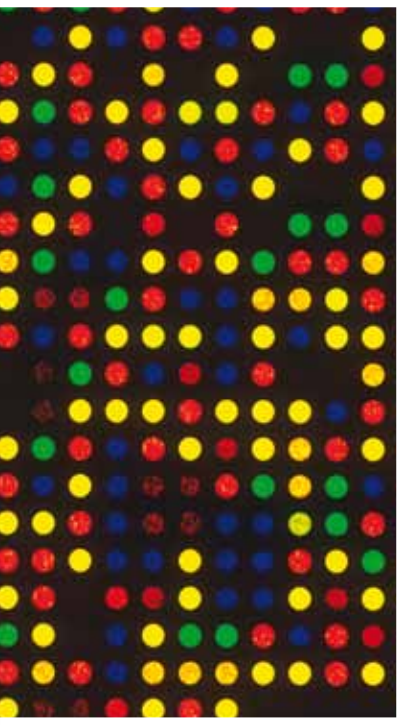
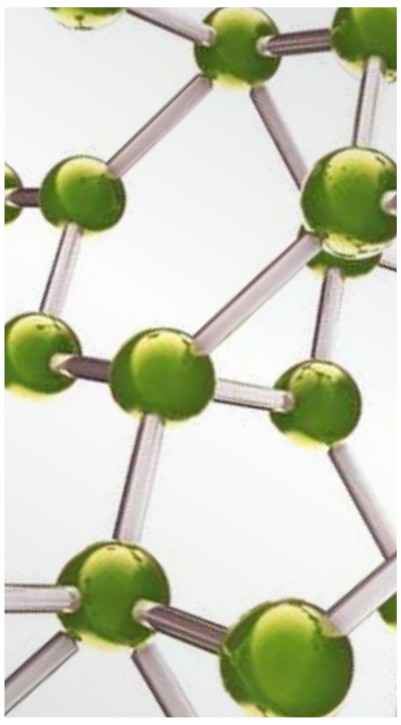
eCAM Evidence-Based Complementary and Alternative Medicine

Integrating Complementary and Alternative Medicine with Primary Health Care

Guest Editors: Annie Shirwaikar, Raghavan Govindarajan, and Ajay Kumar Singh Rawat



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Editorial

Integrating Complementary and Alternative Medicine with Primary Health Care

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Complementary/alternative medicine (CAM) has been described as "diagnosis, treatment, and/or prevention which complements mainstream medicine by contributing to a common whole, satisfying a demand not met by orthodoxy, or diversifying the conceptual frameworks of medicine" [1]. Approximately, 1500 articles on CAM are published annually in the literature covered by MEDLINE [2]. In the United Kingdom, the market for herbal and homoeopathic remedies and aromatherapy oils increased by 41% between 1992 and 1996 [3]. In Germany, a herbal remedy (St. John's wort) is now the most frequently prescribed drug for depression. In the USA, the sales of St. John's wort rose by 2800% between 1997 and 1998, and the total market for medicinal botanicals was worth US$ 3.87 billion in 1998 [4]. Most experts agree that the interest in and practice of CAM are driven mainly by consumer pressure. A 1990 national survey of alternative medicine prevalence in US, costs, and patterns of use [5] demonstrated that alternative medicine has a substantial presence in the US health care system. Data from a survey in 1994 [6] and a public opinion poll in 1997 [7] confirmed the extensive use of alternative medical therapies in the United States. An increasing number of US insurers and managed care organizations now offer alternative medicine programs and benefits [8]. The majority of US medical schools now offer courses on alternative medicine [9]. In western Europe and Australia, 20–70% of the population regularly use complementary and alternative medicine [10, 11]. In the USA, it was estimated in 1992 that at least one in three Americans utilized one of those methods, and the number of annual visits to providers of alternative medicine exceeds the number of visits to all primary care physicians [12]. These therapies include acupuncture, chiropractic, herbal medicine and dietary supplements, nutraceuticals, homeopathy, mind-body techniques, spirituality, and faith healing, massage, and therapeutic touch. In a 1998 follow-up study, the percentage of CAM patients had increased to 42% of the US population [13]. Subsequent analyses showed that 67.6% of respondents had used at least one CAM therapy in their lifetime. This trend suggests a continuing demand for CAM therapies that will affect health care delivery for the foreseeable future [14]. The WHO report of 2002 states that at least 70% of the world population still believes in alternative medicine, and therapies which include homeopathy, Ayurveda, Siddha, Unani, Amchi, acupuncture, aromatherapy, herbal medicine in general, dietary supplements, nutraceuticals, Yoga, mind-body techniques, spirituality and faith healing, and massage. The current trend is indicative of a continuing demand for CAM therapies which will definitely have its impact on health care delivery in the future [15].

The term primary healthcare (PHC) has been interpreted in different ways. At its core, PHC is defined as a set of universally accessible services that promote health, prevent disease, and provide diagnostic, curative, rehabilitative, supportive, and palliative services. At the heart of PHC, reform is the goal to establish a holistic health and social-service system that emphasizes health promotion and disease prevention. With an emphasis which is holistic and more personalized, unlike the symptomatic approach of the orthodox system

of medicine, CAM should be able to complement the goals of PHC per se. Many of the concepts inherent to CAM are consistent with those recommended by already established PHC services. Integrating the health services may serve to enhance health care equity wherein all individuals may have access to a full range and combination of health care services that can contribute to reduced sickness and an increased health-related quality of life. To support and facilitate this endeavour, there is a need, as well as potential for an improved public health mandate, to monitor and promote the integration of CAM with PHC.

Despite a few attempts, it has yet to be established how to integrate CAM therapies into the conventional medical system in a systematic way. A logical first step in this direction of integration is to establish guidelines for the proper integration of CAM into primary care, supported by appropriate research and clinical experience. Unfortunately, the research data on this issue are quite limited. Recently, in the USA, the Federation of State Medical Boards developed and adopted new model guidelines for the use of complementary and alternative therapies in medical practice [16].

A summary of 26 surveys across 13 countries concluded that the prevalence of CAM use by cancer patients overall was 31.4% (range: 7% to 64%) [17]. Most cancer patients combine CAM with conventional therapy [18, 19]. Oncologists are becoming increasingly aware that patients use CAM, yet few oncologists discuss these therapies with patients. Instead, the established medical community is demanding regulation and evaluation of CAM [20]. Some groups insist that CAM poses serious health risks and cite poor outcomes for patients who reject proven conventional cancer treatment for CAM approaches [19]. The increasing interest in CAM among cancer patients may be due to limitations of conventional cancer treatment, increased advertising and media coverage of CAM, or the desire for holistic or natural treatments. Cancer patients want more information, and some patients believe that access to CAM should be part of standard cancer treatment [21]. As cancer incidence increases and survival time lengthens, the population seeking information about an access to CAM is likely to increase. A review examining the overlap between participation in allopathic breast cancer early detection activities and CAM use has been presented.

Herbal medicine (HM) is one of the most widely used complementary and alternative medicine (CAM) therapies used throughout the world. In many countries, HM has a long tradition and the knowledge about local medical plants is ingrained into cultural memory. The WHO estimates that 70–90% of the rural population in developing countries use HM to meet, in part or completely, their health needs [22]. Also, in many developed countries, HM as an element of CAM is highly popular. Therefore, HM is recognized as an essential component of primary healthcare by the WHO [22]. In this context, the perspectives and experiences of patients using HM in a primary care context have also been presented. Herbal medicines automatically take us to traditional Chinese medicine (TCM) which has always been popular in China and also among the Chinese in other parts of the western world. Hence, a study describing the utilization of TCM services by those attending the community health centres has been presented in detail including the prevalence and frequency of TCM use and health-related characteristics of TCM users. Unique dual medical system in the Republic of Korea has resulted in the emergence of dual licensed medical doctors (DLMD) who have both traditional Korean medicine (KM) and western medicine (WM) licenses. There have been few studies on DLMD in spite of their growing number and importance within the medical system. Hence a survey of the current status and attitudes of DLMD to assess their role in integrative medicine has been presented.

There are two major ways of bioprospecting natural products for investigation. First, the classical methods that rely on phytochemical factors, serendipity, and random screening approaches. Second, the use of traditional knowledge and practices as a drug discovery engine, which is known as the ethnopharmacological approach, which is a time and cost effective approach and could lead to better success than random screening [23, 24]. Traditional methods, for example, Chinese medicine, Japanese Kampo, and Indian Ayurveda, are becoming important bioprospecting tools [25]. Ethnopharmacological validations of some herbal/traditional medicine using modern pharmacological tools have been presented.

Most of the herbal drugs produced currently in the developing countries lack proper quality specification and standards and therefore have no consistency in quality in batch to batch products. Most of these drugs do not have well-defined and characterized composition. A well-experienced traditional physician in the past used to have specific knowledge and special ability to collect the right plants having the therapeutically useful agents from certain specific habitats. These experienced medicinal plant collectors had intimate knowledge of plant species and could identify therapeutically effective plant from a population of a species. With such unique expertise, they were able to maintain certain level of standards in the therapeutic quality of the herbal drugs. There had been a decline or almost extinction of such experienced plant collectors by the turn of the 20th century itself due to a variety of reasons. One of the reasons was the transformation of traditional medicine from the individualized system to a commercial manufacturing system. It is now well known that the therapeutic activity of a medicinal plant is due to the presence of certain biologically active chemical constituents, which are either primary or secondary metabolites. The expression of many of these compounds particularly those of the secondary metabolite category is controlled and conditioned by a variety of factors such as its genetic predisposition, habitat of the plant, agroclimatic conditions, season, and also the stage of growth and development of the plant. Finally, a method for quality evaluation of herbal drugs has also been presented.

Thus, the integration of the traditional and complementary medicine into PHC involves, understanding the CAM, knowing the need gaps in the existing system of medicine, validation of the activity of the traditional medicine to induce confidence in the practitioner/patient, and finally

the chemical standardization of the drugs to ensure proper quality of the herbal drugs.

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Research Article

Integrating Traditional Chinese Medicine Services in Community Health Centers: Insights into Utilization Patterns in the Pearl River Region of China

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In China's healthcare reform, community health centers (CHCs) are designed to take a pivotal role in providing primary care. Whilst about 20% of all outpatient care in China is delivered by the traditional Chinese medicine (TCM) sector, hospitals, instead of CHCs, are major providers. Using current patterns of patient utilization this study aims to inform CHCs on how they may strengthen access to TCM services. Three thousand three hundred and sixty CHC patients from six cities within the urban Pearl Delta Region were enumerated using multistage cluster sampling. Fifty-two percent had visited herbalists within three months with a mean visit frequency of 1.50 times. Herbal treatments, which are cheaper than western medicines, were more popular amongst those who needed to pay out of pocket including the uninsured. Herbal medicines appeared to be an alternative for those who are underinsured. Acupuncturists and massage therapists were visited by smaller proportions, 6.58% and 5.98%, respectively, with a mean three-month visit of 0.27 and 0.26 times. Access was restricted by lack of social insurance coverage. Whilst increasing provision of TCM in CHCs might respond to patient demand, increasing insurance coverage for TCM needs to be evaluated using current evidence on safety and effectiveness.

1. Introduction

1.1. Primary Care as the Cornerstone of Healthcare Reform in China. Strengthening primary care as a foundation and entry point of the healthcare system is the cornerstone of China's healthcare reform. Current reform proposals aim to position community health services as major providers of first-line care for common clinical problems [1]. Across China, the number of community health service facilities increased by threefold during 2001 to 2008 [1]. By 2010, China had established 33,000 community health services organizations across the nation, with some 29,500 employees [2, 3]. Amongst these organizations, Community Health Centers (CHCs) have been established and are usually buildings with areas of more than 1,000 square meters and with fewer than 50 inpatient beds. CHCs are purposefully established for providing medical and preventive outpatient services to a population of 30,000–50,000 [4]. Together with conventional biomedicine (BM), traditional Chinese medicine (TCM) is a formally recognized part of the Chinese healthcare system [5], and the central government has listed TCM as a required service in all CHCs [6].

1.2. Contribution of Traditional Chinese Medicine in Primary Care Service Provision. According to a 2004 national survey that covered both urban and rural China, 14% of the enumerated households identified TCM as their typical source of care [7]. This figure is consistent with the 2006 official data, which estimated that 10–20% of all healthcare services in China were provided by the TCM sector [8]. If only outpatient services were counted, the figure would fall in the higher end of this range. A large scale survey of 739,600 healthcare organizations undertaken in China in 2009 [9] reported that 19.2% of all outpatient consultations were managed by

TCM clinicians, which translates into 0.67 billion visits/year. Surprisingly, as little as 5.1% of this enormous volume of visits were to CHCs [9].

