediatrics, ophthalmology, etc.), organ, treatment strategy, and composites of the above. The art of the combining is by putting together two or more medicinal substances so as to promote therapeutic effectiveness, to minimize toxicity or side effects, to accommodate complex clinical situations, and to alter the actions of the substances. Chinese herb medicines are usually prepared as decoctions (tang, literally soups) the most common form in which CHM is taken in China, but in the USA there is resistance to using decoctions.

Traditionally, certain combinations of herbs were avoided because they either reduce each other's effectiveness or lead to toxicity or undesirable side effects. There are reported cases of mutual antagonism and cases of mutual incompatibility in the Chinese Classic of the Materia Medica. For example, Radix Ginseng antagonizes the herb Excrementum Trogopterori seu Pteromi which is used for gynecological disorders with energy deficiency and blood stasis. Also cortex cinnamomi cassine (cinnamon bark) antagonize halloysitum rubrum (kaolin) that is used for chronic dysenteric diarrhea, whereas radix glycyrrhizae uralensis (licorice root) is compatible with radix euphorbiae sue knoxie (Japanese thistle) and seaweed. There are also certain foods to be avoided by patients taking certain medical substances and, in general, patients taking herbs should avoid cold, greasy, or other hard to digest foods [10].

5. Methods of Herbal Detoxification

Since the early nineteenth century, scientific research has attempted to understand the actions and properties of CHM herbal substances. It was also during this time that most modern drugs were developed. Many herbs sold in the USA are harvested and ground from the original plant form without any TCM processing or preparation. Without the processing to remove toxicity or undesirable side effects, such products could be toxic. In CHM, there are several processing methods to achieve the desired medicinal substances from herbal plants. For example, using alcohol to process *Angelicae Sinensis* to extract its volatile oils and treating Rhizoma Corydalis Yanhusuo with salt allow its alkaloids to dissolve in water. Radix Rehmanniae Glutinosae (sheng di huang, the dried form of the herb) is cooling in nature and can be cooked in wine and then dried to become Radix Rehmanniae Glutinosae Conquitae for usage as a tonic. Other methods to prepare herbs include dry frying, browning, frying with honey or vinegar, baking, roasting, steaming, and boiling with water. Heating and boiling frequently denature the toxic proteinous parts of the herbs [10].

6. Discussion

In the last 50 years, tremendous amount of acupuncture research has been done in Europe, Asia, and the United States to unlock the mysteries of acupuncture. Those researches have helped the modern medicine to know more about how human body works. Whereas acupuncture is well received by both American medical establishments and the public alike, the attitude to herbal medicine is entirely a different matter.

The increased use of herbs as supplements is problematic as consumers often do not have understanding of the therapeutic effects of these herbs. In fact, some herbal supplement use is prevalent in the facial/cosmetic surgery population, especially in older female patients. Some herbs cause coagulopathy, hypertension, or dry eyes. Abstaining from herb usage two weeks before surgery is recommended; nearly half of patients reported taking herbs, which cause intraoperative bleeding [11]. In addition, in China, many of these herbs were not traditionally meant to be used therapeutically as a single unprepared product, and it is questionable whether they share the same effect as the preparations used in CHM concoction by mouth. Now the unconventional intravenous injection of herbal preparations bypassing the gastrointestinal tract has caused some allergic reaction in some patients, which is relatively uncommon if the properly prepared herbs are taken by mouth. In fact, a total of 109 varieties of Chinese medicine injections have been approved by the State Food and Drug Administration of China, all of which have the potential to induce adverse drug reactions that include systemic anaphylaxis, anaphylactic shock, acute intravascular hemolysis, hepatorenal damage, skin lesions, cardiac damage, respiratory distress, and GI disorders [12]. Furthermore, the tendency for consumers to use multiple unprepared herbs in conjunction with one another and in addition to prescription medications is problematic as most consumers and, in fact, most physicians and pharmacists are unaware of their potential drug interactions. These single unprepared products, if taken without professional monitoring, can pose potential risks to patients and those products can cause interdrug reactions with the prescribed medications the patients are taking (see Table 1). The lack of information concerning these cross-interactions and a general lack in training are a potentially significant health issue. This issue is perpetuated by the listing of these herbs as supplements, which do not require significant manufacturer quality control and limit the FDA's oversight concerning possible adverse effects and drug interactions between these herbs and various medications.

The status of herbal supplements as dietary supplements with little oversight is designed to increase the availability and accessibility of these supplements to the general public. This has increased the incorrect widespread use of Chinese herbs by mainstream consumers. However, unless proper precautions, studies, and oversight of the herbs are developed there lies the possibility that these drugs may become less utilized when the potential hazards of these herbs become more publicized.

Qualitative and quantitative testing of the active ingredients of traditional herbal preparations is also necessary for the effective utilization of Chinese herbal medicine. Lack of quality control is a major problem as unscrupulous manufacturers of herbal medicine have blended western medicine into CHM to exaggerate its therapeutic effect without labeling it as such. This is because pure CHM is milder and requires longer time to take effect than western medicines, which reach peak therapeutic effect faster but with more side effects than CHM. For example, acetylsalicylic acid has been added to antipyretic herbals and Butazolidin was added to arthritic herbals to boost their effect.

Herbal doping by athletes to improve performance is less of a problem in the United States than other countries due to stringent testing. Nevertheless, doping usually involves ginseng and in some cases musk pod from the musk deer. Ginseng is used as an adaptogenic throughout the world. In the United States, the use of ginseng is skeptically viewed as an athletic performance enhancer due to studies that have shown varying benefits in fatigued athletes. These results may reflect

Table 1: Some common unprepared Chinese herbs in the USA and their potential risks and interdrug reactions when taken without professional monitoring.

Herbal names	Botanic names	Medical uses	Potential risks
Aloe (other names: hsiang-dan, lu-hui)	*Aloe barbadensis/capensis/vera*	Depresses the action potential generation and conduction at neuromuscular junction processes, analgesic, and anti-inflammatory effects. Increases the collagen content of granulation. Tissue contributed to wound healing, sometimes used to treat AIDS, diabetes, asthma, stomach ulcers, immune weakness, evacuation relief, anal fissures after rectoanal surgery, fungal diseases, constipation, colic, and worm infestations.	Loss of electrolytes, potassium, this hypokalemic effect enhanced in conjunction with thiazide diuretics, loop diuretics, licorice, and corticosteroids (increase the action of cardiac glycosides and antiarrhythmic drugs).
Huang-Qi (other names: superior Chinese astragalus)	*Astragalus* species	Used for respiratory infections, immune depression, cancer, heart failure, viral infections, liver disease, and kidney disease. Hyperthyroidism, hypertension, insomnia, diabetes, genital herpes, AIDS, and the side effects of chemotherapy.	Bleeding when used with other anticoagulant, antiplatelet, or antithrombotic agents. It is incompatible with opiates.
Chinese rhubarb, da-huang	*Rheum palmatum*	Constipation, appetite stimulant, painful teething, delirium, edema, and diarrhea.	Electrolyte loss (especially potassium leading to hyperaldosteronism and enhanced effects of radioactive drugs). Long term use causes arrhythmias, nephropathies, and bond loss.
Dandelion (other names: lion's tooth, endive)	Taraxacam offinae/laevigatum	Dyspeptic conditions, urinary tract infections, liver and gallbladder, loss of appetite, fluid retention, constipation, rheumatism, and diabetes.	Not to be used with diuretics, antihypertensive agents and oral hypoglycemic, and mammal and lactating related problems.
Ephedra, ma huang (other names: natural ecstasy, fen-phen)	*Ephedra sinica*	Used as CNS stimulant for appetite suppressant, a nasal decongestant, bronchial asthma, joint symptoms, inability to perspire edema, and pain in the bones.	May alter effects of MAO inhibitors, ephedrine, B-blockers, phenothiazines, and Sudafed. Higher dosages result in blood pressure and cardiac rhythm disorders; it has an additive effect with caffeine and decongestants and heart rhythm disturbances when used with halothane.
Garlic (other names: da-suan)	*Allium sativum*	For elevated lipid levels, age-related vascular change and arteriosclerosis, inflammatory respiratory conditions, gastrointestinal ailments, diabetes, constipation, and joint pain.	Decrease in hematocrit values and plasma viscosity; concomitant use with Coumadin antiplatelet drugs such as aspirin and dipyridamole could increase the effect of bleeding. Risk of bleeding increased with ginkgo or high-dose vitamin E and may increase serum insulin levels.
Ginkgo, *Ginkgo biloba* (other names:xGinkgo)		For organic brain dysfunction intermittent claudicating, vertigo and tinnitus, improving concentration, asthma, hypertonic, erectile dysfunction, and angina pectoris.	Spontaneous bleeding due to potent inhibitory effect on platelet-activating factor; care when used with aspirin and other anticoagulant hypertension with thiazide diuretics.
Ginseng (other names: Chinese Red Panax)	*Panaxginseng/quinquefolius*	For fatigue and debility, concentration, loss of appetite, cachexia, anxiety, impotence and sterility, neuralgia, and insomnia.	Hypoglycemic effects, hypotension resulting with prolonged high-dose ginseng with caffeine, adverse effects with oral hypoglycemic and MAO inhibitors, concomitant use with aspirin, NSAIDs, heparin, and warfarin should be avoided.
Green tea (other names: Chinese matsu-cha)	*Camellia sinensis*		Vitamin K in green tea interferes with Coumadin, decreases the absorption of alkaline drugs.

Table 1: Continued.

Herbal names	Botanic names	Medical uses	Potential risks
Licorice	*Glycyrrhiza glabra*	For cough/bronchitis and gastritis, also used for appendicitis, constipation, increase milk production, micturition, gastric ulcers, headache, sore throat, spleen disorders, dehydration, and chronic fatigue syndrome.	Hypokalemia, hypernatremia, edema, hypertension, and cardiac complaints. Additive effect with furosemide and thiazide diuretics. Hypokalemic effects potentiate digitalis toxicity. Severe ventricular tachycardia of the torsade de pointes type resulted with the concomitant use of antiarrhythmic agents and may prolong the half-life of cortical increasing its effectiveness and its side effects.
Papaya	*Carica papaya*	For gastrointestinal digestive complaints, athletic injuries, and herniated vertebral disks.	Fibrinolytic effect, tendency to bleed due to interaction with warfarin and increased INR levels.
Prickly ash (other names: toothache tree, stanberry)	*Zanthoxylum americanum*	For toothache, intestinal gas, to promote circulation, and rheumatism.	Promote bleeding when used with aspirin or other blood thinners.
Turmeric (other names: Jiang huang)	*Curcuma longa*	Dyspeptic complaints and loss of appetite, also cancer, gallstones, intestinal gas osteoarthritis, and rheumatoid arthritis.	Alter the action of Coumadin, NSAIDs, and immune system suppressants.
Wild Yam (other names: China root)	*Dioscorea villosa*	For rheumatic conditions, gall bladder colic, dysmenorrheal, and cramps.	Decrease the anti-inflammatory effect of indomethacin, additive effect with estrogen.

that some commercially available ginseng products contain little to no active ingredient. Some ginseng products have even been shown to contain added anabolic steroids [13, 14].

The use of and demand for herbs as supplements continue to rise and when used as isolated products, it is questionable whether they have the same effects as CHM concoctions. The authors remain hopeful about the future of combining western and eastern medicines to attain their full potential for the benefit of patients. Regulatory agencies in the United States and China will need to cooperate. Continuing education courses and dialogue should be developed for pharmacists, physicians, dentists, and practitioners of traditional Chinese medicine to keep up with current research. Herbal medicine and drug interactions need to be thoroughly understood in order to safeguard public safety and health [15–17].

To address the issues of inadequate knowledge base and understanding of herbal medicine by health professionals, we recommend increased exposure to herbal medicine in the curriculums of medical, dental, pharmacy, and nursing schools. Increased sponsorship of clinical studies to study and address the comparative and acceptable uses of herbals would better clarify their safe and effective use in an objective format more easily accepted and understood by health care professionals. For qualified and interested health professionals, postcertification programs might be developed to unify fields of study, such as a dietary certificate with an emphasis on supplemental nutrition and herbal medicine.

Herbs are not patentable and knowledge acquired through research would soon become public domain. It is desirable that either a special division of the FDA or a separate regulating agency be created to regulate herbs (both CHM herbs and traditional herbs) used in the United States.

7. Perspectives and Conclusion

Herbal medicine and western medicine may be seen as totally separate distinctive entities and disciplines, but, in fact, both can be combined together for better results. They can be quite complementary to each other for synergizing the therapeutic effects. With increasing public awareness of herbal medicine, it is the hope of the authors that complementary and alternative medicines can be added to mainstream schools of health sciences by developing a view of the human body that includes oriental concepts. In addition, a public health campaign can be launched to educate about the merits and hazards of indiscriminate use of herbs.

Disclosure

Dominic P. Lu is the President of American Society for Advancement of Anesthesia and Sedation in Dentistry.

Conflict of Interests

There is no conflict of interests and the authors received no financial support or grant from any outside source.

References

[1] C. W. Fetrow and J. R. Avita, "Understanding and using herbal medicine," in *The Complete Guide to Herbal Medicine*, pp. 1–3, Simon & Schuster, New York, NY, USA, 2000.

[2] Y. He, A. Lu, Y. Zha et al., "Correlations between symptoms as assessed in traditional Chinese medicine (TCM) and ACR20 efficacy response: a comparison study in 396 patients with

rheumatoid arthritis treated with TCM or Western medicine," *Journal of Clinical Rheumatology*, vol. 13, no. 6, pp. 317–321, 2007.

[3] C.-Q. Zhao, H.-T. Gu, and Y. Cheng, "Applying Fuzheng Huayu Gantang comprehensive therapeutic program for treatment of post-hepatitis B liver cirrhosis complicated with glycometabolic abnormality," *Chinese Journal of Integrated Traditional and Western Medicine*, vol. 28, no. 1, pp. 24–27, 2008.

[4] Y. F. Yang, Z. X. Chen, and T. Xu, "Randomized controlled study on effect of Quxie Capsule on the median survival time and quality of life in patients with advanced colorectal carcinom," *Chinese Journal of Integrated Traditional and Western Medicine*, vol. 28, no. 2, pp. 111–114, 2008.

[5] W. I. Lu, D. P. Lu, G. P. Lu, and P. K. Lu, "Americanization of traditional Chinese medicine and some issues of medical concern," *American Journal of Traditional Chinese Medicine*, vol. 7, no. 2, pp. 64–70, 2006.

[6] E. Call, "Consensus conference on protecting medicinal plants and animals in oriental medicine," *American Journal of Traditional Chinese Medicine*, vol. 4, no. 1, pp. 44–47, 2003.

[7] K. Baker, "Impact of herbal medicine and supplements on clinical dentistry," Outlines of Lecture Presented at Lehigh Valley Hospital, Allentownm, Pa, USA, 2004.