In general, distrust in the quality of care is a possible reason for deterring patients from choosing CHC services [10, 11]. In a recent patient satisfaction survey in Dalian, 91% of users had low trust in doctors working in CHCs, and 75% had no confidence in the quality of their services [12]. Another large scale survey found that only 35% of patients consider CHCs as a safe source of care [1]. Hence, hospitals remain to be the major providers of TCM outpatient services [13], instead of CHCs. However, under the context of privatization since 1990s, incentives to provide TCM care in hospitals are decreasing as herbal medicine prescriptions, acupuncture and massage therapies are not considered to be "revenue generating" in secondary care settings. TCM clinicians are inclined to replace their traditional practice with BM treatments [14]. As a result, there is a greater potential of promoting TCM in CHC settings.

1.3. Strengthening Traditional Chinese Medicine Services in Community Health Centers. To redress the mismatch of TCM underutilization in CHCs and overutilization in hospitals, top down policies from the central government have been promulgated. In 2006, the Ministry of Health and the State Administration of Traditional Chinese medicine jointly announced a mandate that each CHC must have at least one clinician specializing in TCM, together with a herbal pharmacy and equipment support [15]. Nevertheless, statistics from 2009 showed that only 51.6% of the nation's CHCs possessed the infrastructure for TCM service provision [9], and only 22% of the CHC clinicians offered TCM treatment [1]. Consequent on this lack of progress, improving the role of TCM in enhancing the nation's basic healthcare system has been restated as a goal in the national 12th Five-Year Plan and is also listed as one of the top 10 national health priorities in 2011 [16, 17]. If implemented successfully, the capacity of CHCs in providing TCM services would be enhanced significantly in the future. However, regardless of service location, TCM outpatient services are not fully covered by the three major healthcare insurance schemes in China, namely, New Rural Cooperative Medical Scheme (NCMS); Urban Employee Basic Medical Insurance (UEBMI); and Urban Residents Basic Medical Insurance (URBMI) [18]. It is however covered by the government sponsored care scheme [19].

The Pearl River Delta (PDR) region of the urban Guangdong province is an economic power house of China. It consists of six major cities: Shenzhen, Guangzhou, Foshan, Zhongshan, Zhuhai, and Dongguan, with a total of nearly 1000 CHCs providing care for a population of about 43.2 million, many of whom are migrant workers. Consistent with the central directives, the Guangdong government is keen to strengthen TCM services amongst CHCs within the PDR region [20]. In order to inform service redesign, a deeper understanding of the characteristics and health seeking pattern of patients who choose to use TCM services in the community is needed. This will allow the design of appropriate improvement strategies that are responsive to patients' choice.

1.4. Aim of the Study. This study described the utilization of TCM services by those attending CHCs. We investigated the (i) prevalence and frequency of TCM use as well as the (ii) demographic and health-related characteristics of TCM users as compared to those who only utilize BM services. In addition, we also examine the patients' (iii) reason for consultation and their perceived effectiveness of TCM treatments.

2. Methods

2.1. Sampling and Data Collection. CHCs for inclusion in the study were selected using multistage cluster sampling in six major cities in the Pearl River Delta: Guangzhou, Shenzhen, Dongguan, Zhuhai, Foshan, and Zhongshan. In the 1st stage, 4 districts in each city were randomly selected. In the 2nd stage, 1 neighborhood in each of the 4 districts was randomly selected. In the 3rd stage, one CHC was selected in each of the 4 neighborhoods. We estimated a total sample size requirement of 3360, which was calculated in accordance with the requirement for conducting multivariate analyses [21]. For each city except Guangzhou, data collection continued until the sample size of 480 was reached. Given its larger population and geographical size, we collected data from Guangzhou until a larger sample size of 960 was achieved.

In each CHC, all service users aged ≥18 were invited to participate in a face-to-face interview during the opening hours. We invited all patients who attended the CHCs until the required sample size was reached. A cash incentive of RMB $25 was offered to all participants who completed the questionnaire. Written informed consent was obtained from patients prior to the interview. Ethics approval was obtained from the Survey and Behavioral Research Ethics Committee of the Chinese University of Hong Kong.

2.2. Questionnaire Design. The questionnaire used in the interview consisted of two parts. The first part aimed to collect data on the respondents' demographic and health-related characteristics, including their gender, age, household registry status (*Hukou*), education level, household income, occupation, insurance status, self-perceived health status, and chronic disease status. In the second part, we assessed the use of BM and TCM using a modified International Complementary and Alternative Medicine Questionnaire (I-CAM-Q) [22]. Specifically, respondents were asked to indicate whether they had visited the following types of clinicians in the past 12 months, regardless of location: (i) western trained biomedical doctors (BMD); (ii) TCM herbalists; (iii) TCM acupuncturists; and (iv) TCM massage (Tuina) therapists. If the respondents provided a positive response, they were asked to specify consultation frequency in the past 3 months. Moreover, they were invited to indicate the main reason for visiting in the latest consultation. Four options were provided: (i) for acute condition that lasted <1 month; (ii) for chronic

condition that lasted ≥1 month; and (iii) for improving well-being. Finally, they were asked to evaluate treatment effectiveness on a scale including very helpful, somewhat helpful, not helpful at all, and do not know.

2.3. Data Analysis. Prevalence and its 95% confidence interval (CI) of past year consultation was reported for each type of clinician, together with their corresponding frequency of visits in the past three months. Multiple logistic regression analyses were conducted to identify demographic and health-related characteristics related to TCM use, with reference to those who only consulted BMD in the past year. A separate multiple logistic regression analysis was conducted for each of the modalities. Proportions and 95% CIs of main reason for consultation (visiting for acute conditions, chronic conditions, and well-being improvement) were calculated. Chi-square goodness of fit test was performed to test the equality of frequencies among different reasons for each type of consultation. For significant results of Chi-square goodness of fit test, post hoc one-sample Chi-square tests were conducted for pairwise comparison of two categories by setting the hypothesized frequencies of other categories as the observed frequencies. In addition, the proportions of self-reported treatment effectiveness were calculated together with their respective 95% CIs.

3. Results

3.1. Prevalence and Frequency TCM Service Use amongst CHCs Patients. Three thousand three hundred and sixty patients were interviewed within the prespecified quota from all six cities. The overall response rate was 86.1%. Table 1 displays the demographic and health-related characteristics of our respondents. In the past 12 months, prevalence of consulting BMD at least once is 91.37% (95%CI: 91.36%, 91.37%), with a mean three-month visit frequency of 3.35 (SD = 5.72). The prevalence for visiting a herbalist in the past year is 51.70% (95%CI: 51.68%, 51.71%), with a mean three-month visit frequency of 1.50 (SD = 3.62). Acupuncturists and massage therapists were visited by a smaller proportion of 6.58% (95%CI: 6.57%, 6.58%) and 5.98% (95%CI: 5.98%, 5.99%). The mean three-month visit frequencies for the two modalities were 0.27 (SD = 1.95) and 0.26 (SD = 1.69), respectively.

3.2. Demographic and Health-Related Characteristics of TCM Users. Compared to respondents who were financially covered by government sponsorship, multiple logistic regression analyses (Table 2) showed that those who paid by other means, including UEBMI, URBMI, NCMS, and out of pocket, were less likely to consult acupuncturists or massage therapists. Interestingly, those who paid out of pocket were more likely to use herbal medicine services, compared to those who were entitled to government sponsored care. Those who rated their health status as poor were also less likely to use acupuncture services compared to those who perceived to be in good health. Older respondents were more likely to consult herbalists, and those who completed secondary education were less likely. In all three multiple logistic regression analyses, we have more than 10 utilization events per independent variable; thus our sample size is sufficient for conducting such analyses [21].

Table 1: Demographic and health-related characteristics of respondents.

Characteristics	Number of respondents (%)
Gender	
Male	1423 (42.4%)
Female	1933 (57.6%)
Household registry[†]	
Resident with Hukou	1726 (51.4%)
Resident without Hukou	1284 (38.2%)
Nonresident without Hukou	349 (10.4%)
Education level	
Tertiary education or above	828 (24.7%)
Secondary education	1975 (58.9%)
Primary education or below	551 (16.4%)
Household income (¥)	
<1000	272 (9.9%)
1000–2000	903 (33.0%)
2001–3000	708 (25.9%)
3001–4000	333 (12.2%)
4001–5000	235 (8.6%)
>5000	285 (10.4%)
Insurance status[#]	
Government sponsored medical care	249 (7.5%)
UEBMI	905 (27.1%)
URBMI	707 (21.2%)
NCMS	470 (14.1%)
Commercial insurance	22 (0.7%)
Out of pocket	982 (29.4%)
Self-perceived health status	
Excellent	207 (6.2%)
Very good	735 (21.9%)
Good	886 (26.4%)
Fair	1378 (41.0%)
Poor	152 (4.5%)
No. of chronic diseases	
0	2194 (65.3%)
1	830 (24.7%)
2	239 (7.1%)
3	83 (2.5%)
4	12 (0.4%)
5	2 (0.1%)
Mean age (SD)[*]	43.4 (17.05)

[*]Data are presented as mean (SD).
[†]Hukou: household registration; resident with hukou: permanent resident. Resident without hukou: temporary resident without local household registration but lived in the city ≥6 months. Nonresident without hukou: temporary resident without local household registration and lived in the city <6 months.
[#]UEBMI: urban employee basic medical insurance; URBMI: urban resident basic medical insurance; NCMS: new cooperative medical scheme.

Table 2: Association between TCM usage and demographic and health characteristics: multiple logistic regression analyses.

TCM modalities	Herbalists		Acupuncturists		Massage therapists	
Demographic characteristics	Adjusted OR (95% CI)	*P* value	Adjusted OR (95% CI)	*P* value	Adjusted OR (95% CI)	*P* value
Gender						
Male (reference)	1.000		1.000		1.000	
Female	1.155 (.988, 1.351)	.070	.822 (.603, 1.119)	.213	.936 (.676, 1.297)	.692
Age	1.007 (1.000, 1.014)	.039	1.008 (.995, 1.021)	.255	1.007 (.993, 1.020)	.348
Hukou status						
Resident with Hukou (reference)	1.000		1.000		1.000	
Resident without Hukou	.870 (.714, 1.061)	.169	.985 (.657, 1.478)	.943	.851 (.551, 1.315)	.468
Non-Resident without Hukou	.829 (.614, 1.119)	.221	1.432 (.807, 2.540)	.219	.787 (.388, 1.594)	.505
Education level						
Tertiary education (reference)	1.000		1.000		1.000	
Secondary education	.764 (.622, .940)	.011	.820 (.550, 1.223)	.331	.802 (.532, 1.209)	.292
Primary education	.783 (.575, 1.066)	.121	.637 (.341, 1.190)	.157	.550 (.284, 1.066)	.077
Monthly household income	1.005 (.947, 1.067)	.860	1.091 (.967, 1.232)	.157	1.040 (.919, 1.178)	.534
Health insurance status						
Government sponsored care (reference)	1.000		1.000		1.000	
Out of pocket	1.507 (1.045, 2.174)	.028	.411 (.215, .786)	.007	.333 (.176, .631)	.001
Urban employee basic medical insurance	1.127 (.811, 1.567)	.476	.506 (.291, .881)	.016	.356 (.210, .601)	.000
Urban resident basic medical insurance	1.063 (.748, 1.509)	.734	.542 (.297, .987)	.045	.356 (.198, .641)	.001
New cooperative medical scheme	1.119 (.764, 1.637)	.565	.498 (.253, .982)	.044	.210 (.098, .452)	.000
Self-reported health status						
Good or above (reference)	1.000		1.000		1.000	
Fair	1.020 (.864, 1.203)	.817	.959 (.693, 1.327)	.802	1.189 (.843, 1.676)	.323
Poor	1.087 (.731, 1.617)	.680	.196 (.047, .824)	.026	.701 (.284, 1.734)	.442
Number of chronic disease						
0 (reference)	1.000		1.000		1.000	
1	1.174 (.960, 1.436)	.119	1.092 (.739, 1.614)	.658	.927 (.605, 1.422)	.730
2	1.004 (.715, 1.410)	.982	.692 (.333, 1.436)	.323	.904 (.463, 1.766)	.767
≥3	1.178 (.728, 1.907)	.505	1.116 (.470, 2.648)	.804	1.533 (.717, 3.279)	.271

3.3. Main Reason for Consultation and Perceived Effectiveness. Figure 1 shows the main reasons for consultation for the most recent visit within the past twelve months, stratified by treatment modalities. For all modalities, the null hypotheses of Chi-square goodness of fit tests (all categories of main reason for consultation occur with equal probabilities) were rejected at $P < 0.001$. Majority consulted BMD for treatment of acute conditions, which is a significantly higher proportion ($P < 0.001$) compared to patients visiting for chronic conditions. A very low proportion of patients consulted a BMD for improving well-being. The inverse pattern was observed for massage therapy. A very low proportion received massage for acute conditions, and the figure is significantly ($P < 0.001$) lower than the proportions of patients who visited for chronic condition or well-being improvement. The distribution for acupuncture was similar. Compared with patients seeking help for their chronic conditions, proportion of those consulting acupuncturists for acute conditions is significantly lower ($P < 0.001$); but not the proportion seeking improvement in wellbeing ($P = 0.153$). Although TCM herbalists appeared to have a more balanced patient profile, proportion of consultation for acute conditions is significantly ($P = 0.032$) lower than that for chronic condition. A much lower proportion consulted herbalists for well-being improvement. Regardless of modality, the majority of patients found the treatment very helpful or somewhat helpful (Figure 2).