[8] M. K. Ang-Lee, J. Moss, and C.-S. Yuan, "Herbal medicines and perioperative care," *The Journal of the American Medical Association*, vol. 286, no. 2, pp. 208–216, 2001.

[9] D. P. Lu, "Controversy over the dental patient taking anticoagulant," *Valley Forge Dental Journal*, pp. 13–15, 2001.

[10] D. Bensky and A. Gramble, *Chinese Herbal Medicine: Materia Medica*, Eastland Press, Seattle, Wash, USA, 1993.

[11] S. J. Swievel, M. Lee, B. Alleyne, and B. Guyuron, "The incidence of vitamin, mineral, herbal, and other supplement use in facial cosmetic patients," *Plastic and Reconstructive Surgery*, vol. 132, no. 1, pp. 78–82, 2013.

[12] T. Li, "Avoiding adverse drug reactions to Chinese medicine injections," *Journal of Evidence-Based Medicine*, vol. 3, no. 1, pp. 44–49, 2010.

[13] S. Talbott, "Ginseng—the root of improving athletic performance?" Competitor, 2013.

[14] G. Anand, "A special report on doping," *Athletics Journal*, 2012.

[15] M. T. Goupil, "Herbal therapies," in *Medical Emergencies in Density*, J. D. Bennett and M. B. Rosenberg, Eds., pp. 461–476, WB Saunders, Philadelphia, Pa, USA, 2002.

[16] R. Ko, "Adverse reactions to watch for in patients using herbal remedies," *Western Journal of Medicine*, vol. 171, no. 3, pp. 181–186, 1999.

[17] L. G. Miller, "Herbal medicinals," *Archives of Internal Medicine*, vol. 158, no. 20, pp. 2200–2211, 1998.

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Research Article

Anti-Inflammatory Effects of 81 Chinese Herb Extracts and Their Correlation with the Characteristics of Traditional Chinese Medicine

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Inducible nitrogen oxide synthase (iNOS) is the primary contributor of the overproduction of nitric oxide and its inhibitors have been actively sought as effective anti-inflammatory agents. In this study, we prepared 70% ethanol extracts from 81 Chinese herbs. These extracts were subsequently evaluated for their effect on nitrogen oxide (NO) production and cell growth in LPS/IFNγ-costimulated and unstimulated murine macrophage RAW264.7 cells by Griess reaction and MTT assay. Extracts of *Daphne genkwa* Sieb.et Zucc, *Caesalpinia sappan* L., *Iles pubescens* Hook.et Arn, *Forsythia suspensa* (Thunb.) Vahl, *Zingiber officinale* Rosc, *Inula japonica* Thunb., and *Ligusticum chuanxiong* Hort markedly inhibited NO production (inhibition > 90% at 100 μg/mL). Among active extracts (inhibition > 50% at 100 μg/mL), *Rubia cordifolia* L., *Glycyrrhiza glabra* L., *Iles pubescens* Hook.et Arn, *Nigella glandulifera* Freyn et Sint, *Pueraria lobata* (Willd.) Ohwi, and *Scutellaria barbata* D. Don displayed no cytotoxicity to unstimulated RAW246.7 cells while increasing the growth of LPS/IFNγ-costimulated cells. By analyzing the correlation between their activities and their Traditional Chinese Medicine (TCM) characteristics, herbs with pungent flavor displayed potent anti-inflammatory capability. Our study provides a series of potential anti-inflammatory herbs and suggests that herbs with pungent flavor are candidates of effective anti-inflammatory agents.

1. Introduction

Inflammation is a self-protection mechanism aiming at removing harmful stimuli, including damaged cells, irritants, or pathogens, and beginning the wound repair process. However, inflammation sometimes induces further inflammation, leading to self-perpetuating chronic inflammation that can cause severe cellular injury and tissue damage [1]. Chronic inflammation has been linked to a wide variety of diseases such as atherosclerosis [2], Alzheimer's disease [3], diabetes [4], and carcinogenesis [5–7].

Nitric oxide (NO), which is mainly generated by inducible nitric oxide synthase (iNOS) under the inflammatory conditions [8–10], plays a key role in each step of the pathological processes during inflammation [11–14]. Selective inhibitors of iNOS have been shown to be both anti-inflammatory and tissue-protective in various inflammatory animal models [15–17] and are thus regarded as promising agents for treating inflammatory diseases. High expression of iNOS can often be detected in human tumors, supporting the notion that chronic inflammation is actively involved in tumor progression [18–21]. In fact, nonsteroidal anti-inflammatory drugs (NSAIDs), including aspirin [22] and tolfenamic acid [23], are currently used for both cancer prevention and treatment [24].

A variety of natural products have been reported to possess anti-inflammatory and anticancer effects in experimental animal models. For example, curcumin has been shown to inhibit cyclooxygenase 2 (COX2) expression and is actually in clinical use as a chemoprevention agent [25–27]. Because of the promises in curcumin, extensive efforts have also been exerted to identify compounds capable of targeting inflammatory mediators [28–30]. A recent study by Liao et al. investigated the potential association between antioxidation capability and the characteristics of Traditional Chinese Medicine (TCM) in 45 commonly used Chinese

herbs, in which antioxidation capability of Chinese herbs was found to be correlated with their flavor characteristics [31]. Their findings are very encouraging because it indicates that effective anti-inflammatory agents may potentially be identified from Chinese herbs based on their TCM characteristics.

In our effort to identify effective anti-inflammatory agents, we prepared 70% ethanol extracts from 81 Chinese herbs and subsequently tested their abilities to suppress NO production in murine macrophage RAW264.7 cells costimulated with LPS and IFNγ. Moreover, we also analyzed the correlation between anti-inflammatory capacity and TCM characteristics among these herbs. We conclude that herbs with pungent flavor are the strongest in their anti-inflammatory capability.

2. Materials and Methods

2.1. Chemicals. IFNγ was purchased from EMD Millpore Chemicals (Billerica, MA, USA). Bovine serum albumin (BSA), lipopolysaccharide (LPS, *E. coli* 0111: B4), N-(1-naphthyl)-ethylenediamine dihydrochloride (L-NIL), 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium (MTT), naphthylethylenediamine, sulfanilamide, and sodium bicarbonate were all obtained from Sigma-Aldrich Co (St. Louis, MO, USA). RPMI 1640 and trypsin-EDTA were purchased from Life Technologies (Grand Island, NY, USA). Fetal bovine serum (FBS) was purchased from Hyclone Thermo Fisher Scientific (Waltham, MA, USA).

2.2. Preparation of 70% Ethanol Extracts of Chinese Herbs. All herbs were obtained from YANG He Tang and Kangqiao Co (Shanghai, China). All 81 herbs chosen for our study have been reported or suggested to have potential anti-inflammatory activities by either TCM literatures or current pharmacological reports. Botanical identification of these herbs was performed by Shanghai Institute for Food and Drug Control (SIFDC). To prepare ethanol extracts, 100 g of each dried herbs was sliced and extracted with 1 L of 70% ethanol at 80°C for three times. Obtained ethanol extracts were evaporated under reduced pressure at temperature 60°C and stored at −80°C. Extracts were dissolved with DMSO before use.

2.3. Measurement of Nitrite Production. RAW264.7 cells were plated in 96-well plates (5×10^3 cells per well) for overnight and then replenished with FBS-free medium for 10 h followed by adding 100 μg/mL herb extracts into each well. Cells were costimulated with 100 ng/mL LPS and 10 U/mL IFNγ for 24 h, and media were then collected and analyzed for the amount of nitrite, a stable oxidative metabolite and faithful NO indicator, by the Griess reaction as previously described [32]. To do it, 100 μL of Griess reagent (0.1% naphthyl-ethylenediamine and 1% sulfanilamide in 5% phosphoric acid) was mixed with 100 μL of collected medium in a 96-well plate. Mixture was incubated for 10 min at room temperature and then read at 540 nm. The amount of nitrite was calculated based on a standard curve generated with sodium nitrite. Percent inhibition in NO production was calculated with the formula {[(nitrite with herb extract) − (nitrite without herb extract)]/(nitrite without herb extract)} × 100.

2.4. Analysis of Cell Viability. Cell viability was determined by MTT assay as previously described [33]. Briefly, RAW264.7 cells were incubated with MTT (5 mg/mL in phosphate-buffered saline, pH = 7.4) for 4 h. Formed MTT formazan was solubilized with 50 μL of 0.01 M HCl buffer containing 10% SDS and 5% isobutanol. Cell growth was determined by reading plates at 570 nm in a microplate reader. The cell viability of control group is considered as 100%.

2.5. Statistical Analysis. The direction and magnitude of correlation between variables was done using analysis of t-test. P values less than 0.05 were considered statistically significant ($^*P < 0.05$).

3. Results

3.1. Effect of Herb Extracts on NO Production and Cell Growth. With the aid of Griess assay, we analyzed ethanol extracts of 81 herbs for their anti-inflammatory activity. A wide range of inhibition in NO production was observed with these extracts (Table 1). Extracts of 7 herbs [*Daphne genkwa* Sieb.et Zucc, *Caesalpinia sappan* L., *Iles pubescens* Hook.et Arn, *Forsythia suspensa* (Thunb.) Vahl, *Zingiber officinale* Rosc, *Inula japonica* Thunb., and *Ligusticum chuanxiong* Hort] blocked over 90% NO production in LPS/IFNγ-stimulated RAW264.7 cells (Table 1). Among the extracts that elicited over 50% inhibition in NO production, *Rubia cordifolia* L., *Glycyrrhiza glabra* L., *Iles pubescens* Hook.et Arn, *Nigella glandulifera* Freyn et Sint, *Pueraria lobata* (Willd.) Ohwi, and *Scutellaria barbata* D. Don showed no cytotoxicity to unstimulated RAW264.7 cells while significantly increased the viability of LPS/IFNγ-stimulated cells (Table 1). However, *Daphne genkwa* Sieb.et Zucc, which has the strongest inhibitory effect on NO production, was moderately toxic to RAW264.7 cells (Table 1).

3.2. Correlation between Anti-Inflammatory Potency and TCM Characteristics of Herbs. Analyzing the TCM characteristics of 10 herbs that display the strongest inhibitory effect on NO production in LPS/IFNγ-stimulated RAW264.7 cells, we found that most of them are in the categories of bitter or pungent flavor, warm nature, and lung or liver meridian distributions (Table 2). To correlate the TCM characteristics to anti-inflammatory effect in these herbs, we categorized TCM characteristics of these herbs that were able to abolish 50% of NO production in LPS/IFNγ-stimulated RAW264.7 cells. Table 3 showed that herbs with greater anti-inflammatory effect were distributed in a significantly higher percentage in those characterized as bitter/pungent flavors, warm nature, and liver/lung meridian distributions. These results suggest that anti-inflammatory herbs may possess

Table 1: Effect of herb extracts on NO production and cell viability in simulated and resting RAW264.7 cells.

Plant name and authority	Part used[a]	Stimulation cells		Resting cells		Yield[f]
		Percent inhibition of NO[b]	Cell proliferation (%)[c]	NO production (μM)[d]	Cytotoxicity (%)[e]	
Acanthopanax senticosus (Rupr.et Maxim.) Harms	SR	74.66 ± 0.01	97.64 ± 0.09	4.28 ± 0.01	90.76 ± 0.04	5.55
Acanthopanax gracilistylus W. W. Smith	BK	12.11 ± 0.01	66.29 ± 0.01	1.00 ± 0.01	99.16 ± 0.01	23.75
Achyranthes bidentata Bl.	RT	−11.62 ± 0.02	68.78 ± 0001	4.13 ± 0.03	73.25 ± 0.03	31.25
Acorus tatarinowii Schott.	SR	11.82 ± 0.01	47.40 ± 0.09	0.92 ± 0.01	98.57 ± 0.01	17.75
Actinidia arguta (Sieb.et Zucc.) Planch.ex Miq.	RT	54.67 ± 0.02	40.79 ± 0.03	1.18 ± 0.01	103.69 ± 0.01	7.42
Actinidia valvata Dunn	RT	32.11 ± 0.01	51.09 ± 0.01	2.94 ± 0.01	22.07 ± 0.01	7.36
Alisma orientalis (Sam.) Juzep.	ST	46.63 ± 0.04	53.01 ± 0.02	3.86 ± 0.02	101.11 ± 0.04	5.69
Allium macrostemon Bge.	ST	25.43 ± 0.03	73.53 ± 0.04	2.11 ± 0.01	93.74 ± 0.02	38.87
Aloe barbadensis Miller	LF	15.31 ± 0.10	35.02 ± 0.02	3.86 ± 0.02	94.60 ± 0.04	10.71
Amomum villosum Lour.	FR	35.56 ± 0.02	61.15 ± 0.04	4.57 ± 0.01	100.19 ± 0.03	5.18
Artemisia annua L.	HR	12.03 ± 0.03	29.48 ± 0.03	1.34 ± 0.01	105.08 ± 0.01	13.62
Artemisia anomala S. Moore	HR	59.56 ± 0.05	74.25 ± 0.01	1.61 ± 0.01	102.92 ± 0.02	11.04
Artemisia capillaris Thunb.	HR	41.2 ± 0.03	64.62 ± 0.03	4.02 ± 0.01	104.40 ± 0.04	17.36
Astragalus membranaceus (Fisch.) Bge.	RT	13.96 ± 0.01	53.76 ± 0.03	4.30 ± 0.01	93.30 ± 0.05	47.06
Bambusa tuldoides Munro.	ST	27.32 ± 0.01	75.23 ± 0.01	3.19 ± 0.01	110.67 ± 0.03	1.14
Bletilla striata (Thunb.) Reichb. f.	ST	77.52 ± 0.01	95.17 ± 0.08	4.29 ± 0.01	14.37 ± 0.02	18.63
Caesalpinia sappan L.	HW	94.27 ± 0.01	103.70 ± 0.01	3.75 ± 0.01	30.92 ± 0.01	10.66
Carpesium abrotanoides Linn.	HR	74.85 ± 0.03	53.24 ± 0.02	2.85 ± 0.01	102.95 ± 0.02	11.52
Carthamus tinctorius L.	FL	38.89 ± 0.02	89.78 ± 0.02	4.22 ± 0.01	104.26 ± 0.04	45.76
Celastrus orbiculatus Thunb.	RT	−6.06 ± 0.01	55.62 ± 0.01	1.30 ± 0.01	101.88 ± 0.03	2.78
Cinnamomum cassia Presl.	TW	38.43 ± 0.02	86.03 ± 0.03	4.38 ± 0.01	107.18 ± 0.06	9.32
Cinnamomum cassia Presl.	BK	68.31 ± 0.02	97.01 ± 0.04	4.54 ± 0.01	60.18 ± 0.04	11.16
Curcuma longa L.	ST	89.32 ± 0.02	108.09 ± 0.05	4.61 ± 0.01	51.43 ± 0.02	9.36
Codonopsis pilosula (Franch.) Nannf.	RT	−13.92 ± 0.01	60.45 ± 0.01	1.42 ± 0.01	101.83 ± 0.02	36.04
Corydalis yanhusuo W. T. Wang	ST	7.36 ± 0.01	25.78 ± 0.01	0.74 ± 0.01	95.26 ± 0.01	11.14
Chrysanthemum indicum L.	FL	−2.87 ± 0.01	97.74 ± 0.01	1.65 ± 0.01	101.07 ± 0.02	26.1
Curculigo orchioides Gaertn.	ST	8.81 ± 0.01	29.58 ± 0.02	1.25 ± 0.01	106.48 ± 0.02	8.01
Curcuma wenyujin Y. H. Chen et C. Ling	RT	−8.31 ± 0.01	45.33 ± 0.01	0.59 ± 0.01	99.01 ± 0.02	9.423
Curcuma phaeocaulis Val.	ST	18.87 ± 0.03	43.53 ± 0.02	3.10 ± 0.01	98.00 ± 0.05	46.14
Dalbergia odorifera T. Chen	HW	77.38 ± 0.04	88.27 ± 0.07	1.93 ± 0.01	86.12 ± 0.02	17.6
Daphne genkwa Sieb.et Zucc.	FL	99.17 ± 0.01	40.83 ± 0.03	4.25 ± 0.01	70.25 ± 0.04	20.55
Daphne tangutica Maxim.	BK	76.12 ± 0.01	91.32 ± 0.16	4.56 ± 0.01	85.67 ± 0.11	4.75
Drynaria fortunei (Kunze) J. Sm.	ST	6.46 ± 0.04	54.58 ± 0.01	1.15 ± 0.01	99.59 ± 0.02	11.458
Epimedium brevicornum Maxim.	LF	−43.84 ± 0.02	135.36 ± 0.02	1.06 ± 0.01	101.69 ± 0.02	13.78
Euodia rutaecarpa (Juss.) Benth.	FR	56.35 ± 0.02	23.68 ± 0.01	1.80 ± 0.01	41.84 ± 0.03	33.89
Forsythia suspensa (Thunb.) Vahl	FR	91.93 ± 0.01	34.44 ± 0.02	4.24 ± 0.01	27.32 ± 0.01	26.12
Gardenia jasminoides Ellis	FR	15.89 ± 0.01	50.48 ± 0.03	4.27 ± 0.01	129.77 ± 0.09	29.8
Glycyrrhiza glabra L.	SR	66.62 ± 0.01	107.8 ± 0.07	0.76 ± 0.01	109.65 ± 0.03	18.57
Iles pubescens Hook.et Arn.	RT	65.3 ± 0.02	106.52 ± 0.04	4.15 ± 0.01	117.70 ± 0.10	7.09
Ilex latifolia Thunb.	LF	32.33 ± 0.09	79.67 ± 0.01	3.42 ± 0.01	54.11 ± 0.04	19.14
Inula japonica Thunb.	FL	91.19 ± 0.01	84.48 ± 0.03	0.86 ± 0.01	100.42 ± 0.01	17.7
Inula linariifolia Turez.	HR	76.43 ± 0.01	129.93 ± 0.19	4.04 ± 0.01	84.41 ± 0.03	10.91
Isatis indigotica Fort.	LF	47.61 ± 0.02	86.83 ± 0.05	1.56 ± 0.01	106.24 ± 0.02	24.43
Isatis indigotica Fort.	RT	26.48 ± 0.02	53.51 ± 0.08	1.00 ± 0.01	103.22 ± 0.01	26.78