4. Discussion

Our results indicated that not only is provision of TCM a government policy but also it is a popular choice for patients, used by many CHC attendees. Other studies have found that patients purposefully chose these modalities to fill the perceived effectiveness gaps of BM [23]. Herbal medicine, acupuncture, and massage therapies are preferred in the treatment of chronic conditions which have lasted for more than 1 month, while BM remained to be the most popular option for acute conditions. More than 20% of acupuncture and massage service users sought to improve well-being, but only very low proportion of visits to western trained doctors was for this purpose. This is a common pattern and the challenge to policy makers and service providers is to develop services which reflect the different choices patients make for

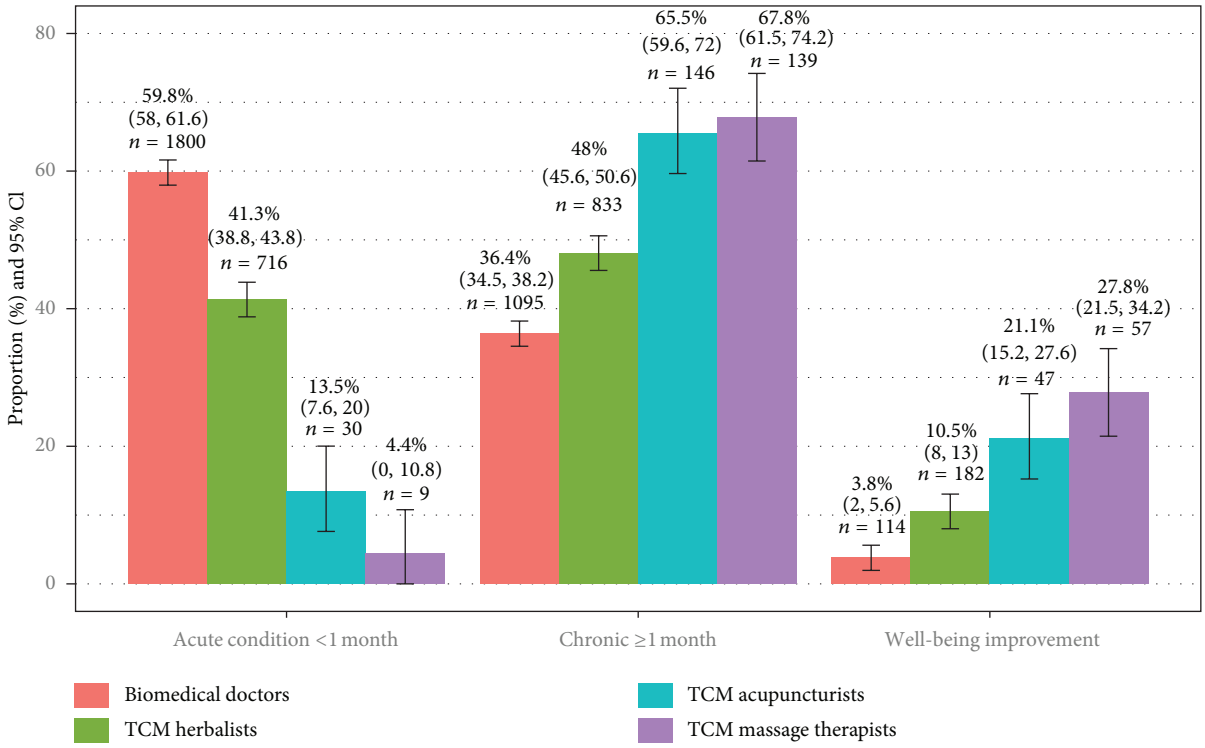

Figure 1: Main reason for visiting in the latest consultation, stratified by treatment modalities.

services at different stages of ill health. This does, however, raise interesting questions about the evidence of effectiveness between modalities and the influence of affordability in the trade-offs made by patients.

4.1. Chinese Herbal Medicine Services as an Accessible Form of Care for the Uninsured or Underinsured. Chinese herbal medicine appeared to be the most popular form of TCM amongst those attending CHCs. More than half had consulted a TCM herbalist in the past year. This is consistent with previous studies which have found that herbal medicine services are more popular amongst older segment of the population, as well as the better or poorer educated [7, 24]. Since in China a qualified TCM herbalist can prescribe both conventional western drugs as well as herbs in a single consultation [14], users of herbalist services included both acute and chronic patients. This contrasts with findings from the West where herbalists are often consulted to complement BM treatment amongst chronic disease patients [25].

Of note, herbalist service appeared to be more popular amongst those who needed to pay out of pocket, which includes those who have no or minimal outpatient insurance coverage and who choose to use herbal medicine as an alternative to more expensive BM drugs. The inclusion of Chinese herbal medicines under the 2009 National Essential and Health Insurance Drug Lists has made them financially affordable to most patients. Amongst the 307 medications on the Chinese National Essential Drug List, 102 are herbal products and they are sold at the guaranteed lowest price. The National Health Insurance Drug List has also included a total of 683 herbal medications, of which the maximum co-payment is only 10% [13, 19].

4.2. Financial Barriers Hinder Access to Acupuncture and Massage Services. In contrast to herbal medicine, prevalence of acupuncture and massage service use is much lower. CHC attendees seem to reserve these options for the treatment of chronic conditions or well-being improvement, instead of using them for managing acute conditions. This pattern is consistent with previous studies in China, where acupuncture was perceived to have special strength in certain chronic disorders [26] and was less useful in managing common ailments [27]. Also, it is believed that patients with poorer health are more susceptible to adverse effects of acupuncture [28]. This may explain why respondents with poor self-rated health are less likely to consult an acupuncturist.

In addition to patients' perception of the appropriateness and effectiveness of the TCM treatment, financial barriers offer additional explanation for lower utilization rates of acupuncture and massage services. Our results demonstrated that those who possess NCMS, UEBMI, and URBMI are less likely to use these services, as the reimbursement mechanisms of these plans often fail to cover treatment costs. In the majority of NCMS and UEBMI plans, outpatient services are often paid through personal medical saving accounts (MSA), and patients are expected to pay out of pocket when the MSA fund is exhausted. For URBMI, pooled funds only cover outpatient services for targeted chronic or catastrophic diseases [29]. The financial pressure of paying out of pocket for acupuncture and massage services, which often require

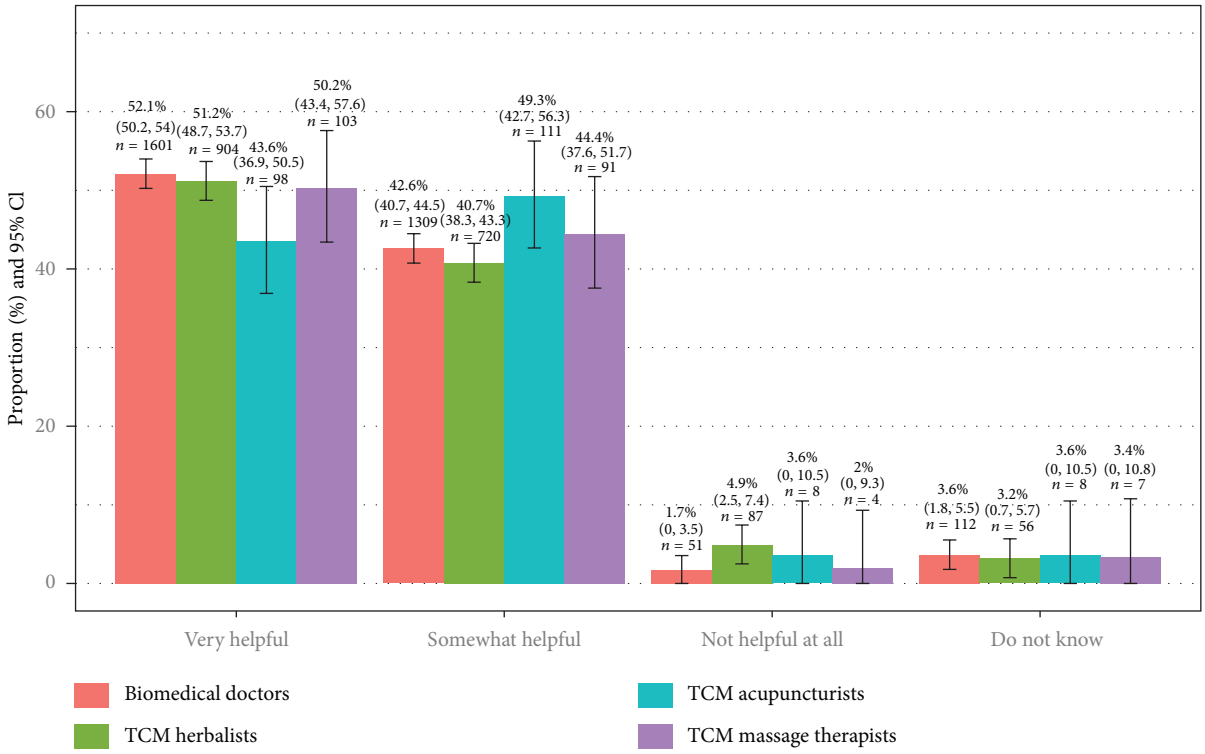

Figure 2: Perceived effectiveness of treatment received in the latest consultation episode, stratified by modalities.

multiple sessions of treatment, may have deterred patients from using them.

4.3. Strengths and Weaknesses of This Study. The most notable strength of this study is that we avoided selection bias by using a multistage cluster sampling strategy. This allowed us to draw a representative sample of CHCs attendees in all six major cities within the urban PDR region. Meanwhile, our study has a number of limitations. First, cross-sectional design has inhibited us from drawing a causal conclusion. A cohort study is needed to ascertain whether the associations observed between TCM use and various health and demographic factors are causal. Second, CHCs attendees were asked to recall the outpatient service utilization and thus recall bias could have led to inflation or deflation of visit frequencies. The use of pretested I-CAM-Q items has allowed us to collect standardized utilization data that allows international comparison [30]. It has demonstrated strong face validity and acceptability in our sample but formal assessment on the Chinese version's reliability and validity is needed in the future. Finally, although we achieved a high response rate, we were unable to assess the potential impact of nonresponse bias due to a lack of sampling frames that contain background information of nonrespondents.

4.4. Further Research. Policy makers in China are adopting strategies to facilitate the channeling of TCM patients from hospitals to CHCs. Encouraging current hospital attendees to use locally based TCM services could be a first step in moving patients to the community. Future studies are needed to compare TCM users' profiles in CHCs and outpatient departments of hospitals and investigate factors that facilitate or hinder the use of CHCs based TCM services. In a similar vein, studies that examine why some patients only use BM, but not TCM services in CHCs settings is also warranted. Finally, future investigations using qualitative method is needed to triangulate our current quantitative findings. For example, focus groups or in-depth interviews maybe conducted for exploring how patients managed the impact of different financial barriers on the use of different TCM modalities.

5. Conclusion

TCM is popular amongst CHCs attendees in the urban PDR region, especially services provided by herbalists. Due to the lower cost of herbal medicines, such service may be regarded an alternative treatment of choice for those who need to pay out of pocket. CHCs in the region may have the potential of increasing herbal medicine use by up-scaling their herbalist services. However, since the efficacy and safety of many Chinese herbal medicines remains to be uncertain [31], evidence-based decision making in this area is a complex problem. Large scale randomized controlled trials of herbal medicines included in the Essential and Health Insurance Drug Lists should be given research priority to enable better understanding of on their efficacy and safety, either prescribed alone or with allopathic drugs [32]. On the other hand, while acupuncture and massage appear to have a relatively stronger evidence base supporting their efficacy and

safety [33, 34], their access is limited by financial barriers. This implies that simply strengthening provision of these services at CHCs may not increase utilization until NCMS, UEBMI, and URBMI extend their coverage on outpatient services. In the longer term, decisions on planning and funding of TCM services in CHCs should be made after considering clinical evidence, cost effectiveness, and patients' choice.