Table 1: Continued.

Plant name and authority	Part used[a]	Stimulation cells		Resting cells		Yield[f]
		Percent inhibition of NO[b]	Cell proliferation (%)[c]	NO production (μM)[d]	Cytotoxicity (%)[e]	
Ligusticum chuanxiong Hort.	SR	91.13 ± 0.01	79.46 ± 0.05	3.88 ± 0.01	81.82 ± 0.04	28.1
Lonicera japonica Thunb.	FL	47.87 ± 0.02	86.17 ± 0.04	1.38 ± 0.01	107.12 ± 0.01	39.55
Magnolia biondii Pamp.	FL	−15.35 ± 0.01	82.89 ± 0.01	3.27 ± 0.01	102.22 ± 0.03	15.39
Morus alba L.	TW	50.78 ± 0.01	72.21 ± 0.06	4.69 ± 0.01	93.67 ± 0.03	7.88
Nelumbo nucifera Gaertn.	FR	21.96 ± 0.02	95.84 ± 0.11	4.53 ± 0.04	105.37 ± 0.04	17.55
Nigella glandulifera Freyn et Sint.	SD	78.56 ± 0.01	95.88 ± 0.04	2.58 ± 0.01	113.01 ± 0.01	10.05
Oldenlandia diffusa (Willd.) Roxb.	HR	43.62 ± 0.02	62.44 ± 0.05	4.12 ± 0.01	69.83 ± 0.02	11.58
Ophiopogon japonicus (L.f.) Ker-Gawl.	RT	9.31 ± 0.01	65.68 ± 0.05	0.62 ± 0.01	96.24 ± 0.01	39.34
Paeonia veitchii Lynch	RT	61.27 ± 0.05	49.03 ± 0.01	2.95 ± 0.01	101.88 ± 0.03	22.17
Paeonia lactiflora Pall.	RT	−8.32 ± 0.01	77.45 ± 0.01	1.16 ± 0.01	98.85 ± 0.03	16.01
Paeonia suffruticosa Andr.	BK	31.64 ± 0.04	64.88 ± 0.02	4.15 ± 0.01	70.23 ± 0.01	28.7
Panax ginseng C. A. Mey.	SR	26.73 ± 0.04	71.25 ± 0.06	0.92 ± 0.01	101.26 ± 0.01	36.617
Perilla frutescens (L.) Britt.	HR	11.22 ± 0.05	64.07 ± 0.02	2.23 ± 0.01	103.16 ± 0.01	12.36
Peucedanum praeruptorum Dunn	RT	66.44 ± 0.02	102.58 ± 0.17	4.51 ± 0.01	20.67 ± 0.07	13.07
Polygonatum odoratum (Mill.) Druce	ST	−3.64 ± 0.02	47.88 ± 0.01	1.00 ± 0.01	97.64 ± 0.01	32.28
Polygonum multiflorum Thunb.	RT	36.49 ± 0.02	63.84 ± 0.02	4.91 ± 0.01	73.7 ± 0.02	12.57
Poria cocos (Schw.) Wolf	SC	56.75 ± 0.04	12.61 ± 0.06	1.34 ± 0.01	49.13 ± 0.03	2.21
Psoralea corylifolia L.	FR	41.35 ± 0.04	93.39 ± 0.04	1.16 ± 0.01	7.93 ± 0.01	5.34
Pueraria lobata (Willd.) Ohwi	RT	58.64 ± 0.03	93.10 ± 0.08	0.68 ± 0.01	101.30 ± 0.02	20.25
Pyrola calliantha H. Andres.	HR	20.09 ± 0.07	50.68 ± 0.02	3.04 ± 0.01	106.48 ± 0.04	11.6
Rehmannia glutinosa Libosch.	RT	−14.78 ± 0.01	38.15 ± 0.01	0.45 ± 0.01	96.41 ± 0.01	39.67
Rosa laevigata Michx.	FR	29.37 ± 0.02	69.39 ± 0.03	4.40 ± 0.01	91.48 ± 0.06	22.8
Rubia cordifolia L.	SR	69.99 ± 0.03	113.22 ± 0.12	5.30 ± 0.01	102.03 ± 0.06	12.67
Salvia miltiorrhiza Bge.	SR	7.35 ± 0.01	82.25 ± 0.14	2.02 ± 0.01	100.35 ± 0.01	40.42
Santalum album L.	HW	36.59 ± 0.02	61.80 ± 0.03	4.61 ± 0.01	63.65 ± 0.16	7.25
Saposhnikovia divaricata (Turcz.) Schischk.	RT	6.73 ± 0.01	56.66 ± 0.01	3.02 ± 0.01	92.08 ± 0.10	20.51
Scutellaria baicalensis Georgi	RT	23.55 ± 0.01	69.68 ± 0.01	3.07 ± 0.01	100.93 ± 0.01	47.06
Scutellaria barbata D. Don	HR	53.51 ± 0.03	98.59 ± 0.03	4.28 ± 0.01	101.75 ± 0.04	21.39
Satsstrea japonica (Thunb.) De.	BK	70.55 ± 0.01	126.05 ± 0.14	4.19 ± 0.01	91.61 ± 0.03	4.66
Spatholobus suberectus Dunn.	ST	33.79 ± 0.01	27.24 ± 0.01	4.98 ± 0.01	92.21 ± 0.07	16.07
Stephania tetrandra S. Moore	RT	52.29 ± 0.06	8.38 ± 0.01	2.06 ± 0.01	98.80 ± 0.03	11.03
Tribulus terrestris L.	FR	73.48 ± 0.02	71.80 ± 0.09	4.15 ± 0.01	87.66 ± 0.05	8.44
Trichosanthes kirilowii Maxim.	PE	−2.38 ± 0.03	29.54 ± 0.01	1.07 ± 0.01	101.00 ± 0.01	35.97
Typha angustifolia L.	PL	78.99 ± 0.05	48.80 ± 0.01	3.66 ± 0.01	85.85 ± 0.02	7.09
Typhonium giganteum Engl.	ST	7.41 ± 0.01	47.14 ± 0.01	0.46 ± 0.01	94.68 ± 0.02	24.56
Xanthium sibiricum Patr.	HR	76.34 ± 0.04	94.41 ± 0.07	4.46 ± 0.01	83.82 ± 0.06	5.73
Zingiber officinale Rosc.	SR	91.28 ± 0.01	98.31 ± 0.05	2.20 ± 0.01	41.37 ± 0.04	10.10
L-NIL[g]		35.2 ± 0.01	84.29 ± 0.01	3.22 ± 0.01	99.95 ± 0.03	

[a]HR: herb; RT: root; ST: stem; LF: leaf; TW: twig; FL: flower; FR: fruit; SD: seed; SC: sclerotium; HW: heartwood; SR: stem and root; PE: pericarp.
[b]Percent inhibition of NO production: Griess reaction was carried out to measure the production of nitrite in LPS/IFNγ-stimulated RAW264.7 cells in the absence or presence of 100 μg/mL herb extracts.
[c]Cell growth: MTT was performed to measure cell growth. The growth rate of control (no herb extract treatment) was considered as 100%.
[d]NO production: Griess reaction was used to measure the amount of nitrite in unstimulated RAW264.7 cells in the absence and presence of 100 μg/mL herb extracts.
[e]Cell cytotoxicity: MTT assay was performed to determine cell cytotoxicity of unstimulated RAW264.7 cells treated with herb extracts. Untreated group was considered as 100%.
[f]Percent yield of extract obtained from 70% ethanol extraction of each 100 g dry herb.
[g]Percent inhibition of iNOS activity at the test concentration of 50 μM.

Table 2: Characteristics (flavor, nature, and meridian distributions) of the 10 most potent anti-inflammatory herbs.

Plant name and authority	Flavors[a,b]	Natures[a,b]	Meridian distributions[a,b]
Daphne genkwa Sieb.et Zucc.	Bitter, pungent	Warm	Lung, spleen, kidney
Caesalpinia sappan L.	Sweat, salty	Moderate	Heart, liver, spleen
Forsythia suspensa (Thunb.) Vahl	Bitter	Litter cold	Lung, heart, intestinum tenue
Zingiber officinale Rosc.	Pungent	Hot	Spleen, stomach, kidney, heart, lung
Inula japonica Thunb.	Bitter, pungent, salty	Little warm	Lung, spleen, stomach, intestinum crassum
Ligusticum chuanxiong Hort.	Pungent	Warm	Liver, gallbladder, pericardium meridian
Curcuma longa L.	Pungent, bitter	Warm	Spleen, liver
Typha angustifolia L.	Sweat	Moderate	Liver, pericardium meridian
Nigella glandulifera Freyn et Sint.	Sweat, pungent	Warm	Liver, kidney
Bletilla striata (Thunb.) Reichb.f.	Bitter, sweet, astringent	Little cold	Lung, liver, stomach

[a]Based on Chinese Pharmacopoeia (2010).
[b]Based on Chinese Materia Medica (1998).

Table 3: Percentage distribution of herbs with the ability to inhibit over 50% NO production in each TCM characteristics.

TCM characteristic	Hit extracts (inhibition over 50%)	Percentage of effective herbs (32)	Herbs sharing same flavors	Percentage (in 81 herbs)
Four properties				
Cold	9	28.13	30	37.04
Cool	1	3.13	2	2.47
Warm	**11**	**34.38**	33	40.74
Hot	3	9.38	4	4.94
Moderate	8	25	12	14.81
Five flavors				
Pungent	**20**	**62.5**	42	51.85
Sweet	9	28.13	30	37.04
Bitter	**20**	**62.5**	47	58.02
Sour	0	0	3	3.70
Astringent	2	6.25	6	7.41
Salty	3	9.38	3	3.70
Mild	2	6.25	3	3.70
Meridian distributions				
Liver	**18**	**56.25**	43	53.09
Lung	**17**	**53.13**	35	43.21
Spleen	13	40.63	29	35.80
Heart	10	31.25	30	37.04
Kidney	8	25	25	30.86
Stomach	7	21.88	22	27.16
Intestinum crassum	4	12.5	9	11.11
Urinary bladder	2	6.25	7	8.64
Gallbladder	2	6.25	6	7.41
Intestinum tenue	1	3.13	2	2.47

common characteristics that are of pungent/bitter flavor, warm nature, and lung/liver meridian.

3.3. Correlation between Cell Protective Effect and TCM Characteristics of Herbs. Chronic inflammation often leads to cell damage and thus agents capable of deterring this process are actively sought. Examining 21 herbs with the TCM characteristic of pungent flavor, we observed that, under the costimulation of LPS and IFNγ, RAW264.7 cells treated with these herb extracts displayed 90% increase in cell viability (Table 4). Moreover, herbs with pungent flavor also conferred the highest degree of cell protection in LPS/IFNγ-stimulated cells in comparison with herbs with other flavors (Figure 1).

Table 4: Percentage distribution of herbs with cell protective capability in each TCM characteristics.

TCM characteristics	Hit herbs[a]	Percentage (21 herbs)	Hit herbs[b]	Percentage (43 herbs)
Four natures				
Cold	5	23.81	**19**	44.19
Cool	1	4.76	1	2.33
Moderate	5	23.81	5	11.63
Warm	**8**	38.10	**15**	34.88
Hot	2	9.52	1	2.33
Five flavors				
Pungent	**15**	71.43	**21**	48.84
Sweet	9	42.86	14	32.56
Bitter	**13**	61.90	**22**	51.16
Sour	0	0	0	0
Astringent	2	9.52	2	4.65
Salty	2	9.52	1	2.33
Mild	0	0	2	4.651
Meridian distributions				
Liver	**9**	42.86	**20**	46.51
Lung	**11**	52.38	**18**	41.86
Spleen	**10**	47.62	12	27.91
Heart	8	38.10	14	32.56
Kidney	8	38.10	12	27.91
Stomach	4	19.05	13	30.23
Intestinum crassum	3	14.29	3	6.977
Urinary bladder	1	4.76	6	13.95
Gallbladder	0	0	5	11.63
Intestinum tenue	0	0	1	2.326

[a]Herbs with over 90% cell protective capability in stimulated RAW264.7 cells.
[b]Herbs with ability to increase over 90% cell proliferation in resting RAW264.7 cells.