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Research Article

Medical Practices and Attitudes of Dual-Licensed Medical Doctors in Korea

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Unique dual medical system in Korea has resulted in the emergence of dual-licensed medical doctors (DLMDs) who have both traditional Korean medicine (KM) and Western medicine (WM) licenses. There have been few studies on DLMDs in spite of their growing number and importance within the medical system. We surveyed the current status and attitudes of DLMD to assess their role in integrative medicine. Questionnaires were administered to the members of the association of DLMD. Data from 103 DLMD were collected and statistically analyzed. 41.4% of DLMD were copracticing both WM and KM at a single clinic, preferring the WM approach for physical examinations, laboratory tests, and education for patients—and the KM approach for treatment and prescription. Musculoskeletal, gastroenterologic, and allergic diseases were considered to be effectively treated with co-practice. DLMD highly agreed on the efficiency of copractice for disease control and patients' satisfaction. On the other hand, they regarded the lack of health insurance coverage for copractice and increased medical expenditure as major problems in providing co-practice. To expand the role of DLMD as mediators of integration in primary health care, the effectiveness of their co-practice should be evaluated and a corresponding health insurance reimbursement system should be established.

1. Introduction

Though the National Center for Complementary and Alternative Medicine declared that complementary and alternative medicine (CAM) is not to be considered an integral part of conventional western medicine (henceforth abbreviated "WM") at present [1], since the introduction of CAM, the terms and concepts of CAM have gradually been integrated into mainstream medicine in many Western countries; likewise disease-centered biomedicine has shifted to holistic patient-centered medicine [2–5]. An increasing number of physicians in Western countries have interest in studying and practicing CAM nowadays [6–9], and many medical schools have included CAM-related content in their compulsory curriculum in the United States [10].

Some Asian countries, however, where traditional medicine exists as a whole medical system, such as Ayurveda or traditional Chinese medicine (TCM), possess their own dedicated and independent medical system due to idiosyncratic historical factors [11]. For example, in China and Taiwan, TCM and WM exist harmoniously and function complementarily while their integration is an ongoing project supported nationally [12, 13]. Ayurveda in India is practiced conventionally and independently from WM on a national scale [11], and recently the government set more rigorous standard requirements for Ayurvedic education and practice than in the past [14, 15].

Korea has a unique dual system where WM and traditional medicine exist on equal terms with exclusive practice boundaries. Traditional Korean medicine (referred to in this paper as "KM") is not regarded as CAM, but as a part of conventional medicine, mainly due to having its own education and licensing system since the 1950s. Though this kind of system has been advantageous in preserving traditional medicine, it has given rise to institutionalized conflict between the two medical disciplines [16]. In spite of the conflict, KM doctors have tried to combine KM and WM since the inception of KM hospitals in the 1970s; and dual-licensed medical doctors (DLMDs) who had both KM and WM licenses began to emerge and their ranks have grown

steeply during the 2000s. Since there have not been special license examinations or integrated education programs such as those in Taiwan and China, it is even more difficult to become DLMD in Korea. The portion of DLMD among medical doctors at large is much smaller than that of Taiwan [12] and the number of an DLMD is around 200 for now. Nevertheless, they draw more and more attention and are expected to assuage the conflicts and to mediate between KM and WM.

CAM trained medical doctors in Western countries are regarded as mediators in the integration of distinct and different modalities, and studies on their role and attitudes are emphasized [2, 12, 17]. There have been some studies on the attitudes of WM or KM doctors towards the cooperation of KM and WM [18–22]. The studies, however, on DLMD are rare [23]. In this study, we introduced the current situation of DLMD in Korea, investigated their experiences with cooperative practices, and discussed their roles in the future.

2. Methods

2.1. Sample and Data Collection. This study is a cross-sectional survey. The questionnaires on the characteristics of the medical practices and attitudes toward copractice were developed and administered to both DLMD and medical students who were already KM or WM doctors and preparing to obtain a second medical license. After obtaining informed consent, the questionnaires were sent by e-mail based on the members' information received from the association of DLMD. The questionnaires were sent twice in January 2011 to 190 members whose e-mail addresses had been verified.

2.2. Questionnaire. The questionnaire consists of questions about the following topics: demographics, motive for obtaining dual medical licensure (DML), medical practice, and attitude toward co-practice of the two medical disciplines. Questions about the topic "medical practice and attitude" were measured mainly with 5-point Likert scale (1 = strongly disagree, 3 = neutral, and 5 = strongly agree).

Some terms were defined as follows so as to meet the purpose of this study. "DLMD" refers to those who already have obtained the other license additionally after having obtained a WM or KM license, or students who are preparing for their second license. We included students (prospective DLMD) because they are supposed to obtain DML in the near future, and many of them were doing part time practice and identified themselves as DLMD. "DLMD duration" refers to the duration (in years) from the acquisition of the second license to the moment of participating in the survey. Those who answered as students were assigned a value of 0. "Co-practice" represents the practice in which a single DLMD combines KM and WM procedures or drugs for any given patient. In contrast, "cooperation" denotes the same practice except done by two practitioners—a WM doctor and a KM doctor.

2.3. Statistics. SPSS 18.0 software was used for statistical calculations. Missing values were clearly presented in the results of each question. Response percentage was presented for questions with no responses or questions with multiple responses ranging from 1 to 5 answers.

χ^2 test and t-test were used in order to compare the characteristics and attitudes of participants, and 95% confidence interval or "mean ± SD" was indicated.

3. Results

Among the 190 members of the Association of DLMD receiving the questionnaire, 103 replied within 4 weeks (54.2% response rate). The general characteristic of respondents is presented in Table 1. We made two-group designations to classify the participants: "KM-based DLMD" and "WM-based DLMD." KM-based DLMD refers to who obtained KM doctor licenses first, while WM-based DLMD are those who first obtained WM doctor licenses.

Most respondents were male (82.5%); 24.5% were students; and the average age was 40 years old (40.35 ± 8.373). Mean age of WM-based DLMD was approximately 5 years higher than that of KM-based DLMD. More than 60% of DLMD replied that they obtained DML within the last five years. As for DLMD duration, the most frequently occurring response among the WM-based group was less than 5 years (48.3%), whereas KM-based DLMD were most likely students preparing for DML (45.2%). 41.4% of respondents have opened clinics where they practiced both WM and KM at a single site (hereafter, such a site will be called a "WM-KM clinic"). Nearly half of the WM-based DLMD (48.3%) and 27.6% of KM-based DLMD were working in WM-KM clinics. The percentage of respondents who have obtained or are scheduled to obtain board certification as WM specialists (37.4%) is greater than that of those having obtained or pursuing the KM one (6.9%). The percentage of KM-based DLMD pursuing or already having obtained WM specialist qualifications is significantly higher than that of their WM-based counterparts.

The values for motives for obtaining DML were measured with a 5-point Likert scale and the average was calculated (Table 2). All of the means were close to "agree." "I have been interested in using KM (WM) modalities in my practice." scored the highest (4.17 ± 0.071), followed by "I thought obtaining DML would give me a competitive advantage over other doctors" (3.78 ± 0.086), and "I wanted to formulate a new medical discipline integrating both WM and KM" (3.70 ± 0.098). WM-based DLMD showed the same order of strength in the motives as the whole group. However, there was a significant difference ($P < 0.01$) in the responses for the question "I thought WM (or KM) by itself has limitations in diagnosis and treatment" between KM-based and WM-based DLMD. KM-based DLMD more strongly agreed (3.79 ± 0.645) than WM-based DLMD (3.25 ± 1.092), and consequently, it has turned out to be the second most important motive in the KM-based DLMD.

The disease condition considered to be the most effectively treated with copractice was musculoskeletal disease (71 responses), followed by gastroenterologic (61 responses)

Table 1: Characteristics of respondents.

	Total $n = 103$	WM based $n = 60$	KM based $n = 43$	χ^2 or T
Gender	($n = 103$)	($n = 60$)	($n = 43$)	
Male	82.5%	85.0%	79.1%	0.611
Female	17.5%	15.0%	20.9%	
Age				
Mean (±SD)	40.35 (±8.373)	42.13 (±6.721)	37.86 (±9.795)	2.627*
DLMD duration (yr)	($n = 102$)	($n = 60$)	($n = 42$)	
0 (student)	24.5%	10.0%	45.2%	
≤5	40.2%	48.3%	28.6%	
6–10	20.6%	26.7%	11.9%	20.371***
11–15	10.8%	13.3%	7.1%	
>5	3.9%	1.7%	7.1%	
Mean (±SD)	4.74 (±5.667)	5.23 (±6.721)	4.02 (±7.141)	1.061*
Work place	($n = 87$)	($n = 58$)	($n = 29$)	
WM- KM clinic	41.4%	48.3%	27.6%	
WM institution	26.4%	29.3%	20.7%	11.201*
KM institution	21.8%	19.0%	27.6%	
Others	10.3%	3.4%	24.1%	
WM specialist (present or prospective)	($n = 99$)	($n = 59$)	($n = 40$)	
Yes	37.4%	27.1%	52.5%	6.561*
KM specialist (present or prospective)	($n = 101$)	($n = 59$)	($n = 42$)	
Yes	6.9%	3.4%	11.9%	2.758

* $P < 0.05$, *** $P < 0.001$.

Table 2: Motives for obtaining dual medical licensure.

	Mean (±SD)			T
	Total	WM based	KM based	
(1) I have been interested in using KM (WM) modalities in my practice	4.17 ± 0.719	4.23 ± 0.698	4.07 ± 0.745	0.265
(2) I thought obtaining DML would give me a competitive advantage over other doctors	3.78 ± 0.860	3.88 ± 0.796	3.64 ± 0.932	0.176
(3) I wanted to formulate new medical discipline integrating both WM and KM	3.70 ± 0.980	3.81 ± 0.972	3.56 ± 0.983	0.210
(4) I wanted to help mediate between WM and KM	3.64 ± 0.944	3.67 ± 0.886	3.60 ± 1.027	0.723
(5) I thought WM (KM) by itself has limitations in diagnosis and treatment	3.48 ± 0.965	3.25 ± 1.092	3.79 ± 0.645	0.003**

Different set of statements was given to each of the two groups.
To WM-based DLMD: (1) "I have been interested in using KM modalities in my practice."
(5) "I thought WM by itself has limitations in diagnosis and treatment."
To KM-based DLMD: (1) "I have been interested in using WM modalities in my practice."
(5) "I thought KM by itself has limitations in diagnosis and treatment."
** $P < 0.01$.
Note: 5-point Likert scale (1 = strongly disagree, 3 = neutral, and 5 = strongly agree).

and allergic disease (53 responses) in multiple responses (Figure 1).

We asked about the use of WM and KM modalities in usual practice with 5-point Likert scale (−2 = strongly WM approach; 0 = both equally; 2 = strongly KM approach) (Figure 2). Participants have preferred the WM approach over the KM approach for "physical examinations", "laboratory tests," and "education for patients," while they preferred the KM approach slightly over the WM approach for "treatment" and "prescription," especially in KM-based DLMD. WM-based DLMD preferred WM modalities for "treatment" (−0.02 ± 1.239), while KM-based DLMD more often used KM

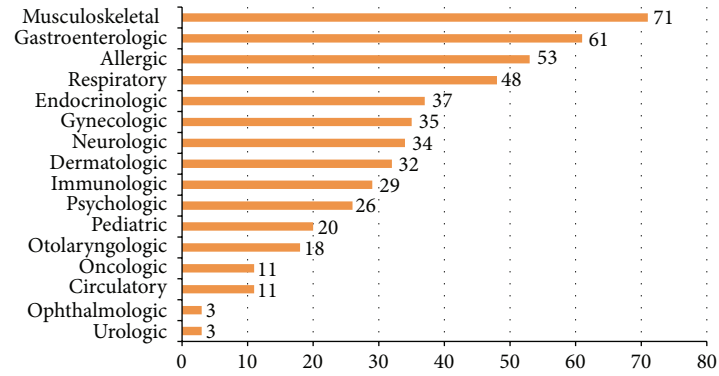

Figure 1: Disease conditions considered to be most effectively treated with copractice by DLMDNote. Multiple response(s): $n = 103$.

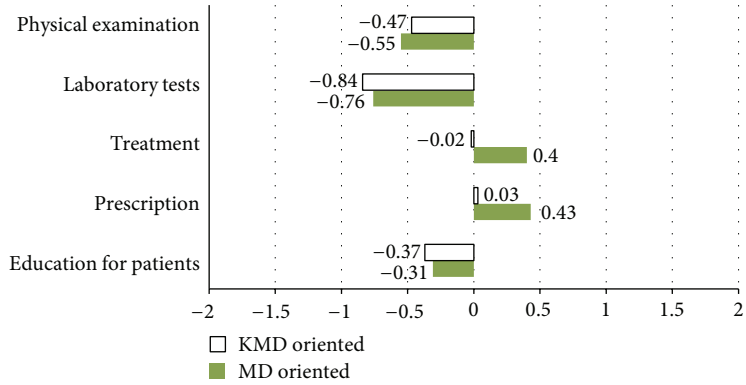

Figure 2: Use of WM and KM modalities in DLMD' practice. Note: 5-point Likert scale (−2 = strong WM approach, 0 = both equally, and 2 = strong KM approach).

modalities (0.40 ± 1.270). The two groups showed opposite, albeit not statistically significant, tendencies.

Figure 3 shows that DLMD generally took a positive view of the effect of co-practice. They thought that co-practice is more efficient in most of disease control (3.92 ± 0.788) and that patients were more satisfied with co-practice than WM or KM alone (3.88 ± 0.637). On the other hand, they identified some problems in providing co-practice: lack of health insurance coverage for co-practice (4.34 ± 0.682); increased patient's medical expenditures (3.32 ± 0.867); and difficulties in maintaining facilities and space for co-practice (3.24 ± 0.818). Intellectual incompatibility between WM and KM does not seem to be a great difficulty in co-practice (2.62 ± 0.985).

4. Discussion

Although KM shares a common origin with TCM, Korea has developed distinctive traditional disciplines and practices [24]. KM is not regarded as CAM in Korea, but rather as a subgroup within conventional medicine, legally speaking. However, many of the KM modalities are not covered by the national health insurance system yet, and KM comprises only a small portion of national insurance expenditures—around 4% of the total. While the medical system of China allows TCM doctors to freely use WM drugs and examinational instruments, in stark contrast, the use of WM modalities by KM doctors is prohibited completely by medical law in Korea. The number of DLMD in Korea has risen since the late 1990s and has rapidly increased during the 2000s [23]. According to the official government statistics in 2010, the total number of WM doctors was 101,307; KM doctors numbered 19,055; and DLMD 206 [25]. Rising interest in the rival discipline can be attributed to the following factors: (1) increased interest in cooperation and the number of cooperating hospitals since the 1990s [26]; (2) the steep climb in the number of doctors, resulting in intense competition; and (3) regulations limiting KM doctors' practices.

Compared to Taiwan, where 7.6% of WM doctors are DLMD [12], the number of DLMD is still very small in Korea. DLMD constitute approximately 0.2% and 1%, respectively, of WM doctors and KM doctors, at large. Nevertheless, the recent increase in the number of DLMD has become a catalyst for legal and systemic change, resulting in the 2009 legislation making provisions for WM-KM clinics run by DLMD. Before this legislation, DLMD were not allowed to practice WM and KM simultaneously in one clinic, so they had to exclusively choose one or the other for their practice. There are still limitations on DLMD' practicing both modalities in hospital settings.

4.1. Current Status. 85.3% of the respondents obtained DMLs in less than 10 years as shown in Table 1, which means many have done it recently or are scheduled to. As stated in the

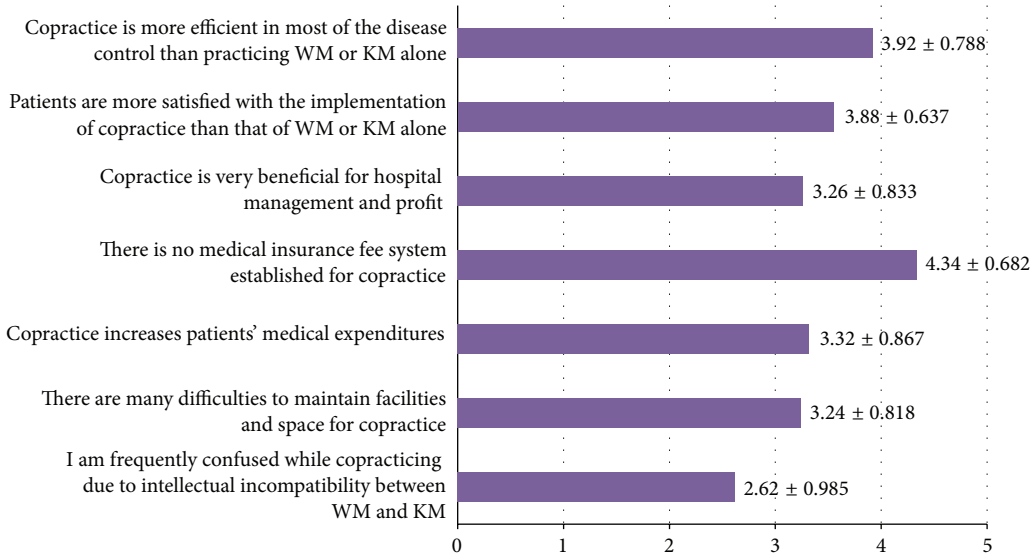

Figure 3: Attitudes of DLMD toward copractice of WM and KM. Note: 5-point Likert scale (1 = strongly disagree, 3 = neutral, and 5 = strongly agree).

preceding study, the number of DLMD is expected to increase continuously [23]. The percentage of WM-based DLMD was 58.8% and the student portion among them was 10.0%. In comparison, the percentage of KM-based DLMD was 41.2% and students constituted 45.2% of them. The number of KM-based DLMD is growing faster than WM-based DLMD. This implies that KM-based DLMD can become a major constituent of DLMD in the not-too-distant future. The percentage of DLMD that are present or prospective WM specialists is 37.4%, which is significantly lower than the percentage of specialists among WM doctors at large—around 70% in 2010. The number of KM specialists is also lower than that of KM doctors as a whole—around 10% in 2010 [25]. We can conservatively predict steady growth in the number of qualified medical specialists among DLMD.

Considering that approximately 41% are working or willing to work in WM-KM clinics notwithstanding the fact that the law was legislated only 2 years ago, the number of DLMD working in WM-KM clinics is expected to increase as well. Hospitals and clinics established by DLMD will have a competitive advantage over other medical institutions; and if the ranks of DLMD grow, they will position themselves as a third classification of medical doctors within the dual medical system of Korea. This change may accelerate discussion on the topic of integrated medicine, particularly when governmental efforts toward integration are not enough to produce good results.

4.2. Practice of DLMD. The disease conditions considered to be the most effectively treated with co-practice by DLMD were shown to be musculoskeletal, gastroenterologic, and allergic diseases—which are common in primary health care. This result is similar to the previous study [26], where doctors working in cooperative hospitals indicated that musculoskeletal and immunologic diseases are more effectively treated with cooperation than other disease categories. By contrast, this result is different from other studies on the attitudes of doctors toward cooperation, where cerebrovascular (circulatory) disease ranked the highest [18, 21]. We assume that since our respondents were mainly working at local clinics, they were unlikely to indicate "cerebrovascular disease," the majority of which is observed in inpatients.

As for the results of the use of WM and KM modalities in practice, there exist different tendencies according to the areas of practice. The reason for WM being preferred for examinations and laboratory tests is that KM doctors are legally restricted from using diagnostic devices, thus having limitations in examination and diagnosis. Moreover, it seems that participants regarded using WM terms and approach as advantageous in explaining prognosis and progress during education for patients. On the other hand, they showed preference for KM modalities in the treatment and prescription process. This seems most likely due to the fact that KM has various treatment modalities such as acupuncture, moxibustion, cupping, chu-na, and herbal medicine. Similar results are shown in Lee and Yoo's study about attitudes of WM and KM doctors toward cooperation. KM doctors showed high trust in WM laboratory tests, diagnostic tools such as X-ray, CT, MRI, and so forth. In contrast, WM doctors highly valued acupuncture, moxibustion, cupping, and constitutional discerning tools [21].

4.3. Cognition and Attitudes of DLMD. This study also investigated DLMD' attitudes such as motives for obtaining DML and merits and difficulties related to co-practice, which seems important for their prospects.

As for the motives for obtaining DMLs, interest in using other medicinal modalities (4.17 ± 0.719) scored the highest; and limitations of each modality scored relatively low (3.48 ± 0.965) as a whole and among the WM-based

DLMD, however, KM-based DLMD more strongly agreed to the limitations of KM. As we mentioned before, these results seem to reflect the legal restrictions on using WM devices by KM doctors.

The greatest difficulty related to co-practice seems to come from discrepancies between the current medical laws and the insurance system. In the Korean national health insurance system, WM and KM each has its own reimbursement system. Since a reimbursement system for co-practice is yet to be established, a DLMD who has used both WM and KM modalities can only be reimbursed for the predominant treatment, which increased out-of-pocket expenses for the patient.

By contrast, intellectual incompatibility between WM and KM was not considered to be a great difficulty in co-practice (2.62 ± 0.098). This is one of the major findings of our study and a significant divergence from other research pertaining to doctors' attitudes toward cooperation. Other studies identified differing approaches to disease [19–21] and intellectual incompatibility in clinical practice [18] as the greatest difficulties and the reasons for poor cooperation. This means that these aforementioned factors can become obstacles when cooperation is only a parallel implementation of WM and KM modalities, but these hindrances diminish when a single individual practices the two medical modalities. This implies that one of the keys to resolve the conflict between WM and KM lies in training more professionals like DLMD via diverse integrated education courses.

4.4. Limitations and Achievements. One limitation of this study is that we could not survey the whole population of DLMD. Our study included 25 prospective DLMD and 78 present DLMD—around 38% of all present DLMD. Nevertheless, this study is the first to investigate DLMDs' status, attitudes, and perspectives, which may prove invaluable insofar as concrete implications for roles and prospects of DLMD within the Korean medical system may be drawn. Further study soliciting responses from all DLMD would be required to investigate their actual co-practice in detail and formulate an integrative practice model that would be practical and efficient. Comparative effectiveness research on their practice would be also needed for reimbursement by the national health insurance system.

5. Conclusion

Korea faces a less favorable situation than some other countries when it comes to integrating medicine due to its exclusionary, dichotomous medical system. Even though the number of DLMD is still very small, their co-practice can be regarded as a viable way to deal with the situation. Our study suggests that misunderstanding and conflict between different approaches, which are somewhat inevitable between WM and KM doctors, can be mitigated by DLMD. Our study also shows a majority of DLMD have treated common diseases in local clinics by implementing a unique practice model combining modalities of both WM and KM. It is certain that the experiences and suggestions of DLMD will become valuable in the process of integrating medicine especially in primary health care. Development of diverse integrated education courses for both disciplines could accelerate the integration by increasing the number of DLMD-like medical professionals. To expand the role of DLMD as mediators of integration in primary health care, the effectiveness of their co-practice should be evaluated and a corresponding health insurance reimbursement system should be established as soon as possible.

Authors' Contribution

J. Ryu and B. Choi have equally contributed to this paper.

Acknowledgments

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Research Article

Quality Evaluation of Ayurvedic Crude Drug *Daruharidra*, Its Allied Species, and Commercial Samples from Herbal Drug Markets of India

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Berberis aristata known as "*Daruharidra*" in Ayurveda is a versatile medicinal plant used singly or in combination with other medicinal plants for treating a variety of ailments like jaundice, enlargement of spleen, leprosy, rheumatism, fever, morning/evening sickness, snakebite, and so forth. A major bioactive marker of this genus is an alkaloid berberine, which is known for its activity against cholera, acute diarrhea, amoebiasis, and latent malaria and for the treatment of oriental sore caused by *Leishmania tropica*. Although the roots of *B. aristata* are considered as the official drug (Ayurvedic Pharmacopoeia of India), the study revealed that different species of *Berberis,* namely. *B. asiatica*, *B. chitria,* and *B. lycium* are also used under the name of *Daruharidra* in different parts of the country. Detailed physicochemical and phytochemical studies of subjects like total ash, acid insoluble ash, tannins, and total alkaloids were calculated from the shade dried powdered material according to the recommended procedures. Further, heavy metal studies and quantitative estimation of berberine through HPTLC have also been performed as per ICH guidelines. A detailed study of four *Berberis* species, namely *B. aristata*, *B. asiatica*, *B. chitria,* and *B. lycium,* which are implicated as *Daruharidra* and collected from wild and ten commercial samples procured from various important drug markets in India has been carried out, which may be useful to pharmaceutical industries for the authentication of the commercial samples and exploring the possibilities of using other species as a substitute of *B. aristata*.

1. Introduction

Berberis aristata known as *"Daruharidra"* in Ayurveda is a versatile medicinal plant used singly or in combination with other medicinal plants for treating a variety of ailments like jaundice, enlargement of spleen, leprosy, rheumatism, fever, morning/evening sickness, and snakebite, and so forth [1–4]. In addition, the decoction of root or stem of *Berberis* known as "*Rasaut*" is specifically used in eye disease, skin disorders, and indolent ulcers. Its use in the management of infected wounds has also been described in Ayurvedic classical texts [5]. The major alkaloid of the plant is berberine, which is known for its activity against cholera [6], acute diarrhea [7], amoebiasis, and latent malaria and for the treatment of oriental sore caused by *Leishmania tropica* [4].