4. Discussion

Overproduction of NO due to the elevated iNOS expression has been convincingly linked to the pathogenesis of chronic inflammation and cancer [34]. Hence, agents that can selectively suppress iNOS-generated NO production should be effective to treat chronic inflammation and to prevent cancer. In fact, recent studies demonstrate that selective iNOS inhibitors L-NIL and 1400 W are therapeutically effective as anti-inflammation and anticancer drugs [35, 36].

Macrophages play a critical role in regulating inflammation. Macrophages are activated by external stimuli and activated macrophages produce various inflammatory mediators such as NO and reactive oxygen species. Chinese herbs are the rich sources for anti-inflammatory agents and efforts have been made to identify effective components in these herbs [37, 38]. Taking advantage of the well-established RAW264.7 cell model, we evaluated 81 herb extracts for their inhibitory effect on LPS/IFNγ-induced NO production. Among them, the extract of *Daphne genkwa* Sieb.et Zucc showed the strongest inhibitory effect on NO production. The constituents isolated from *Daphne genkwa* Sieb.et Zucc were previously reported to provoke cytotoxic effect to various tumor cell lines and to suppress outgrowth of transplanted mouse sarcoma S180 in mice [39]. We speculate that anticancer effect of *Daphne genkwa* Sieb.et Zucc may be functionally associated with its anti-inflammatory capability. In our study, we found that *Rubia cordifolia* L. and several others decrease LPS/IFNγ-induced NO production without causing significant cytotoxicity to RAW264.7 cells. These herbs may

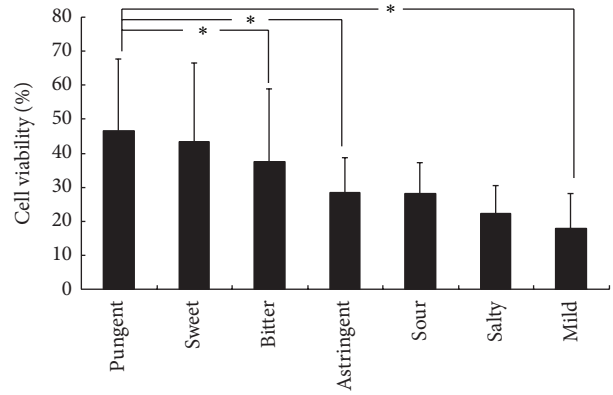

Figure 1: Comparison of herbs with cell viability in different flavors. Average of cell viability of LPS/IFNγ-stimulated RAW264.7 cells treated with herbs belonging to different flavor. $^{*}P < 0.05$.

thus be promising candidates as effective drugs to control inflammation and cancer. Although it is currently unclear how these extracts block LPS/IFN-induced NO production in RAW264.7 cells, the finding that Mollugin suppresses the inflammatory response by blocking the Janus kinase-signal transducers and activators of transcription signaling pathway [40] implicates that herbs may target the different steps of the signaling cascade mediating LPS/IFN-induced NO production to exert their anti-inflammatory roles.

Based on the theory of TCM, we classified these 81 herbs according to distinct flavors (pungent, sweet, sour, bitter, astringent, salty, or mild), natures (cold, cool, moderate, warm, or hot), and meridian distributions (liver, kidney, heart, spleen, etc.). Our study showed that the TCM characteristic of flavor correlated very well with the potency to inhibit NO production—pungent flavor is the strongest, bitter is slightly weaker than pungent, sweet flavors is intermediate, and astringent, salty, mild, or sour flavor is weak or not effective. TCM characteristics of nature and meridian distribution are also associated with the potency to inhibit NO production. For instance, higher pe of herbs with the capability to block NO production has the characteristics of warm nature. Characteristics of liver and lung meridians are the major meridian distributions found in herbs whose extracts can block 50% of NO production. Taken together, we reason that TCM characteristics can potentially be very useful to guide the search for effective anti-inflammatory agents in Chinese herbs.

TCM characteristic is a systematic expression of the distinct property elicited by Materia Medica in humans. Theory of flavors in TCM constitutes the core context of Chinese herb usage guidance. In TCM, the characteristic of flavor is the combination of both real taste and curative effect. According to Shen Nong Ben Cao Jing (Shennong's Classic of Material Medica), an important TCM book firstly written on Chinese herbal flavor and property theory, pungent flavor, which is related to lung meridian, can disperse the internal heat with sudorifics which in turn promote the circulation of Qi and blood. Herbs with pungent flavor have actually been used for thousand years in China to invigorate the circulation of blood and break the block of Qi. The fact that inflammation-related diseases are associated with the symptom of Qi and blood blockage may explain the effectiveness of herbs with pungent flavor to suppress inflammation.

Our study was limited to the investigation of 81 herb extracts on their effect on LPS/IFNγ-induced NO production and cell growth in macrophage RAW264.7 cells. The results generated from this study nevertheless support a close association between modern pharmacology/biomedical science and TCM theory. TCM theory was developed based on thousand years of clinical experience, and the material and pharmacological basis of TCM remains to be explained by the modern biomedical science. We believe that this study has contributed toward this goal.

Conflict of Interests

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

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References

[1] G. Y. Chen and G. Nuñez, "Sterile inflammation: sensing and reacting to damage," *Nature Reviews Immunology*, vol. 10, no. 12, pp. 826–837, 2010.

[2] R. Ross, "Atherosclerosis—an inflammatory disease," *The New England Journal of Medicine*, vol. 340, no. 2, pp. 115–126, 1999.

[3] H. L. Weiner and D. Frenkel, "Immunology and immunotherapy of Alzheimer's disease," *Nature Reviews Immunology*, vol. 6, no. 5, pp. 404–416, 2006.

[4] C. Garcia, B. Feve, P. Ferré et al., "Diabetes and inflammation: fundamental aspects and clinical implications," *Diabetes and Metabolism*, vol. 36, no. 5, pp. 327–338, 2010.

[5] L. M. Coussens and Z. Werb, "Inflammation and cancer," *Nature*, vol. 420, no. 6917, pp. 860–867, 2002.

[6] H. Clevers, "At the crossroads of inflammation and cancer," *Cell*, vol. 118, no. 6, pp. 671–674, 2004.

[7] A. Mantovani, P. Allavena, A. Sica, and F. Balkwill, "Cancer-related inflammation," *Nature*, vol. 454, no. 7203, pp. 436–444, 2008.

[8] C. J. Lowenstein and E. Padalko, "iNOS (NOS2) at a glance," *Journal of Cell Science*, vol. 117, no. 14, pp. 2865–2867, 2004.

[9] C. V. Suschek, O. Schnorr, and V. Kolb-Bachofen, "The role of iNOS in chronic inflammatory processes *in vivo*: is it damage-promoting, protective, or active at all?" *Current Molecular Medicine*, vol. 4, no. 7, pp. 763–775, 2004.

[10] G. C. Brown, "Mechanisms of inflammatory neurodegeneration: iNOS and NADPH oxidase," *Biochemical Society Transactions*, vol. 35, no. 5, pp. 1119–1121, 2007.

[11] H. H. Schmidt and U. Walter, "NO at work," *Cell*, vol. 78, no. 6, pp. 919–925, 1994.

[12] K. Bian and F. Murad, "Nitric oxide (NO)—biogeneration, regulation, and relevence to human diseases," *Frontiers in Bioscience*, vol. 8, pp. d264–d278, 2003.

[13] H. Ohshima and H. Bartsch, "Chronic infections and inflammatory processes as cancer risk factors: possible role of nitric oxide in carcinogenesis," *Mutation Research*, vol. 305, no. 2, pp. 253–264, 1994.

[14] J. R. Kanwar, R. K. Kanwar, H. Burrow, and S. Baratchi, "Recent advances on the roles of NO in cancer and chronic inflammatory disorders," *Current Medicinal Chemistry*, vol. 16, no. 19, pp. 2373–2394, 2009.

[15] S. Heemskerk, R. Masereeuw, F. G. Russel, and P. Pickkers, "Selective iNOS inhibition for the treatment of sepsis-induced acute kidney injury," *Nature Reviews Nephrology*, vol. 5, no. 11, pp. 629–640, 2009.

[16] V. Kostourou, J. E. Cartwright, A. P. Johnstone et al., "The role of tumour-derived iNOS in tumour progression and angiogenesis," *British Journal of Cancer*, vol. 104, no. 1, pp. 83–90, 2011.

[17] C. Hesslinger, A. Strub, R. Boer, W.-R. Ulrich, M. D. Lehner, and C. Braun, "Inhibition of inducible nitric oxide synthase in respiratory diseases," *Biochemical Society Transactions*, vol. 37, no. 4, pp. 886–891, 2009.

[18] M. Lechner, P. Lirk, and J. Rieder, "Inducible nitric oxide synthase (iNOS) in tumor biology: the two sides of the same coin," *Seminars in Cancer Biology*, vol. 15, no. 4, pp. 277–289, 2005.

[19] B. Fitzpatrick, M. Mehibel, R. L. Cowen, and I. J. Stratford, "iNOS as a therapeutic target for treatment of human tumors," *Nitric Oxide*, vol. 19, no. 2, pp. 217–224, 2008.

[20] G.-Y. Yang, S. Taboada, and J. Liao, "Induced nitric oxide synthase as a major player in the oncogenic transformation of inflamed tissue," *Methods in Molecular Biology*, vol. 512, pp. 119–156, 2009.

[21] C. M. Ulrich, J. Bigler, and J. D. Potter, "Non-steroidal anti-inflammatory drugs for cancer prevention: promise, perils and pharmacogenetics," *Nature Reviews Cancer*, vol. 6, no. 2, pp. 130–140, 2006.

[22] E. J. Jacobs, "Will an aspirin a day help keep fatal cancer away?" *The Lancet*, vol. 377, no. 9759, pp. 3–4, 2011.

[23] R. Basha, C. H. Baker, U. T. Sankpal et al., "Therapeutic applications of NSAIDS in cancer: special emphasis on tolfenamic acid," *Frontiers in Bioscience*, vol. 3, pp. 797–805, 2011.

[24] B. B. Aggarwal, R. V. Vijayalekshmi, and B. Sung, "Targeting inflammatory pathways for prevention and therapy of cancer: short-term friend, long-term foe," *Clinical Cancer Research*, vol. 15, no. 2, pp. 425–430, 2009.

[25] A. Murakami and H. Ohigashi, "Targeting NOX, INOS and COX-2 in inflammatory cells: chemoprevention using food phytochemicals," *International Journal of Cancer*, vol. 121, no. 11, pp. 2357–2363, 2007.

[26] S. Bengmark, "Curcumin, an atoxic antioxidant and natural NF-κB, cyclooxygenase-2, lipooxygenase, and inducible nitric oxide synthase inhibitor: a shield against acute and chronic diseases," *Journal of Parenteral and Enteral Nutrition*, vol. 30, no. 1, pp. 45–51, 2006.

[27] V. B. Patel, S. Misra, B. B. Patel, and A. P. Majumdar, "Colorectal cancer: chemopreventive role of curcumin and resveratrol," *Nutrition and Cancer*, vol. 62, no. 7, pp. 958–967, 2010.

[28] C. H. Hong, S. K. Hur, O.-J. Oh, S. S. Kim, K. A. Nam, and S. K. Lee, "Evaluation of natural products on inhibition of inducible cyclooxygenase (COX-2) and nitric oxide synthase (iNOS) in cultured mouse macrophage cells," *Journal of Ethnopharmacology*, vol. 83, no. 1-2, pp. 153–159, 2002.

[29] A. Murakami, "Chemoprevention with phytochemicals targeting inducible nitric oxide synthase," *Forum of Nutrition*, vol. 61, pp. 193–203, 2009.

[30] C. Cerella, C. Sobolewski, M. Dicato, and M. Diederich, "Targeting COX-2 expression by natural compounds: a promising alternative strategy to synthetic COX-2 inhibitors for cancer chemoprevention and therapy," *Biochemical Pharmacology*, vol. 80, no. 12, pp. 1801–1815, 2010.

[31] H. Liao, L. K. Banbury, and D. N. Leach, "Antioxidant activity of 45 Chinese herbs and the relationship with their TCM characteristics," *Evidence-Based Complementary and Alternative Medicine*, vol. 5, no. 4, pp. 429–434, 2008.

[32] L. C. Green, D. A. Wagner, J. Glogowski, P. L. Skipper, J. S. Wishnok, and S. R. Tannenbaum, "Analysis of nitrate, nitrite, and [15N] nitrate in biological fluids," *Analytical Biochemistry*, vol. 126, no. 1, pp. 131–138, 1982.

[33] B. Ayissi Owona, N. F. Njayou, S. Laufer, P. F. Moundipa, and H. J. Schluesener, "A fraction of stem bark extract of *Entada africana* suppresses lipopolysaccharide-induced inflammation in RAW 264.7 cells," *Journal of Ethnopharmacology*, vol. 149, no. 1, pp. 162–168, 2013.

[34] Y. Wu, S. Antony, J. L. Meitzler, and J. H. Doroshow, "Molecular mechanisms underlying chronic inflammation-associated cancers," *Cancer Letters*, 2013.

[35] A. G. Sikora, A. Gelbard, M. A. Davies et al., "Targeted inhibition of inducible nitric oxide synthase inhibits growth of human melanoma *in vivo* and synergizes with chemotherapy," *Clinical Cancer Research*, vol. 16, no. 6, pp. 1834–1844, 2010.

[36] K. Engels, S. K. Knauer, S. Loibl et al., "NO signaling confers cytoprotectivity through the survivin network in ovarian carcinomas," *Cancer Research*, vol. 68, no. 13, pp. 5159–5166, 2008.

[37] K. S. Kim, X. Cui, D. S. Lee et al., "Anti-inflammatory effect of neoechinulin A from the marine fungus *Eurotium* sp. SF-5989 through the suppression of NF-κB and p38 MAPK pathways in lipopolysaccharide-stimulated RAW264.7 macrophages," *Molecules*, vol. 18, no. 11, pp. 13245–13259, 2013.

[38] H. J. Lee, Y. J. Jeong, T. S. Lee et al., "Moringa fruit inhibits LPS-induced NO/iNOS expression through suppressing the NF-κ B activation in RAW264.7 cells," *The American Journal of Chinese Medicine*, vol. 41, no. 5, pp. 1109–11023, 2013.

[39] S. Li, G. Chou, Y. Hseu, H. Yang, H. Kwan, and Z. Yu, "Isolation of anticancer constituents from flos genkwa (*Daphne genkwa* Sieb.et Zucc.) through bioassay-guided procedures," *Chemistry Central Journal*, vol. 7, article 159, 2013.

[40] Z. G. Zhu, H. Jin, P. J. Yu, Y. X. Tian, J. J. Zhang, and S. G. Wu, "Mollugin inhibits the inflammatory response in lipopolysaccharide-stimulated RAW264.7 macrophages by blocking the Janus kinase-signal transducers and activators of transcription signaling pathway," *Biological & Pharmaceutical Bulletin*, vol. 36, no. 3, pp. 399–406, 2013.

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Research Article

Capilliposide Isolated from *Lysimachia capillipes* Hemsl. Induces ROS Generation, Cell Cycle Arrest, and Apoptosis in Human Nonsmall Cell Lung Cancer Cell Lines

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Several data has reported that capilliposide, extracted from a traditional Chinese medicine, *Lysimachia capillipes* Hemsl. (LC) could exhibit inhibitory effect on cell proliferation in various cancers. The current study investigated the antitumor efficacy of *Capilliposide* and elucidated its potential molecular mechanism involved in vivo and vitro. Our results indicated that LC capilliposide inhibited proliferation of lung cancer cells in a dose-dependent manner. LC capilliposide induced cell cycle arrest at the S stage and enhanced apoptosis in NSCLC cells. Treatment with LC capilliposide increased the intracellular level of ROS, which activated the mitochondrial apoptotic pathway. Blockage of ROS by NAC highly reversed the effect of LC capilliposide on apoptosis. Xenograft tumor growth was significantly lower in the LC-treated group compared with the untreated control group ($P < 0.05$). The results also show that LC treatment does not produce any overt signs of acute toxicity in vivo. These findings demonstrate that LC capilliposide could exert an anti-tumor effect on NSCLC through mitochondrial-mediated apoptotic pathway and the activation of ROS is involved.

1. Introduction

Lung cancer has been the most common malignant tumor worldwide and the leading cause of human cancer-related deaths for several decades [1]. Nonsmall cell lung cancer (NSCLC) accounts for nearly 80% of lung cancer cases and approximately two thirds of these patients are diagnosed at an advanced stage. Chemotherapy or radiation therapy is largely ineffective and highly toxic with a low survival profile. Although the prognosis is improved by early diagnosis and treatment, tumor recurrence and progression still plague some patients [2]. Developing novel drugs and therapies with fewer side effects is of significance for prognosis of patients with NSCLC [3].

Reactive oxygen species (ROS) including superoxide anion, hydroxyl radicals, and hydrogen peroxide (H_2O_2) are produced by all aerobic cells, which had important role in variety of various biological processes during physiological and pathological conditions [4]. ROS are thought to play multiple roles in tumorigenesis, progression, and maintenance [5]. On the one hand, cancerous cells have shown a higher level of ROS compared with their noncancerous counterparts. Up-regulation of ROS is usually accompanied with oncogene activation which may contribute to cancer progression. On the other hand, an imbalance between production of ROS and antioxidant depletion results in irreversible oxidative stress. Anticancer drugs and ionizing

radiation may be selectively toxic to cancer cells by increasing oxidant stress and enhancing the already stressed cells beyond their limit [6]. Intracellular ROS burst leads to cell cycle arrest and triggers apoptosis [7].

Lysimachia capillipes hemsl is a traditional medicinal plant that grows in southeastern China. The whole plant is used for treating coughs, menstrual, rheumatalgia disorder and carcinomas. Capilliposide had been extracted from *Lysimachia capillipes* by Tian et al. [8, 9]. Some experimental analysis have proven that LC capilliposide possess anti-cancer properties in different cancer cell lines both in vivo and in vitro, such as prostate and gastric cancer [10, 11]. LC capilliposide exhibited cytotoxicity against human breast cancer cells MCF7 with an IC50 value of 0.3 ug/mL [12]. Although capilliposidecan induce growth inhibition in cancer cells, the molecular mechanism underlying antitumor activity remained poorly understood. This study was, therefore, conducted to investigate the antiproliferative activity of LC capilliposide in nonsmall cell lung cancer (NSCLC) cell lines and its underlying mechanism.

2. Materials and Methods

2.1. Cell Cultures. The lung cancer cell lines A549, H1299, and H460 were obtained from Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). The cells were cultured in RPMI-1640 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Gibco, Carlsbad, CA, USA). The cell lines were maintained in a humidified atmosphere containing 5% CO_2 at 37°C. The culture medium was renewed every 2 to 3 days. Adherent cells were detached by incubation with trypsin. Throughout the experiment, the cells were used in logarithmic phase of growth.

2.2. Chemical Reagents and Antibodies. LC capilliposide was dissolved in double distilled water, presented by professor Tian from Zhejiang University (Hangzhou, China), TS101021. Dimethyl sulfoxide (DMSO), N-acetyl L-cysteine (NAC), cisplatin (DDP), 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphnyl-2H-tetrazolium bromide (MTT), phenylmethylsulfonyl fluoride (PMSF), 5-(and 6)-carboxy-2′7′-dichlorodihydrofluorescein diacetate (DCFDA) and the fluorescent dyes Hoechst 33342, and propidium iodide (PI) were all purchased from Sigma-Aldrich (St. Louis, MO, USA). The monoclonal antibodies against p53 (number 2527), Bax (#5023P), cleaved caspase-3 (#9661s), cleaved caspase-9 (#9505p), cytochrome C (#11940S), GAPDH (#2118), and horseradish peroxidase (HPR)-conjugated goat antirabbit secondary antibody (#7074P2) were obtained from Cell Signaling Technology (Cell Signaling Technology, MA, USA). The monoclonal antibody against Bcl-2 (#sc-492) was obtained from Santa Cruz.

2.3. Cell Viability Assay. To evaluate the effect of LC capilliposide on A549, H1299, and H460 cell growth, cell viability was determined by MTT assay as described [13]. Cells were seeded in a 96-well microplate and treated with LC capilliposide at different concentrations (0–32 μg/mL) for 24 h. After treatment, the MTT reagent was added (1 mg/mL) and cells were incubated for a further 4 h. Subsequently, 150 μL DMSO was added to each well and the absorbance was measured in a microplate reader at the wave length of 570 nm (Thermo Electron Corp, Waltham, MA, USA). The percentage of cell viability was calculated as follows: cell viability (%) = A570 (sample)/A570 (control) × 100%. At least three replicates were performed for each treatment. The IC50 values were calculated using Graph Pad Prism 5.

2.4. Clonogenicity Assay. Clonogenicity assays were performed to determine the effects of LC capilliposide treatment on the colony-forming ability of H460 cells. Cells grew at low density, treated with LC capilliposide at different concentrations (0, 2, 4, and 6 μg/mL) for 6 h. After cultured with fresh medium, cells were allowed to grow for 14 days to form colonies, which then were fixed and stainedwith 0.5% crystal violet (Sigma) in methanol for 30 min. The number of colonies (>50 cells) was scored using a microscopy.

2.5. Cell Cycle Analysis. H460 cells were seeded into 6-well culture plates. After LC capilliposide (0, 2, 4, and 6 μg/mL) treatment for 24 h, the cells were collected and then fixed over night with 70% ethanol. After centrifugation, the cell pellets were incubated with 50 μg/mL PI and 0.1% RNase in PBS for 30 min at room temperature in dark. The samples were measured using FACS flowcytometer (Becton Dicknson, USA).

2.6. Apoptosis Assay. Apoptotic cells by fluorescent staining were determined as previously described [14]. After LC capilliposide (0, 2, 4, and 6 μg/mL) treatment for 24 h, the cells were stained with 5 μL annexin V-fluoirescein isothiocyanate (FITC) and 10 μL propidium iodide (PI) for 30 min at room temperature in dark. Stained cells were immediately measured using FACS Calibur flow cytometer and Cell Quest software.

2.7. Nuclear Double Staining with Hoechst 33342/PI. H460 cells were seeded into 6-well culture plates and treated with LC capilliposide (0, 2, 4, and 6 μg/mL) for 24 h. After treatment, cells were harvested and washed with PBS. Hoechst 33342 (10 μg/mL) was added, followed by PI (2.5 μg/mL), and the cells were further incubated for 15 min at 37°C. Cells of blue and red fluorescence were examined under a fluorescence microscopy (Zeiss, LSM710, Germany) and 100 cells from five random microscope fields were counted.

2.8. Detection of Reactive Oxygen Species (ROS). Intracellular ROS were measured using 2′,7′-dichlorodihydroluorescein diacetate (DCFH-DA). After incubation with LC capilliposide (0, 2, 4, and 6 μg/mL) for 3 h, H460 cells (1×10^6) were washed with PBS and labeled with 10 Um DCFDA for 30 min. Then, excess DCFH-DA was removed by washing the cells in serum-free 1640 RPMI medium. The fluorescence intensities were measured using an FACS flow cytometer.

2.9. Western Blot Analysis. After treatment with various concentrations of LC for 24 h, H460 cells were harvested, washed with PBS, and 0.1 mL of cold lysis buffer (150 mM NaCl, 50 mM of pH 7.4 Tris, 1 mM EDTA, 1% Triton X-100, 0.5% SDS, and 0.01% PMSF). The cell lysate was centrifuged at 4°C and 12000 ×g for 12 min, and the supernatant were collected. Protein concentrations were determined using the BCA protein assay (Beyotime Institute of Biotechnology, Jiangsu, China). Equal amounts of lysate (30 μg) was subjected to 10 %SDS-PAGE at 80 mA and then transferred onto PVDF membranes. The membranes were blocked with a 5% skim milk solution for 1 h and incubated with respective primary antibodies overnight at 4°C. Then the membrane was incubated with a HRP-conjugated secondary antibody for 1 h at room temperature. The protein expression levels were determined by the enhanced chemiluminescence (ECL) system (ECL, Beyotime Institute of Biotechnology, Jiangsu, China).

2.10. In Vivo Studies. Female BALB/c nu/nu mice (16 weeks old, 18–20 g) were provided by Shanghai Experimental Animals Co. The animals were maintained at a specific pathogen-free grade animal facility with a regulated environment (22 ± 1°C, relative humidity 60 ± 5%) and a 12 h light and 12 h dark cycle (08:00–20:00, light). Then, H460 cells (1×10^7) were subcutaneously inoculated into the right flank mice. Therapy was initiate 7 days after tumor inoculation when the mean tumor volume was 50 mm^3. Tumor-bearing mice were divided into four groups (10 mice per group). Group of vehicle were infused with 100 uL physiological saline; group of L-LC were infused with 100 uL LC (40 mg/kg body weight) by oral administration; group of H-LC were infused with 100 uL LC (80 mg/kg body weight) by oral administration; group of DDP were administered with 100 uL cisplatin (1.2 mg/kg body weight) intraperitoneally. The groups of L-LC and H-LC were administered for 16 days, once a day. The DDP group was administered once every 2 days.

After 16 days of treatment, mice from each group were scarified and the weight of tumor mass was measured. The tumor weight of treatment group showed statistically significant differences compared with those of control group. No mice died during the period of treatment. Serum was separated and stored at −20°C for biochemistry analysis. In order to understand the acute side effect of LC treatment on liver and kidney function, the liver and kidneys were fixed in buffered formalin, embedded in paraffin, cut into 2 μm sections, and stained with hematoxylin and eosin (H&E). Blood the biochemical parameters including alanine aminotransferase (ALT), aspartate aminotransferase (AST), albumin, gamma glutamyl transpeptidase (GGT), blood ureanitrogen (Bun), and creatinine (Cr) were measured by an automated biochemical analyzer (Hitachi 7600, Japan).

2.11. Statistical Analysis. Data are expressed as mean ± standard deviation. Statistical comparisons were performed using a one-way analysis of variance followed by the Fisher test. Significant differences between the groups were determined using an unpaired Student *t*-test.

3. Results

3.1. LC Capilliposide Decreased Viability and Inhibited the Proliferation of NSCLC Cells. In order to investigate the effect of LC capilliposide on cell viability of NSCLC cell lines, MTT assay was assayed using A549, H1299, and H460 cell lines. Figure 1(a) indicated that cellular proliferation was inhibited by LC capilliposide for 24 hours in a dose-dependent manner. The IC50 values of LC capilliposide in A549, H1299, and H460 cells were 4.13 μg/mL, 3.76 μg/mL, and 2.85 μg/mL, respectively. The IC50 values of LC capilliposide for 48 h in A549, H1299, and H460 cells were 3.54 μg/mL, 2.61 μg/mL, and 2.08 μg/mL, respectively; the IC50 values of LC capilliposide for 72 h in A549, H1299, and H460 cells were 2.76 μg/mL, 2.03 μg/mL, and 1.58 μg/mL, respectively. H460 cell lines seemed to be more sensitive to LC capilliposide. Thus, we selected H460 cell line as the model system to conduct mechanistic studies.

Clonogenic assays were performed to examine the long-term antiproliferative activity of LC capilliposide in H460 cells. As shown in Figures 1(a) and 1(c), the clone formation were 159 ± 13, 112 ± 10, 91 ± 10, and 68 ± 8 at the concentration of 0, 2, 4, and 6 μg/mL LC capilliposide, respectively. In addition, the clonogenicity of H460 cell lines in the LC capilliposide groups was decreased in a concentration-dependent manner. LC treatment can significantly suppress the colony-forming activity compared the control group ($P < 0.05$). As clonogenic assays in vitro have been reported to correlate very well with in vivo assays of tumorigenicity in nude mice [15], we investigated the antitumor effects of capilliposide in vivo in the following test.

3.2. Capilliposide Causes Apoptosis and Cell Cycle Arrest. To study the nature of LC-induced cell apoptosis, H460lung cancer cells were quantified with annexin V-FITC/PI double staining flow cytometry. As shown in Figures 2(a) and 2(b), LC capilliposide exposure at different concentrations (2, 4, and 6 μg/mL) resulted in higher population of early apoptotic population (18.5 ± 1.8%, 31.7 ± 4.5% to 18.3 ± 2.6%, resp.) and late apoptotic population (11.8 ± 1.4%, 12.6 ± 2.1% to 23.6 ± 2.8%, resp.) compared to the control ($P < 0.01$). The data demonstrated that LC capilliposide induced a dose-dependent apoptosis.

To further understand the effect of LC on induced cell death, H460 cells were stained with Hoechst 33342/PI. Cells that were stained brightly by Hoechst 33342 were considered as early apoptotic cells. On the contrary, cells that were stained with both Hoechst 33342 and PI were considered to be at the late apoptosis. As depicted in Figure 2(b), there were higher percentages of bright blue cells (apoptosis) and red cells (necrosis). These data suggested that LC induced the apoptosis in H460 cells.