Although the roots of *B. aristata* are considered as the official drug [8], the study revealed that different species of *Berberis, namely, B. asiatica, B. chitria* and *B. lycium* are also used as *Daruharidra* in different parts of the country. In southern India, however, *Coscinium fenestratum* is used as "*Daruharidra.*" The study also shown that most of the market material sold as *Daruharidra* consists of mostly the stem parts than the roots of *Berberis* species.

As such there are different alkaloids available to differentiate different *Berberis* species. Several workers have also done molecular analysis of different *Berberis* species including the

Table 1: Comparative botanical analysis of the roots of four *Berberis* species.

Characters	*Berberis aristata*	*Berberis asiatica*	*Berberis chitria*	*Berberis lycium*
Macroscopic	Outer surface of the bark, creamish brown, and the inner surface attached to wood is yellowish brown. Bark 2 mm thick, knotty and brittle. Cut surface of the wood is bright yellow. Fracture hard, odourless and bitter in taste. Fine longitudinal ridges and flakes are present	Outer surface creamish brown but inner surface is muddy yellow. Bark 2 mm thick, friable separated out immediately from woody part. Cut surface of the wood lemon yellow. Fracture very hard, odour phenolic and very bitter in taste	Outer surface light brown, grooved with transverse marks, bark not easily detachable. Bark upto 5 mm thick, split longitudinally. Cut surface bright yellow. Fracture hard, odour faintly phenolic and very bitter in taste	Outer surface grayish brown with shinnings. Bark up to 3 mm thick, brittle, warty and easily detachable. Cut surface deep yellow. Fracture hard, odour phenolic and bitter in taste
Cork cells	Brown, 10–20 layered	Brown, 12–15 layered	Dark brown, 8–10 layered	Dark brown, 8–11 layered
Cork Cambium	2 or 3 layered	1 or 2 layered	1 or 2 layered	2 or 3 layered
Cortical zone	30–35 layered, outer 4 to 6 layers compressed, devoid of stone cells	18–20 layered	12–20 layered	17–22 layered
Sclereids	Solitary or in group of 2 to 10	Rarely solitary but in group of 2 to 12, comparatively more than other three species	2 to 4 in groups	2 to 4 in groups
Pericyclic fibres	Mostly solitary but sometimes in groups of 2 to 10	Interrupted with stone cells	Frequently present comparatively lesser than *B. aristata* and *B. asiatica*	Frequently present comparatively lesser than other three species
Vessels	Solitary or in group of 2 or 3	Solitary or in group of 2 or 5	Solitary or in group of 2 or 3	Solitary or in group of 3 or 4
Medullary Rays	2 to 4 cells broad	2 to 3 cells broad	2 to 4 cells broad	2 to 5 cells broad

Table 2: Comparative Maceration study of the roots of four *Berberis* species.

Macerated elements (in μm)	*Berberis aristata*		*Berberis asiatica*		*Berberis chitria*		*Berberis lycium*	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Tracheids								
Length	491.024	±125.571	658.613	±253.672	348.308	±129.626	282.836	±4.598
Width	13.062	±4.600	13.749	±6.481	12.221	±2.645	12.603	±0.231
Vessels								
Length	259.720	±158.670	494.964	±181.477	160.405	±187.958	140.108	±14.862
Width	25.590	±18.198	20.623	±16.203	19.859	±11.533	24.629	±0.468
Fibres								
Length	645.481	±259.182	522.462	±246.290	476.632	±337.029	517.879	±2.217
Width	14.666	±3.986	11.457	±3.240	10.693	±2.645	10.884	±0.258
Tracheidal fibres								
Length	694.064	±324.992	714.949	±181.477	760.778	±194.440	368.931	±15.691
Width	12.986	±2.916	11.457	±3.240	11.457	±3.240	12.218	±0.250

Table 3: Comparative botanical analysis of the stems of four *Berberis* species.

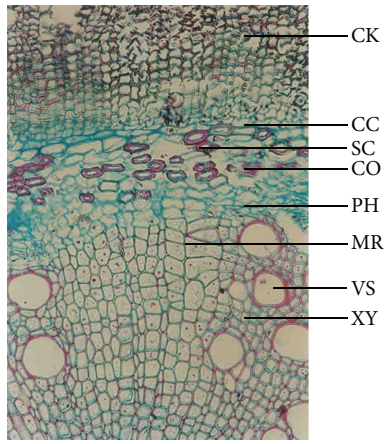
Characters	*Berberis aristata*	*Berberis asiatica*	*Berberis chitria*	*Berberis lycium*
Macroscopic	Outer surface of bark, creamish brown, inner surface yellowish brown, knotty, thin, and brittle. Cut surface light yellow. Fracture hard and bitter in taste	Outer surface of bark grayish brown and friable, fine longitudinal ridges and small warts, inner surface dark brown. Fine longitudinal ridges and small warts below the bark surface leaving dark brown. Cut surface yellowish cream. Fracture very hard and very bitter in taste	Outer surface light brown, split longitudinally, warts comparatively large in size. Whole bark pealed off leaving coffee brown almost smooth inner surface. Cut surface light yellow. Fracture hard and bitter in taste	Outer surface grayish brown with shining. Bark easily detachable, thin, brittle, and twisted. Cut surface canary yellow. Fracture hard and bitter in taste
Cork cells	Brown, 15–25 layered	Brown, 08–10 layered	Dark brown, 8–15 layered	Dark brown, 7–19 layered
Cork Cambium	2 or 3 layered	1 or 2 layered	1 or 2 layered	2 or 3 layered
Cortical zone	20–25 layered, outer 4 to 6 layers compressed, devoid of stone cells	16–18 layered	20–24 layered	20–26 layered
Sclereids	Solitary or in group of 2 to 10	Sometimes solitary but in group of 2 to 4, comparatively more than other three species	Solitary	Scattered or sometimes in linear groups
Pericyclic fibres	Mostly solitary but sometimes in groups of 2 to 10	Interrupted with stone cells	Frequently present comparatively lesser than *B. aristata* and *B. asiatica*	Frequently present comparatively lesser than other three species.
Vessels	In group of 2 to 3 or solitary	Solitary or in group of 2 to 4	Mostly in group of 2 to 3 or solitary	Solitary or in group of 3 or 4
Medullary Rays	2 to 4 cells broad	2 to 5 cells broad	2 to 4 cells broad	1 to 3 cells broad
Pith	Present	Present	Present	Present

Table 4: Comparative Maceration study of the stems of four *Berberis* species.

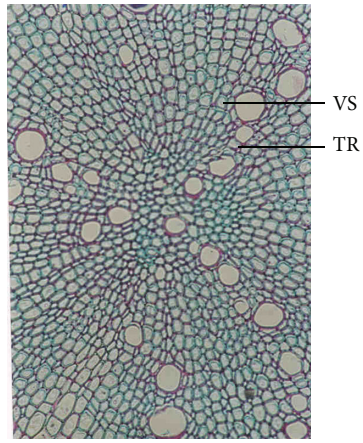
Macerated elements	*Berberis aristata*		*Berberis asiatica*		*Berberis chitria*		*Berberis lycium*	
	Mean	Seed,	Mean	Seed,	Mean	SD	Mean	SD
Trachieds								
Length	439.03	±75.641	625.583	±169.764	360.398	±102.082	262.436	±3.145
Width	12.85	±4.600	12.417	±6.481	12.420	±2.645	11.603	±0.384
Vessels								
Length	459.72	±48.896	594.694	±102.728	468.908	±082.487	440.168	±14.862
Width	20.69	±10.186	18.623	±16.203	19.859	±11.503	20.629	±0.860
Fibres								
Length	627.38	±158.092	543.216	±180.780	424.426	±292.948	497.796	±2.217
Width	13.41	±3.986	11.246	±3.240	10.993	±2.645	18.443	±0.258
Tracheidal fibres								
Length	625.08	±224.887	706.843	±086.878	670.678	±094.087	334.632	±15.691
Width	11.49	±2.916	11.247	±3.240	11.457	±3.240	12.268	±0.250

A flowering twig

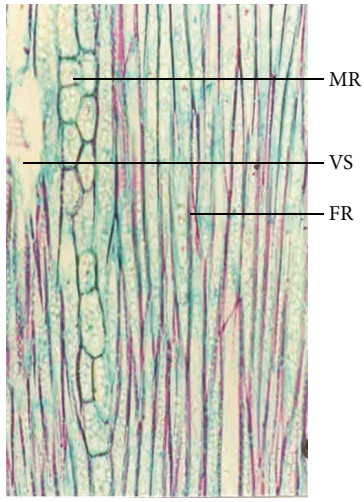
Root+



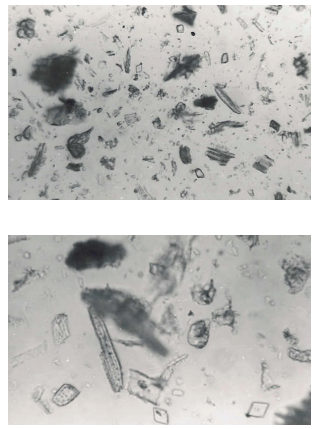
T.S. of root



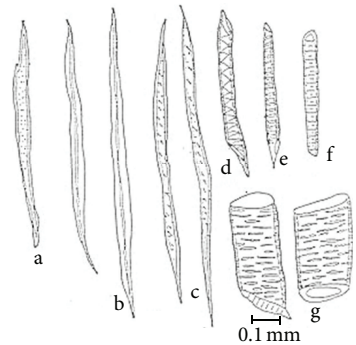
T.S. through central region



T.L.S. of root



Powder study

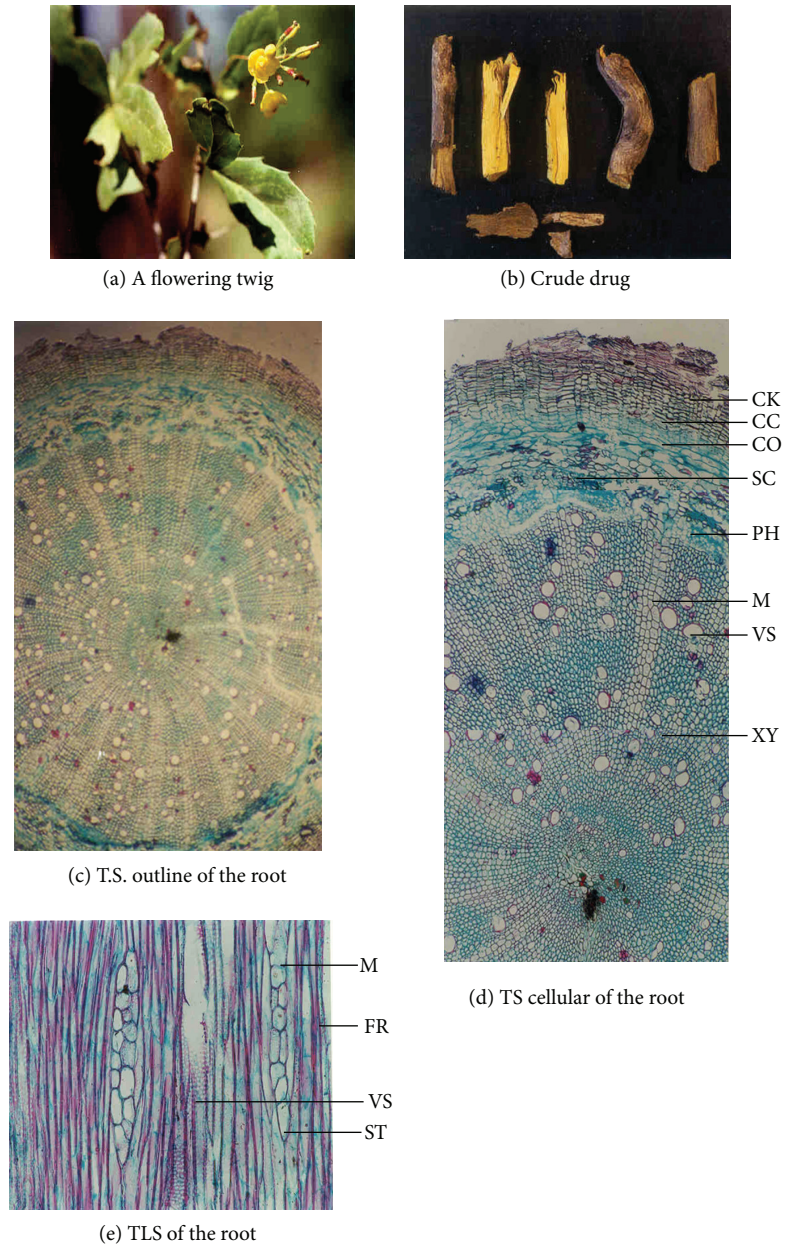


Maceration study

Figure 1: Anatomical characters of *Berberis aristata* root.

presented four species which reflects the use of molecular markers and sequence analysis for identification at inter- and intra-specific level [9–12].