It has been reported that cell cycle arrest may induce apoptosis of cancer cells [16]. To evaluate the effect of capilliposide on the distribution of cell cycle, we performed DNA concentration in cell cycle analysis using flow cytometry. As shown in Figure 3, after 24 h treatment with capilliposide H460 cells were arrested in S-phase in a dose-dependent. Cells treated with 2, 4, or 6 μg/mL LC capilliposide showed

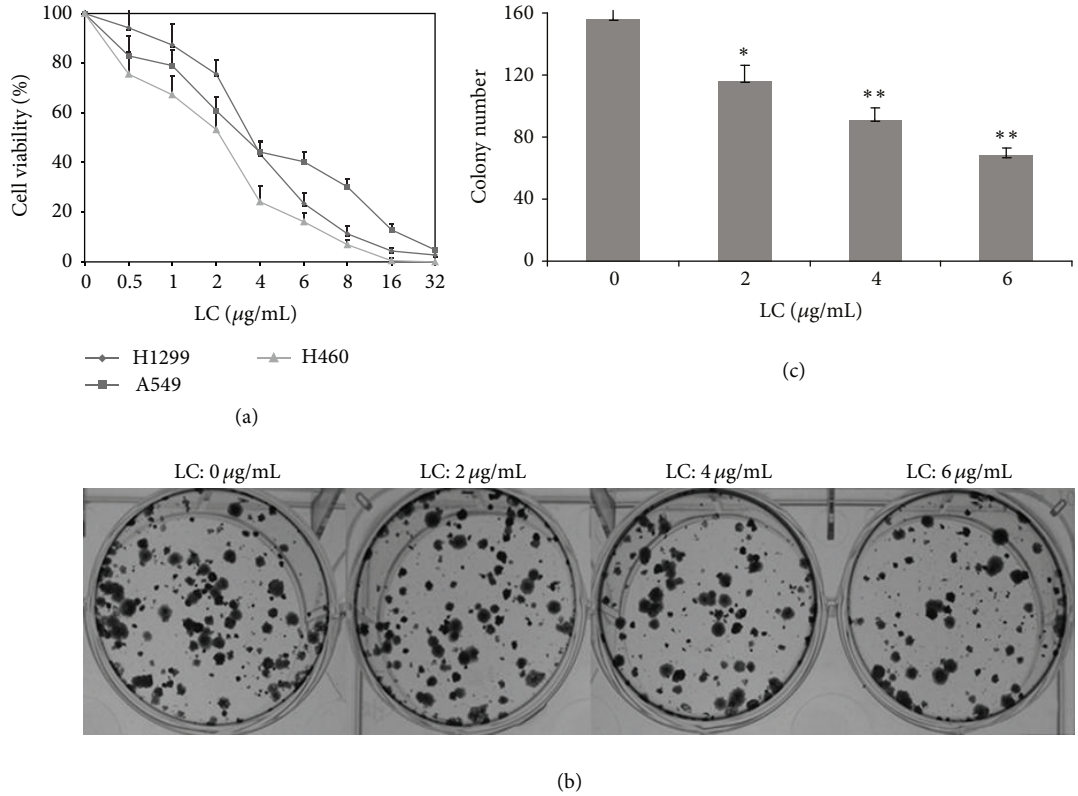

Figure 1: Effects of LC on cell viability and colony formation in of NSCLC cells. (a) Cell viability in LC-treated A549, H292, and H460 cells. The cells were treated with various concentrations (0–32 μg/mL) of LC for 24 h. (b) Influence of LC on the number of colony-forming in H460 cells. Cells were treated with LC capilliposide (0, 2, 4, and 6 μg/mL) for 6 h and allowed to grow for 14 days to form colonies. (c) Summary of colony-forming data in histogram form. All data are representative of at least three independent experiments. $^{*}P < 0.05$ and $^{**}P < 0.01$ indicate statistically significant differences versus control group.

higher S population (22.66%, 34.75%, and 44.02%, resp.) compared with 12.35% in the control ($P < 0.05$). With the increase in the S-phase cell population, cell populations in the G_0/G_1 and G_2/M phase decreased concomitantly.

3.3. LC Capilliposide Induced Apoptosis by ROS Generation in H460 Lung Cancer Cells. Some reports have shown that the generation of ROS in intracellular could induce apoptosis by saponins extracted from different plants [17, 18]. Therefore, we hypothesized that LC capilliposide may cause H460 cells apoptosis via increased ROS production. To test this hypothesis, we investigated whether LC capilliposide treatment has associations with ROS burst in lung cancer cells. As shown in Figure 4(a), H460 cells in various concentration of LC capilliposide had a higher level of ROS-associated mean fluorescence intensity (MFI) compared with control. The level of ROS treated with 2, 4, and 6 μg/mL LC correspondingly increased from 15.81 ± 2.63% to 32.54 ± 4.25% and 54.47 ± 6.21%. To further confirm that ROS was involved in LC capilliposide induced apoptotic pathway of H460 cells, NAC was used to scavenge the over production of ROS from both enzymatic and nonenzymatic mechanisms. Additionally, H460 cells were cultured with LC ± NAC (10 mM) for 24 h, then we analyzed cell viability by MTT and detected the apoptosis rate using Annexin V-FITC/PI double-labeled assay. As shown in Figures 4(b) and 4(c), our data demonstrated that treatment with NAC significantly inhibits the effect of LC capilliposide anti-proliferative and LC-induced apoptosis. Taken together, these results strongly support the hypothesis that LC-induced apoptosis via increased intracellular ROS oxidative stress in H460 cells.

3.3.1. Effect of LC on Expression of Apoptotic-Related Proteins in Lung Cancer Cells. Our results in Figures 2 and 4 demonstrated that LC capilliposide induced cells apoptosis and intracellular ROS accumulation. ROS accumulation was described as an early event of mitochondrial apoptosis. So we hypothesized LC capilliposide induced apoptosis via a mitochondria dependent pathway. To verify this hypothesis, H460 cells were harvested after treatment with 4 μg LC capilliposide for various time periods and total protein levels from each treatment were measured by Western blotting analysis. The Bcl-2 protein family plays a regulatory role in controlling the mitochondrial apoptotic pathway, including antiapoptotic (Bcl-2) and proapoptotic members (Bax) [19]. P53 as a tumor suppressor can regulate the expression of Bcl-2 and Bax protein to mediate mitochondrial apoptosis [20]. As shown in Figure 5(a), treatment with LC upregulated the expression of Bax and P53 whereas the expression of Bcl-2 was downregulated. Release of the cytochrome C from

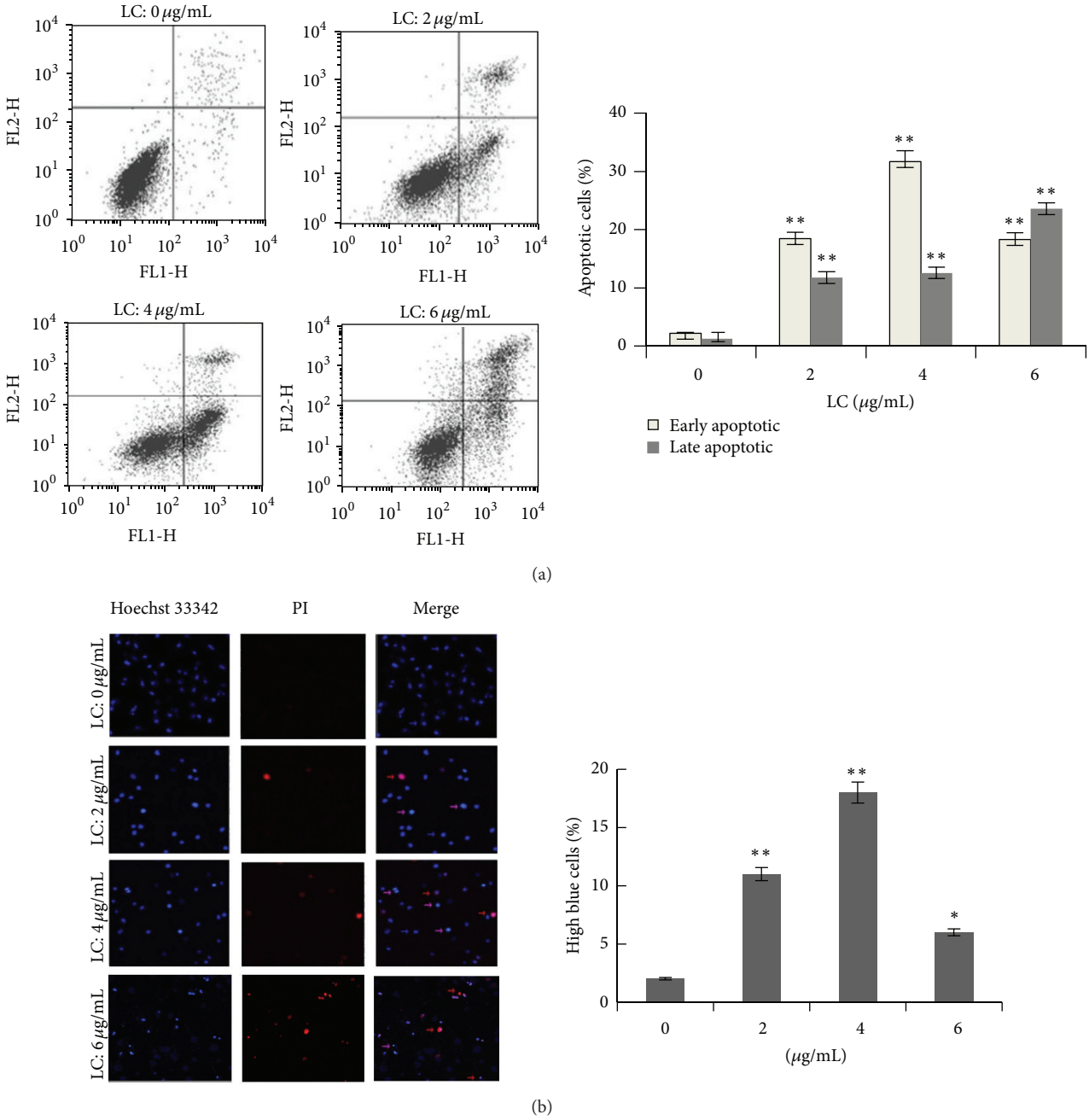

FIGURE 2: LC induces apoptosis in human NSCLC cells. (a) H460 cells treated with 2, 4 and 6 μg/mL LC for 24 hours and apoptosis rates were analyzed by flow cytometry after annexin V/PI staining. (b) Apoptosis was assessed by Hoechst 33342/PI double staining as described in Section 2. High blue fluorescent indicates apoptotic cells (pink arrow), low blue indicates live cells (azury arrow), while red represents dead cells (red arrow). Apoptosis was expressed as a percentage of the total number of nuclei examined. All data are representative of at least three independent experiments. $^{*}P < 0.05$ and $^{**}P < 0.01$ indicate statistically significant differences versus control group.

the mitochondria into the cytosol was a critical process for cells to undergo apoptosis. As shown in Figure 5(b), the level of protein cytochrome C, cleaved caspase-3 and cleaved caspase-9 was elevated after LC treatment in H460 cells. Western blotting analysis also showed that treatment with LC lead a time-dependent increase in the expression of cytochrome C, cleaved caspase-3 and 9 at 6 h, 12 h, and 24 h, suggesting a possible involvement of caspases activation in the apoptotic effect of LC in H460 cells in vitro.

3.4. LC Inhibited the Growth of Human H460 Xenografts. In order to access the therapeutic efficiencies of different concentrations of LC on H460 xenografts, body weights and the tumor sizes were measured by a caliper every 3 days

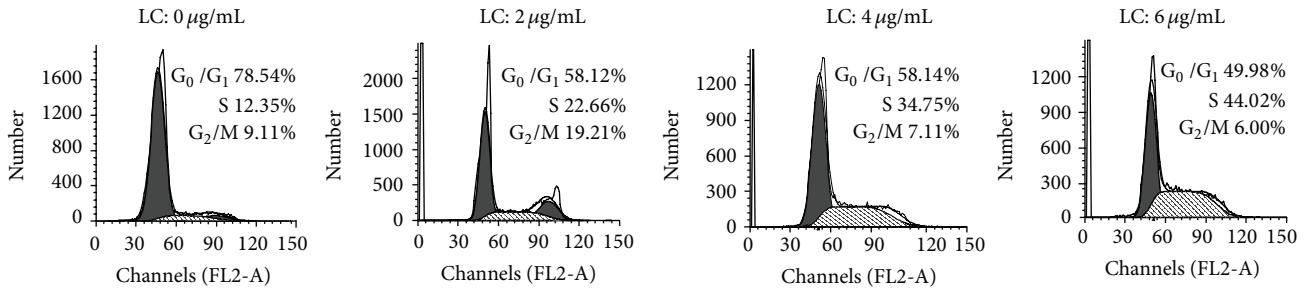

Figure 3: LC induces cell cycle arrest at S stage in H460 lung cancer cells. Cells were harvested and fixed in 70% alcohol and then stained with propidium iodide. Finally the stained cells were analyzed using a flow cytometer.

in vivo. As shown in Figure 6, control group keep a rapid growth all the time due to the lack of anti-cancer drugs and the average relative tumor volumes (V/V_0) was 15.20 ± 2.36; administration of LC (80 mg/kg) or cisplatin significantly decrease the size of tumor formation compared with control groups and their average relative tumor volumes (V/V_0) were 10.75 ± 1.84 and 10.12 ± 2.41 compared with 15.20 ± 2.36 in the control ($P < 0.05$); LC (40 mg/kg) administration also resulted in growth suppression of H460 xenografts, but there is no significant difference compared to the control groups ($P > 0.05$). Consistent with the tumor volumes, tumor weights in Groups H-LC and DDP were significantly lighter than those in Group control ($P < 0.05$). These suggest that oral treatment of LC could significantly inhibit the development and progress of tumor formation in lung cancer model.

3.5. Acute Side Effects of LC. In order to understand acute side effects of LC, we investigated the changes in body weight, blood biochemistry, and histopathology of liver or kidney using BALB/c nu/nu mice. The four groups of mice were in good general state, body weights in the four groups had no obviously difference ($P > 0.05$, date not shown). The histopathological changes in liver and kidneys were assessed using hematoxylin and eosin staining. As shown in Figure 7, no obvious histopathological changes were observed in liver and kidneys structures of Groups L-LC and H-LC compared with control group. Table 1 represents a comparison between the levels of ALT, AST, albumin, GGT, Bun, and Cr of control and treated groups of mice. The results show that the group DDP treated with cisplatin had a slight increase in serum Bun and Cr compared with control group, but the increase was not significantly different ($P > 0.05$). On the otherhand, groups treated with LC did not exhibit obvious hepatotoxicity or nephrotoxicity concerning the serum parameters compared with control group.

4. Discussion

Lung cancer has long been the leading mortality in developed and developing countries. Due to limited efficacies of traditional radiation and chemotherapy, it is urgent to exploit new treatment strategies for lung cancer. An increasing amount of attention has been focused on the use of natural products isolated from Chinese medicinal herbs for lung cancer therapy [21, 22]. The major finding of the present study is that LC capilliposide, a natural compound extracted from *Lysimachia capillipes* Hemsl, effectively decreases human lung cancer cell viability via induction of apoptosis, which suppress the tumor proliferation both in vitro and in vivo. An ideal cancer chemotherapeutic agent must not only kill the cancer cells but must in addition exhibit a high degree of selective toxicity between cancer cells and normal cells [23]. The results also show that LC treatment does not produce any obvious signs of acute toxicity in vivo. It suggests that LC capilliposide may discriminate between normal and cancer cells.

Apoptosis, or programmed cell death, is an essential mechanism through which many types of chemotherapeutic agents inhibit tumor growth [24, 25]. As shown in Figure 2(a), cell population with annexin V positive and PI negative are considered as an early apoptotic population, whereas a cell population with both annexin V and PI positive is considered as a late apoptotic/necrotic population [26]. Our results firstly demonstrate that LC induces both early and late apoptosis in H460 cells in dose-dependent manner. In addition, cells undergoing apoptosis may lead to characteristic morphological changes, such as cell shrinkage, ruling, and chromatin condensation [27, 28]. So Hoechst 33342/PI double staining was used to identify the morphological changes in apoptotic nuclei. As shown in Figure 2(b), the nuclei of cells treated with LC was darkly stained, and thus fluoresced brightly, indicating the condensation of chromatin.

Mitochondria-initiated responses are thought to be the major pathway for apoptosis, and, therefore, targeting the mitochondria is a novel strategy for cancer therapy [29, 30]. Several genes are involved in the regulation of mitochondrial apoptosis, such as the Bcl-2 family and cytochrome C (cyto C). The Bcl-2 gene family, which is significantly involved in the regulation of cell apoptosis, both anti-apoptotic genes (Bcl-2, Bcl-XL) and proapoptotic members (Bax, Bak) [31]. The balance between the expression levels of pro- and anti-apoptotic proteins is critical for cell survival or cell death. Bcl-2 is an upstream effect or molecule in the apoptotic pathway and has been identified as a potent suppressor of apoptosis. As shown in Figure 4, LC treatment significantly downregulated Bcl-2 protein and upregulated levels of Bax protein in H460 cells, leading to an up regulation of the ratio between Bax and Bcl-2 [32]. This indicates the involvement of the Bcl-2

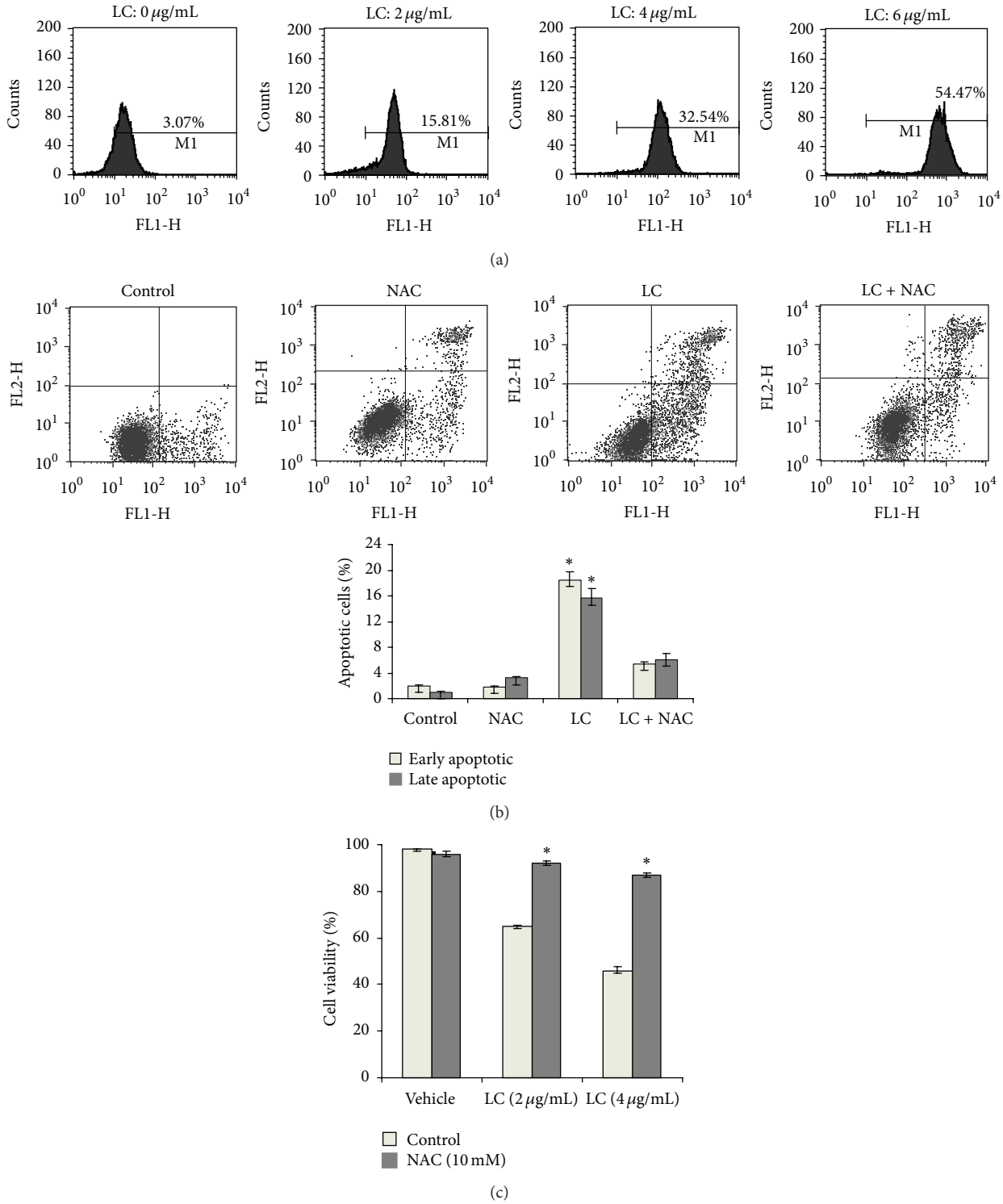

Figure 4: LC leads to ROS-mediated proliferative inhibition and apoptosis in H460 lung cancer cells. (a) ROS levels were determined 3 h later by flow cytometric analysis. The data demonstrated difference in the levels of intracellular ROS in control versus LC treated cells from a representative experiment. (b) The role of ROS in LC-induced apoptosis inhibition was assessed using ROS scavenger NAC. Cells were pretreatment with NAC (10 mM) for 1 h and then cotreated with 4 ug/mg LC for another 24 h. Impact of NAC on the apoptotic value was determined by annexin V-FITC/PI staining. (c) The role of ROS in LC-mediated proliferative inhibition was assessed using ROS scavenger NAC. The influence of NAC on LC-induced cytotoxicity was determined by MTT assay. All data are representative of at least three independent experiments. $^{*}P < 0.05$ and $^{**}P < 0.01$ indicate statistically significant differences versus control group.

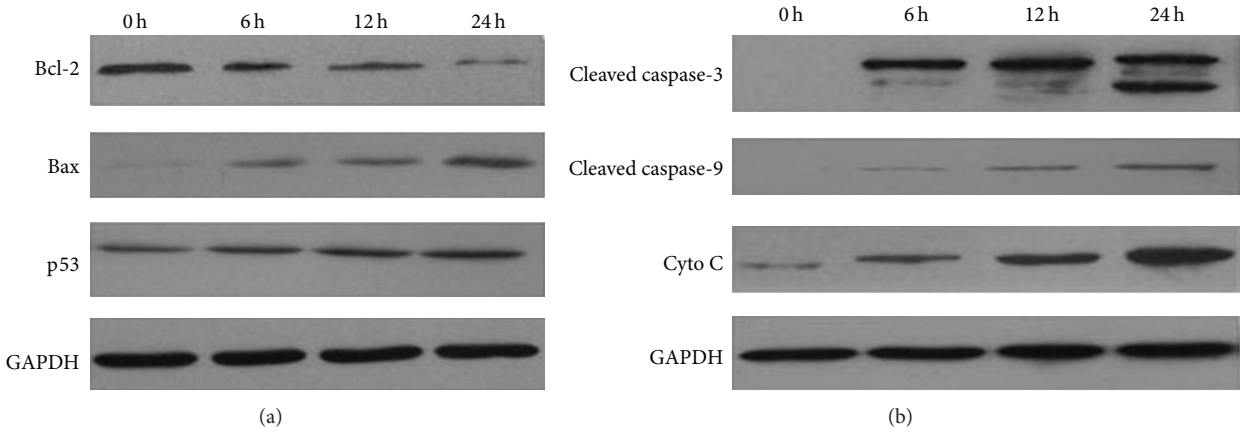

Figure 5: LC suppresses lung cancer cell growth via an apoptosis-independent mechanism. (a) Cells were treated with LC (4 μg/mL) for 6 h, 12 h, and 24 h. Western blot assays were performed to determine the expression of Bcl-2, Bax, and p53 in H460 cells. GAPDH was used as a loading control. (b) Western blot assays were performed to determine the expression of cleaved caspase-3, cleaved caspase-9, and cytochrome C in H460 cells.

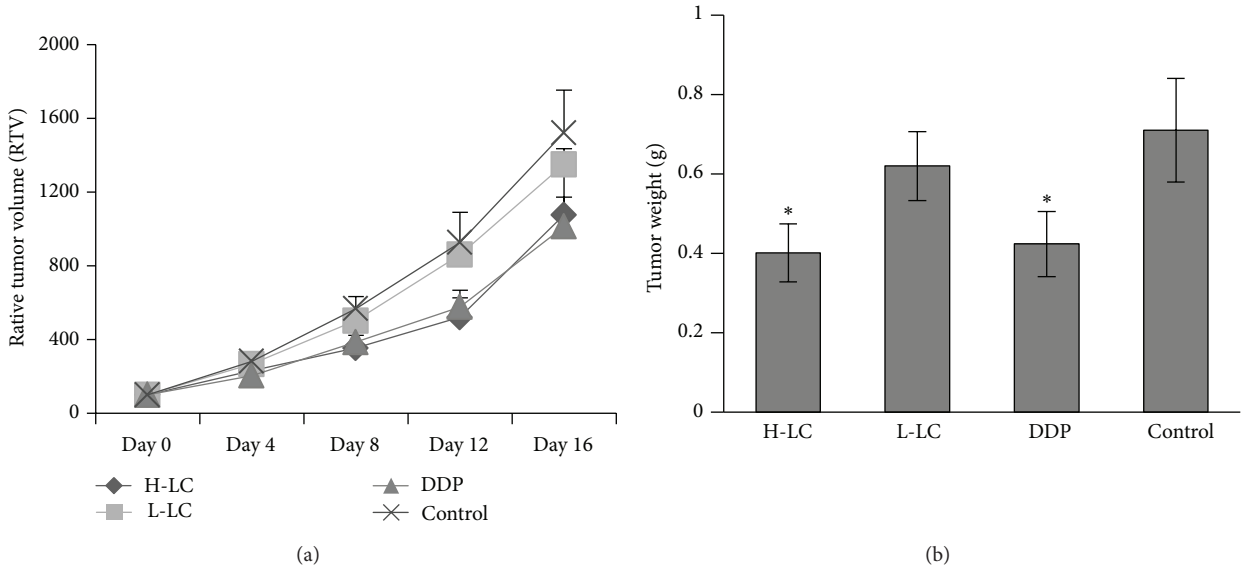

Figure 6: LC inhibited the growth of human H460 xenografts. After mice were injected with H460 cells (2×10^7), they were divided into four groups. Control, L-LC (40 mg/kg) and H-LC (80 mg/kg) were administered orally, each day for 16 days. The DDP group (1.2 mg/kg) was injected once every 2 days. (b) Tumor weights. After 16 days of treatment, mice from each group were scarified and the weight of tumor mass was measured. Values were presented as mean ± SD, ($n = 10$). $^*P < 0.05$ indicate statistically significant differences versus control group.

gene family. Furthermore, up-regulation of the ratio between Bax and Bcl-2 may induce the release of the cytochrome C from the mitochondria into the cytosol, which play a key role in the regulation of caspase-dependent cell death [33, 34]. Our results indicate that LC treatment significantly enhanced the release of the cytochrome C and increased the caspase-9 activity. Both intrinsic and extrinsic pathways converge on common factors including caspase-3. The activation of caspases-3 damages the cell structure and cause functional disorder by proteolysis, final induction of apoptosis. Our Western blot analysis also showed that caspase-9 and caspase-3 were all involved in LC-induced apoptosis in H460 cells. These results clearly indicate that LC induces apoptosis via mitochondrial pathways.

Apart from apoptosis, cell cycle arrest is another cause of growth inhibition. Many anti-cancer agents exhibit anti-proliferation by inhibiting cell cycle progression at a particular check point such as G_0/G_1, S, or G_2/M [35, 36]. Deregulation of cell cycle has been linked with cancer initiation and progression. p53, a tumor suppressor protein, triggers cell cycle arrest to provide time for self-mediated apoptosis through transcriptional activation of cyclin-dependent kinase (CDK) inhibitor p21 [37, 38]. Data presented in Figure 5 showed a significant and progressive increase in the expression of p53 protein in LC-treated Cells. Interestingly, flow cytometry analysis also showed cell cycle was arrested at the S phase after treated with LC, suggesting that LC induce apoptosis of H460 cells via cell cycle arrest in S phase, which

Table 1: Effect of LC on blood biochemistry of control and treated mice.