Over exploitation of *B. aristata* created scarcity of the material that opened new vistas to identify a possible substitute for this species. During the market surveillance of



(a) A flowering twig

(b) Crude drug

(c) T.S. outline of the root

(d) TS cellular of the root

(e) TLS of the root

Figure 2: Anatomical characters of *Berberis asiatica* root.

different herbal drug markets of India, it was observed that almost all the markets either comprise *Berberis lycium* or *Berberis asiatica*. Although a detailed pharmacognostic study of *B. aristata, B. asiatica,* and *B. chitria* is reported by Srivastava et al. [13–15], market surveillance is not yet performed. Hence, the present study has been undertaken, which may be useful to pharmaceutical industries for the authentication of the commercial samples and to explore the possibilities of using other species as a substitute of *B. aristata*.

2. Materials and Methods

The plant materials were collected from the Dhanaulti (Uttaranchal) region of India (LWG 221238-11) and the roots were preserved in 70% ethyl alcohol for histological studies. Procurement of commercial samples was done from various important drug markets of India, *namely,* Aligarh, Amritsar, Bangalore-I, Bangalore-II, Delhi, Hyderabad, Jammu, Lucknow, Trichur, Varanasi, and so forth.

Microtome sections were cut and stained with safranin and fast green and photographed with Nikon F70X camera [16]. Physicochemical and phytochemical studies like total ash, acid insoluble ash, tannins, and total alkaloids were calculated from the shade dried powdered material according to the recommended procedures [17–19]. Further, heavy metal studies and quantitative estimation of berberine through HPTLC have also been performed as per ICH guidelines.

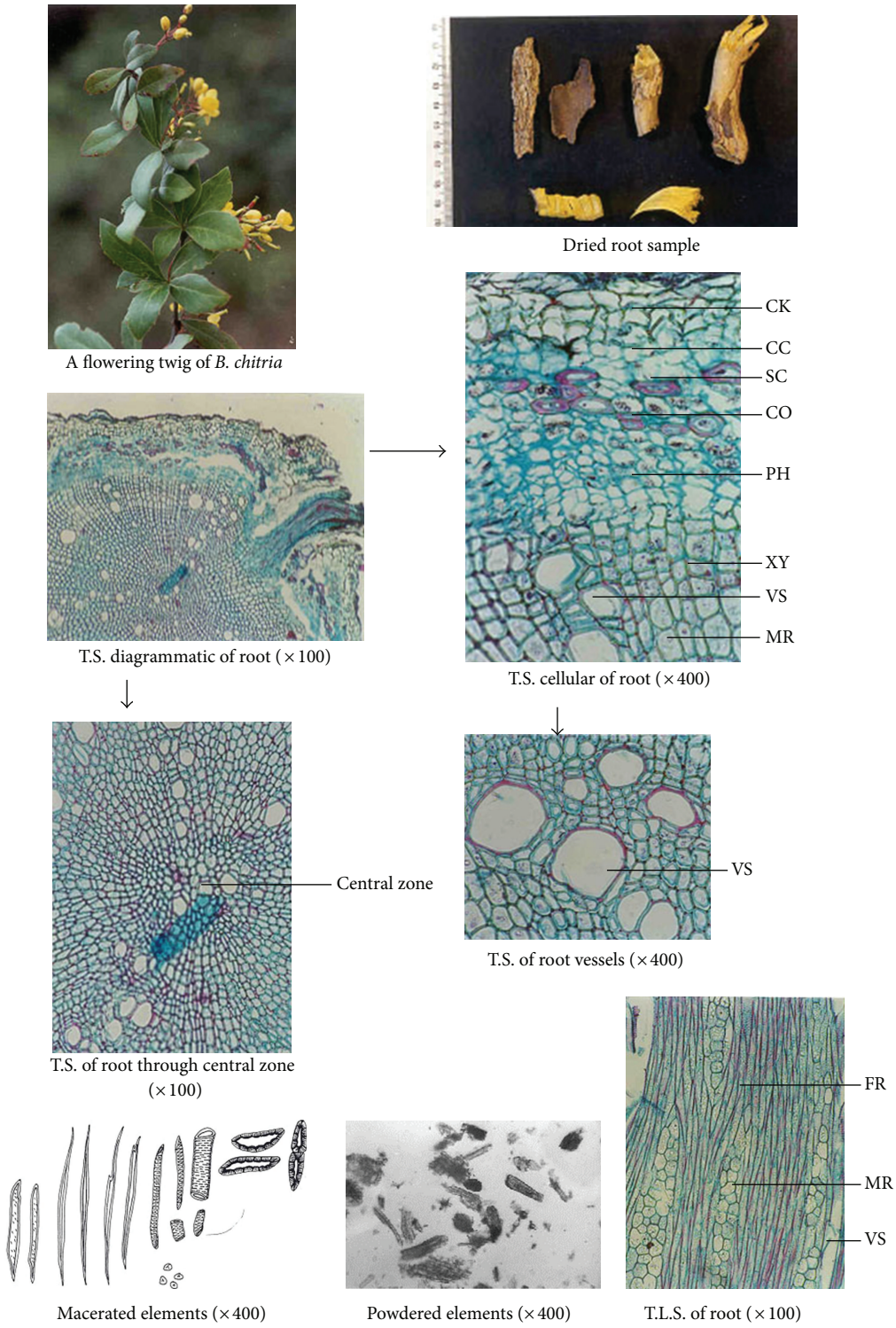
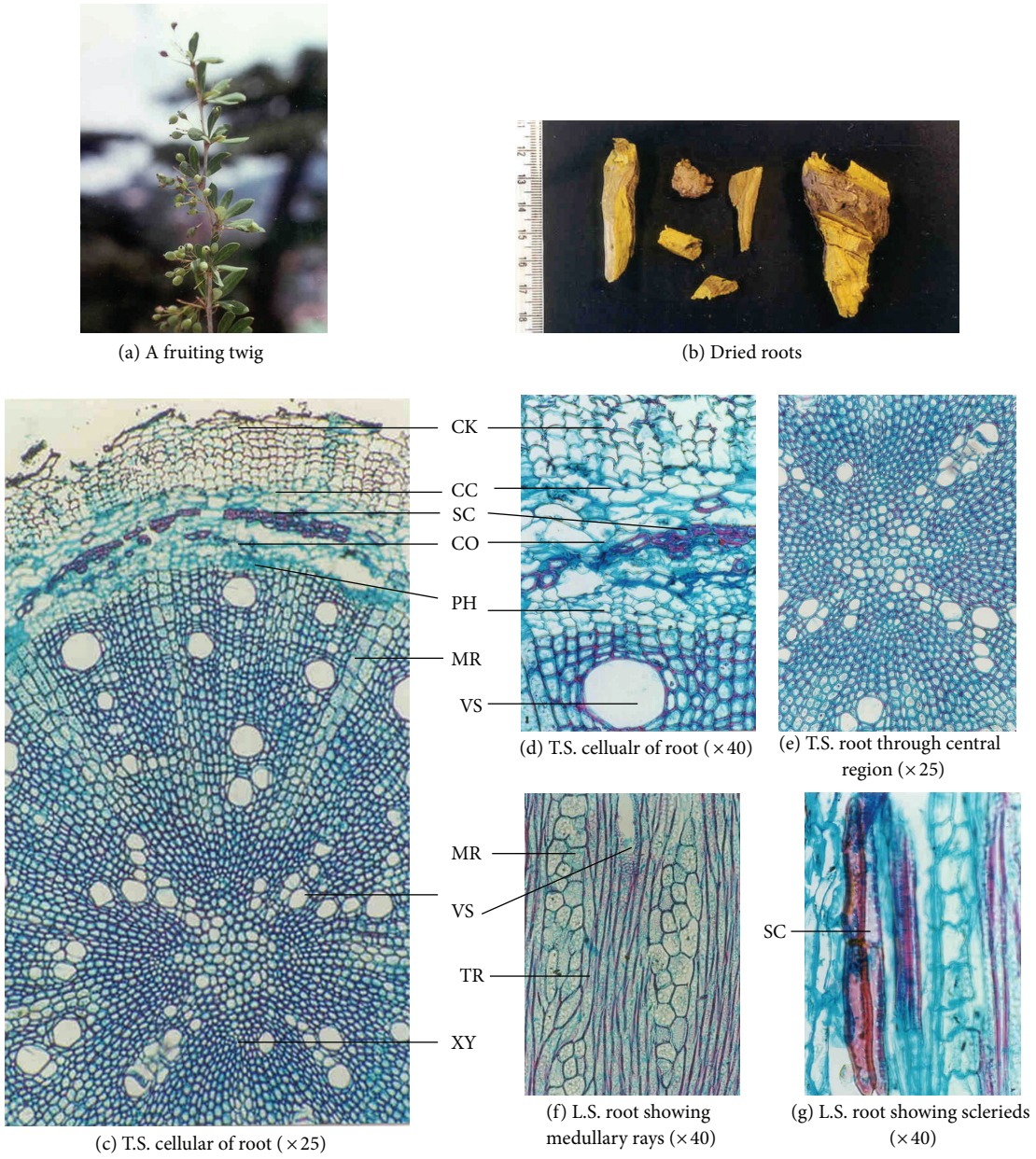


Figure 3: Anatomical characters of *Berberis chitria* root.

3. Results and Discussion

Morphological studies showed certain minor variations in all the four *Berberis* species (see Table 4). For example, in *B. aristata* and *B. chitria* the cut surface is bright yellow while that of *B. asiatica* and *B. lycium* is lemon yellow, and deep yellow, respectively. Similarly the colour of wood bark has also minor variation, namely, it is yellowish brown to



(a) A fruiting twig

(b) Dried roots

(c) T.S. cellular of root (×25)

(d) T.S. cellualr of root (×40)

(e) T.S. root through central region (×25)

(f) L.S. root showing medullary rays (×40)

(g) L.S. root showing sclerieds (×40)

Figure 4: Anatomical characters of *Berberis lycium* root.

yellowish gray in all the three species except in *B. lycium* the colour is grayish white. Likewise the numbers of pericyclic fibres are different in all the four species, for example, the maximum is found in *B. aristata* and minimum in *B. lycium* (Tables 1, 2, and 3; Figures 1, 2, 3, and 4).

A comparative account of all physicochemical values has been depicted in histograms (Figures 5–10). It is quite clear from these studies that no significant variation was observed in total ash of all the four species of *Berberis*. However, the percentage of acid insoluble ash of roots and stem showed significant variations; for example, the highest percentage of 0.26% acid insoluble ash was observed in *B. asiatica* root and the lowest one of 0.05% was noted in *B. aristata* root (Figure 5). It is interesting to note that the percentage of alcohol and water-soluble extractives were higher in root as compared to stem except in *B. chitria* (Figure 6). On the contrary the percentage of starch was higher in stem (14–19%) except in *B. lycium* (root) it was 26.03%. Percentage of tannin was more or less similar in both in root and stem of all the samples (0.7–1.7%).

Similarly, the percentage of successive Soxhlet extractive values revealed that alcohol, acetone, and water extractives were found to be significantly higher in *B. aristata* root, that is, 7.83%, 6.51% and 5.96% respectively. While the roots

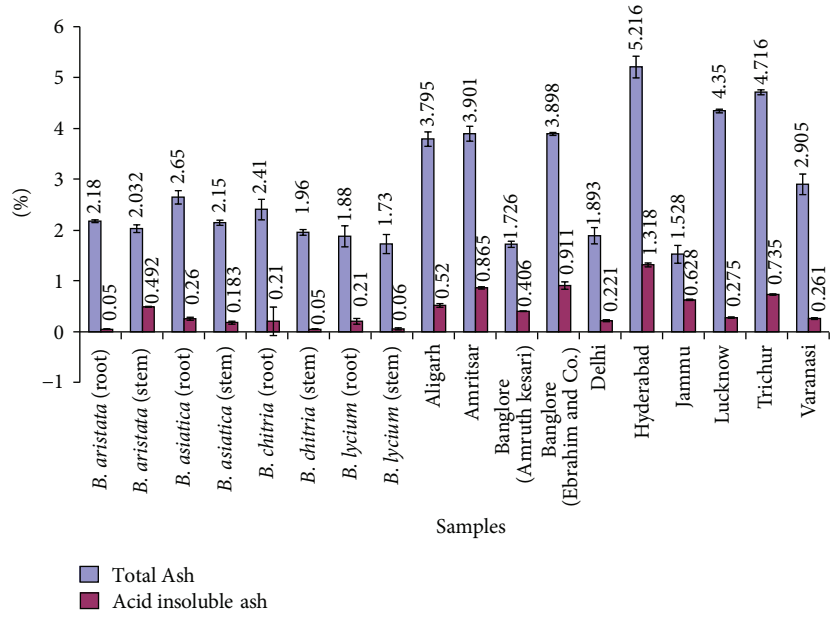


Figure 5: Comparative ash values of different *Berberis* species and market samples of *Daruharidra*.