Group	AST (U/L)	ALT (U/L)	Albumin (U/L)	GGT (U/L)	Cr (umol/L)	BUN (mmol/L)
Control	95.4 ± 7.6	38.3 ± 4.2	27.3 ± 3.2	2.1 ± 0.4	22.3 ± 2.5	9.4 ± 1.1
LC (40 mg/kg)	87.6 ± 6.5	36.6 ± 5.1	30.4 ± 3.5	2.6 ± 0.6	22.6 ± 3.8	8.6 ± 1.4
LC (80 mg/kg)	104.8 ± 8.4	43.7 ± 6.1	25.8 ± 2.7	2.9 ± 0.7	24.1 ± 3.5	10.8 ± 1.5
DDP	106.2 ± 7.9	46.5 ± 5.8	24.6 ± 2.8	2.8 ± 0.3	28.4 ± 4.2	12.3 ± 2.2

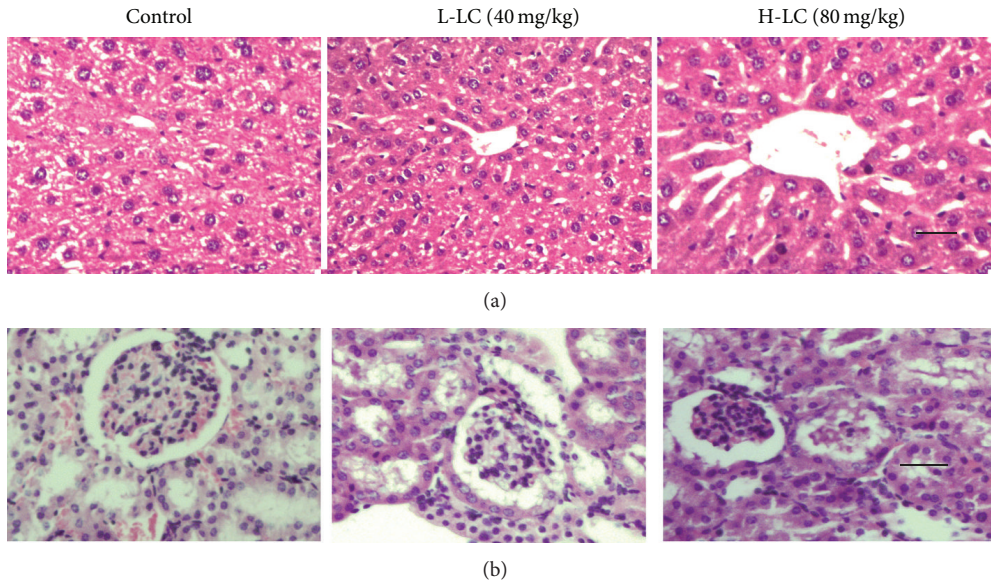

Figure 7: Effect of LC on mice liver and kidneys. The liver and kidneys from control and LC-treated mice were excised and processed for hematoxylin and eosin staining followed established procedures. (a) kidney section, scale bar = 100 μm; (b) liver sections, scale bar = 100 μm.

might be regulated by p53. Besides cell cycle arrest, p53 can induce the expression of several genes involved in apoptosis. For example, the Bcl-2 family has been shown to be a p53 target. An up-regulation of the ratio between Bax and Bcl-2 is involved in the regulation of p53-mediated cell death [39]. Our dates demonstrate that LC treatment increases the expression of Bax and p53 and decrease the expression of Bcl-2 by western blot assay. This suggests that p53 is involved in the apoptotic effect of LC.

ROS, which are the byproducts of normal cellular oxidative processes, have been suggested as regulating the process involved in the initiation of apoptotic signaling. Evidence is accumulating which indicates that many anticancer agents destroy tumor cells by raising the level of ROS above a toxic threshold [40–42]. High level of ROS can destroy the integrity of plasma membrane, affects dynamic of actin cytoskeleton and causes DNA damage, cumulatively known as oxidative stress. To investigate whether LC induce apoptosis is promoted through an increase in ROS production, we measured ROS levels using DCFH-DA staining and flow cytometric assays. The results showed that the apoptotic effect of LC on H460 cells was associated with increased ROS production. Moreover, to further confirm the finding that the apoptotic effect of LC was mediated by ROS, H460 cells were exposed to LC ± NAC and then analyzed for cell viability and apoptosis of H460cells [7]. As shown in Figure 4(b), treatment of H460 cells with NAC led to a significant reduction in LC-induced cell killing and apoptosis. Together, these results suggest that the accumulation of ROS is an important mechanism in the mitochondrial apoptosis pathway. However, the mechanism by which LC generates ROS needs to be further investigated in the future.

In summary, our data provide evidence for the first time that LC induces apoptosis in H460 NSCLC cells via ROS generation resulting p53 activation, increase Bax/Bcl-2 ratio, release of cytochrome C, and cleavage of caspases 9, 3. Finally, our data also showed that the growth of xenograft tumors was remarkably inhibited by oral administration of LC, indicating that the agent also has potential for clinical anticancer activity. Importantly, LC did not induce significant acute toxicity in mouse liver and kidneys. LC therefore has the potential to be a potent agent for non-small lung cancer treatment.

Conflict of Interests

The authors declare that they have no conflict of interests.

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References

[1] R. Siegel, D. Naishadham, and A. Jemal, "Cancer statistics, 2012," *CA Cancer Journal for Clinicians*, vol. 62, no. 1, pp. 10–29, 2012.

[2] Y. Ohe, Y. Ohashi, K. Kubota et al., "Randomized phase III study of cisplatin plus irinotecan versus carboplatin plus paclitaxel, cisplatin plus gemcitabine, and cisplatin plus vinorelbine for advanced non-small-cell lung cancer: four-arm cooperative study in Japan," *Annals of Oncology*, vol. 18, no. 2, pp. 317–323, 2007.

[3] H. Yang, K. R. Landis-Piwowar, D. Chen, V. Milacic, and Q. P. Dou, "Natural compounds with proteasome inhibitory activity for cancer prevention and treatment," *Current Protein and Peptide Science*, vol. 9, no. 3, pp. 227–239, 2008.

[4] R. Paletta-Silva, N. Rocco-Machado, and J. R. Meyer-Fernandes, "NADPH oxidase biology and the regulation of tyrosine kinase receptor signaling and cancer drug cytotoxicity," *International Journal of Molecular Sciences*, vol. 14, no. 2, pp. 3683–3704.

[5] S. S. Myatt, J. J. Brosens, and E. W.-F. Lam, "Sense and sensitivity: FOXO and ROS in cancer development and treatment," *Antioxidants and Redox Signaling*, vol. 14, no. 4, pp. 675–687, 2011.

[6] M. Benhar, D. Engelberg, and A. Levitzki, "ROS, stress-activated kinases and stress signaling in cancer," *EMBO Reports*, vol. 3, no. 5, pp. 420–425, 2002.

[7] X. Shi, Y. Zhao, Y. Jiao, T. Shi, and X. Yang, "ROS-dependent mitochondria molecular mechanisms underlying antitumor activity of *Pleurotus abalonus* acidic polysaccharides in human breast cancer MCF-7 cells," *PLoS ONE*, vol. 8, no. 5, Article ID e64266.

[8] B. Liang, L. Zhang, J. Tian, L. Xu, and S. Yang, "Isolation and characterization of two new saponins from *Lysimachia capillipes*," *Carbohydrate Research*, vol. 341, no. 14, pp. 2444–2448, 2006.

[9] J.-K. Tian, L.-Z. Xu, Z.-M. Zou, and S.-L. Yang, "Two new triterpene saponins from *Lysimachia capillipes*," *Journal of Asian Natural Products Research*, vol. 8, no. 5, pp. 439–444, 2006.

[10] H.-M. Ying, Z.-J. Qi, D.-W. Guo, T.-L. Yu, L. Zhang, and J.-K. Tian, "Quantitative determination of capilliposide B and capilliposide C in *Lysimachia capillipes* by HPLC-ELSD assay," *Chinese Pharmaceutical Journal*, vol. 46, no. 9, pp. 704–706, 2011.

[11] Y. Xu, Y. M. Rong, and T. Jk, "Experimental studies on anti-tumor effects of capilliposide," *Chinese Pharmacological Bulletin*, vol. 28, no. 4, pp. 545–549.

[12] J.-K. Tian, L.-Z. Xu, Z.-M. Zou, and S.-L. Yang, "Three novel triterpenoid saponins from *Lysimachia capillipes* and their cytotoxic activities," *Chemical and Pharmaceutical Bulletin*, vol. 54, no. 4, pp. 567–569, 2006.

[13] M. Khan, B. Yu, A. Rasul et al., "Jaceosidin induces apoptosis in U87 glioblastoma cells through G2/M phase arrest," *Evidence-Based Complementary and Alternative Medicine*, vol. 2012, Article ID 703034, 12 pages, 2012.

[14] C. Y. Looi, M. Imanishi, S. Takaki et al., "Octa-arginine mediated delivery of wild-type Lnk protein inhibits TPO-induced M-MOK Megakaryoblastic leukemic cell growth by promoting apoptosis," *PLoS ONE*, vol. 6, no. 8, Article ID e23640, 2011.

[15] V. H. Freedman and S. Shin, "Cellular tumorigenicity in nude mice: correlation with cell growth in semi solid medium," *Cell*, vol. 3, no. 4, pp. 355–359, 1974.

[16] J. Gao, W. A. Morgan, A. Sanchez-Medina, and O. Corcoran, "The ethanol extract of *Scutellaria baicalensis* and the active compounds induce cell cycle arrest and apoptosis including upregulation of p53 and Bax in human lung cancer cells," *Toxicology and Applied Pharmacology*, vol. 254, no. 3, pp. 221–228, 2011.

[17] J. H. Lee, J. H. Yeon, H. Kim et al., "The natural anticancer agent plumbagin induces potent cytotoxicity in MCF-7 human breast cancer cells by inhibiting a PI-5 kinase for ROS generation," *PLoS ONE*, vol. 7, no. 9, Article ID e45023.

[18] D. E. Reuss, J. Mucha, C. Hagenlocher et al., "Sensitivity of malignant peripheral nerve sheath tumor cells to TRAIL is augmented by loss of NF1 through modulation of MYC/MAD and is potentiated by curcumin through induction of ROS," *PLoS ONE*, vol. 8, no. 2, Article ID e57152.

[19] H.-W. Chiu, Y.-S. Ho, and Y.-J. Wang, "Arsenic trioxide induces autophagy and apoptosis in human glioma cells in vitro and in vivo through downregulation of survivin," *Journal of Molecular Medicine*, vol. 89, no. 9, pp. 927–941, 2011.

[20] H. Gu, S. Rao, J. Zhao et al., "Gambogic acid reduced bcl-2 expression via p53 in human breast MCF-7 cancer cells," *Journal of Cancer Research and Clinical Oncology*, vol. 135, no. 12, pp. 1777–1782, 2009.

[21] W. S. Xu, Y. Y. Dang, X. P. Chen, J. J. Lu, and Y. T. Wang, "Furanodiene presents synergistic anti-proliferative activity with paclitaxel via altering cell cycle and integrin signaling in 95-D lung cancer cells," *Phytotherapy Research*, 2013.

[22] L. Ma, Z.-S. Wen, Z. Liu et al., "Overexpression and small molecule-triggered downregulation of CIP2A in lung cancer," *PLoS ONE*, vol. 6, no. 5, Article ID e20159, 2011.

[23] P. S. Low, W. A. Henne, and D. D. Doorneweerd, "Discovery and development of folic-acid-based receptor targeting for imaging and therapy of cancer and inflammatory diseases," *Accounts of Chemical Research*, vol. 41, no. 1, pp. 120–129, 2008.

[24] A. H. Wyllie, J. F. R. Kerr, and A. R. Currie, "Cell death: the significance of apoptosis," *International Review of Cytology*, vol. 68, pp. 251–306, 1980.

[25] W. A. Cooper, M. R. J. Kohonen-Corish, L. Zhuang et al., "Role and prognostic significance of tumor necrosis factor-related apoptosis-inducing ligand death receptor DR5 in nonsmall-cell lung cancer and precursor lesions," *Cancer*, vol. 113, no. 1, pp. 135–142, 2008.

[26] M. C. Willingham, "Cytochemical methods for the detection of apoptosis," *Journal of Histochemistry and Cytochemistry*, vol. 47, no. 9, pp. 1101–1109, 1999.

[27] M. Aghaei, M. Panjehpour, F. Karami-Tehrani, and S. Salami, "Molecular mechanisms of A3 adenosine receptor-induced G1 cell cycle arrest and apoptosis in androgen-dependent and independent prostate cancer cell lines: involvement of intrinsic pathway," *Journal of Cancer Research and Clinical Oncology*, vol. 137, no. 10, pp. 1511–1523, 2011.

[28] J. H. Ha, H. S. Noh, I. W. Shin, J. R. Hahm, and D. R. Kim, "Mitigation of H_2O_2-induced autophagic cell death by propofol in H9c2 cardiomyocytes," *Cell Biology and Toxicology*, vol. 28, no. 1, pp. 19–29, 2012.

[29] S. Fulda and K.-M. Debatin, "Targeting apoptosis pathways in cancer therapy," *Current Cancer Drug Targets*, vol. 4, no. 7, pp. 569–576, 2004.

[30] H. J. Kim, K. M. Yang, Y. S. Park et al., "The novel resveratrol analogue HS-1793 induces apoptosis via the mitochondrial pathway in murine breast cancer cells," *International Journal of Oncology*, vol. 41, no. 5, pp. 1628–1634.

[31] B. Leber, F. Geng, J. Kale, and D. W. Andrews, "Drugs targeting Bcl-2 family members as an emerging strategy in cancer," *Expert Reviews in Molecular Medicine*, vol. 12, article e28, 2010.

[32] M. Kaparou, D. Choumerianou, C. Perdikogianni et al., "Enhanced levels of the apoptotic BAX/BCL-2 ratio in children with acute lymphoblastic leukemia and high-risk features," *Genetics and Molecular Biology*, vol. 36, no. 1, pp. 7–11.

[33] A. Inoue, K. Takitani, M. Koh, C. Kawakami, T. Kuno, and H. Tamai, "Induction of apoptosis by α-Tocotrienol in human cancer cell lines and leukemic blasts from patients: dependency on bid, cytochrome c, and caspase pathway," *Nutrition and Cancer*, vol. 63, no. 5, pp. 763–770, 2011.

[34] H.-I. Lin, Y.-J. Lee, B.-F. Chen et al., "Involvement of Bcl-2 family, cytochrome c and caspase 3 in induction of apoptosis by beauvericin in human non-small cell lung cancer cells," *Cancer Letters*, vol. 230, no. 2, pp. 248–259, 2005.

[35] H. G. Drexler, "Review of alterations of the cyclin-dependent kinase inhibitor INK4 family genes p15, p16, p18 and p19 in human leukemia-lymphoma cells," *Leukemia*, vol. 12, no. 6, pp. 845–859, 1998.

[36] M. A. Park, K. A. Hwang, H. R. Lee et al., "Benzophenone-1 stimulated the growth of BG-1 ovarian cancer cells by cell cycle regulation via an estrogen receptor alpha-mediated signaling pathway in cellular and xenograft mouse models," *Toxicology*, vol. 305, pp. 41–48.

[37] D. P. Lane, "p53, guardian of the genome," *Nature*, vol. 358, no. 6381, pp. 15–16, 1992.

[38] Q. Wang, L. Su, N. Liu, L. Zhang, W. Xu, and H. Fang, "Cyclin dependent kinase 1 inhibitors: a review of recent progress," *Current Medicinal Chemistry*, vol. 18, no. 13, pp. 2025–2043, 2011.

[39] D. A. Martin and K. B. Elkon, "Mechanisms of apoptosis," *Rheumatic Disease Clinics of North America*, vol. 30, no. 3, pp. 441–454.

[40] M. Dewaele, H. Maes, and P. Agostinis, "ROS-mediated mechanisms of autophagy stimulation and their relevance in cancer therapy," *Autophagy*, vol. 6, no. 7, pp. 838–854, 2010.

[41] B. R. You and W. H. Park, "Zebularine-induced apoptosis in Calu-6 lung cancer cells is influenced by ROS and GSH level changes," *Tumour Biology*, vol. 34, no. 2, pp. 1145–1153.

[42] J. A. Strickertsson, C. Desler, T. Martin-Bertelsen et al., "*Enterococcus faecalis* infection causes inflammation, intracellular oxphos-independent ROS production, and DNA damage in human gastric cancer cells," *PLoS ONE*, vol. 8, no. 4, Article ID e63147.