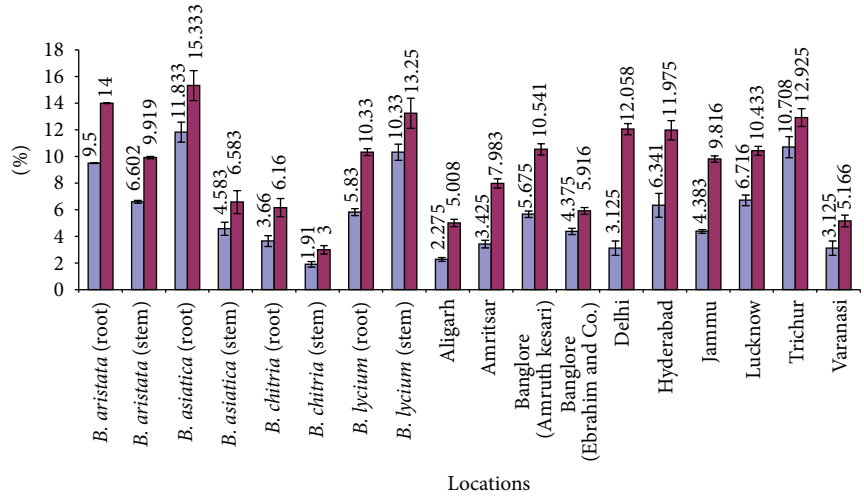


Figure 6: Comparative alcohol and water-soluble extractives values of different *Berberis* species and market samples of *Daruharidra*.

of *B. asiatica* possessed maximum percentage of alcoholic, acetone, and water-soluble matter, that is, 10.30%, 5.92%, and 4.92%, respectively. On the other hand the percentage of successive extractives in stem was maximum in *B. lycium* that is, 5.11% (acetone), 7.20% (alcohol), and 3.20% (water), respectively, and percentage of hexane soluble matter was higher in *B. aristata* (Figure 9).

The percentage of total crude alkaloid percentage was also estimated and it was found that it varied from species to species, that is, maximum in roots of *B. chitria* (3.65%) followed by the roots of *B. lycium* (2.8%), *B. aristata* (2.45%), and *B. asiatica* (2.4%), respectively. Besides, the active constituents berberine one of the major alkaloids was also calculated through HPTLC densitometric method (solvent system, n-propanol: water: formic acid, 90 : 80 : 0.4) and it was found more in roots as compared to stem, that is, 2.25–5.20% and 1.02–2.01%, respectively. Its concentration was also varied from species to species, that is, maximum in roots of *B. chitria* (5.20%) followed by *B. lycium* (3.99%), *B. aristata* (3.55%), and *B. asiatica* (2.25%). Details are depicted in Figure 10.

A comparative study of official drug *B. aristata* sample with that of commercial samples was made (Figures 5–15) and it was found that the Bangalore-I sample has all the similar morphological characters of roots of *B. asiatica,* namely, (i) outer surface grayish brown with 2 mm thick friable bark, which was separated out immediately leaving muddy yellow surface of the wood; (ii) transversely cut surface lemon yellow; (iii) sclerieds mostly in groups of 2–12 rarely solitary and comparatively more than other three

Figure 7: Comparative sugar and starch percentage of different *Berberis* species and market samples of *Daruharidra*.

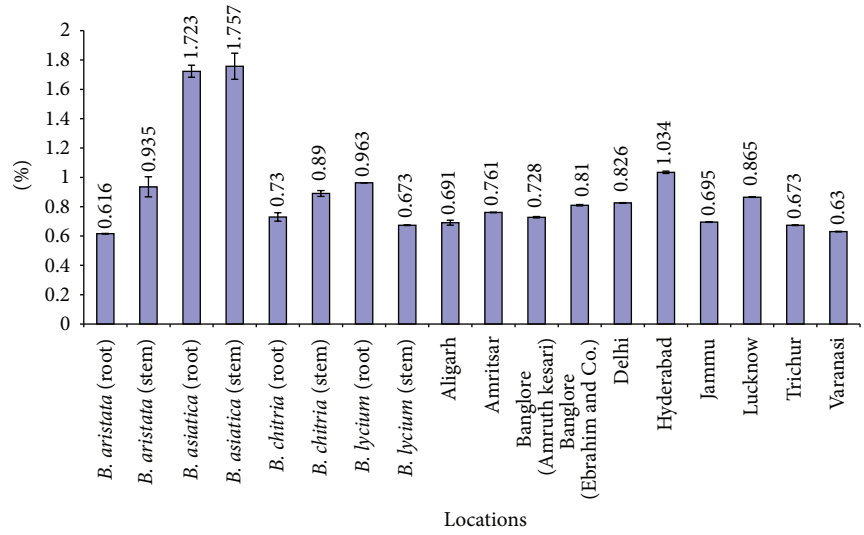


Figure 8: Comparative tannin percentage of different *Berberis* species and market samples of *Daruharidra*.

species; (iv) pericycle fibres interrupted by stone cells; (v) length of the vessel elements much more than the other species, that is, up to 500 μm (±181.0); (vi) physicochemical values are within the prescribed range (Table 6) of Ayurvedic Pharmacopoeia of India [9], hence, identified as roots of *B. asiatica*.

Similarly majority of anatomical characters of Aligarh and Varanasi samples matched with the stem and roots of *B. asiatica* in having (i) some pieces with fine longitudinal ridges and small warts on the outer surface of bark and dark brown outer surface of wood; (ii) transversely cut surface lemon yellow; (iii) sclerieds rarely solitary mostly in groups of 2–12 and comparatively more than other three species; (iv) pericycle fibres interrupted by stone cells; (v) length of the vessel elements much more than the other species up to 600 μm (±181.0); (vi) trachieds up to 680 μ (±167.0) long; (vii) some other pieces have grayish brown outer surface with 2 mm thick friable bark which was separated out immediately leaving muddy yellow surface of the wood; presence of prominent pith as in stem of *B. asiatica*.

Furthermore, the commercial samples of Delhi and Lucknow showed close resemblance with the stem of *B. asiatica* by the presence of (i) fine longitudinal ridges and small warts on the outer surface of bark and yellowish creamy transverse cut surface; (ii) dark brown outer surface of wood as appeared after peeling off the bark; (iii) sclerieds rarely solitary mostly in groups of 2–12 and comparatively more than other three species; (iv) pericycle fibres interrupted by stone cells; (v) trachieds up to 680 μ (±167.0) and vessels up to 600 μ (±102.0) long (vi) pith.

Similarly, the market samples of Amritsar and Jammu were found to be the mixture of stem and root of two different *Berberis* species. Amritsar samples were found to be the stem

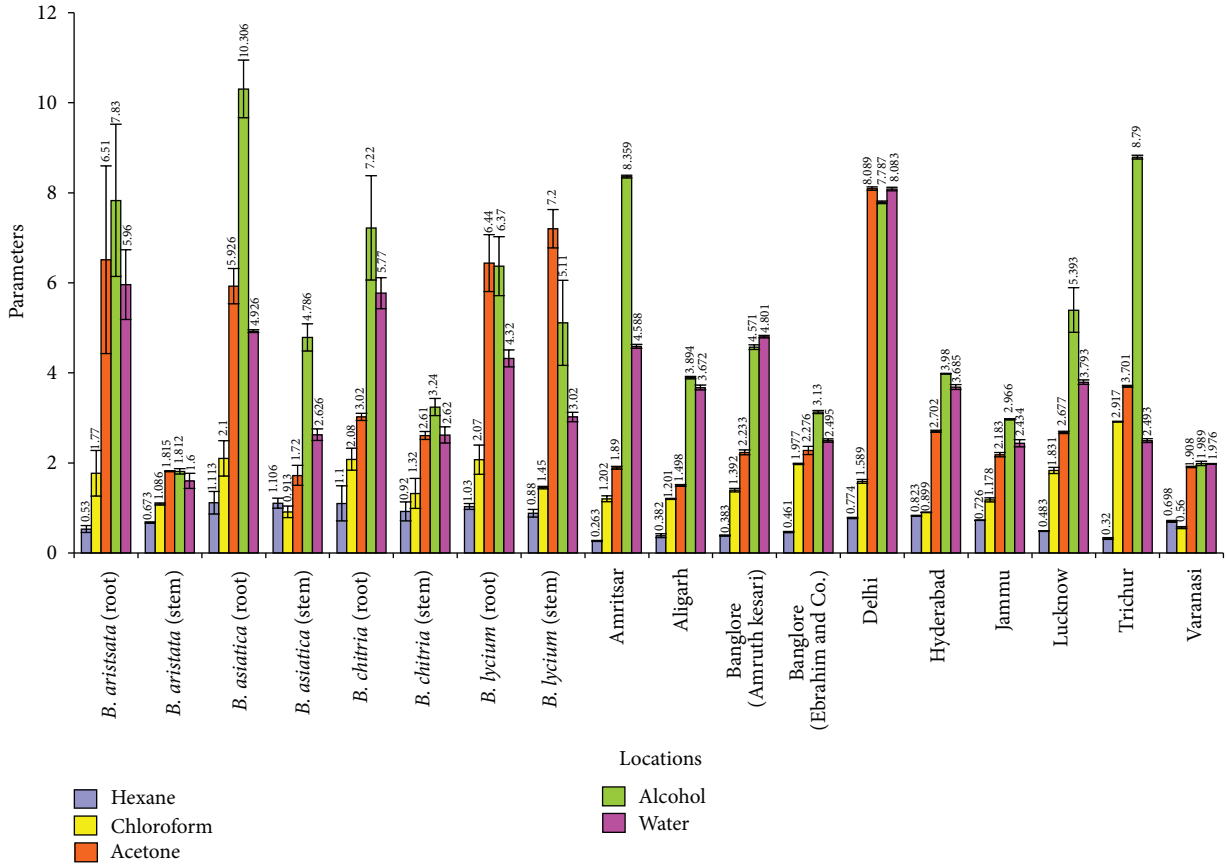


Figure 9: Comparative successive soxhlet extractive values of different *Berberis* species and market samples of *Daruharidra*.

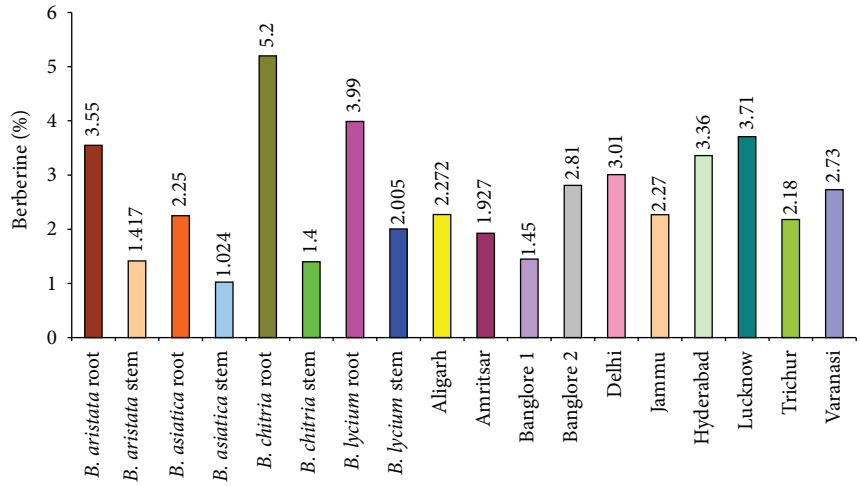


Figure 10: Quantitative estimation of berberine in different species of *Berberis* and market samples of *Daruharidra*.

of *B. aristata* and root of *B. asiatica* while Jammu sample comprised of root of *B. chitra* and stem of *B. asiatica*.

The morphological characters in Amritsar sample are (i) outer surface creamish brown with knots, fine longitudinal ridges, and flakes; (ii) bark very thin and brittle; (iii) transverse cut surface bright yellow; (iv) outer surface of wood which appeared after peeling off the bark was yellowish brown; (v) sclerieds solitary or in a group of 2–10; (vi) pericyclic fiber mostly solitary, rarely in groups of 2–10; (vii) length of the fibres much more, that is, about 630 μm as compared to other three species.

On the other hand some of the pieces of this sample showed close resemblance with that of the stem of *B. asiatica* in having (i) outer surface grayish brown with 2 mm thick

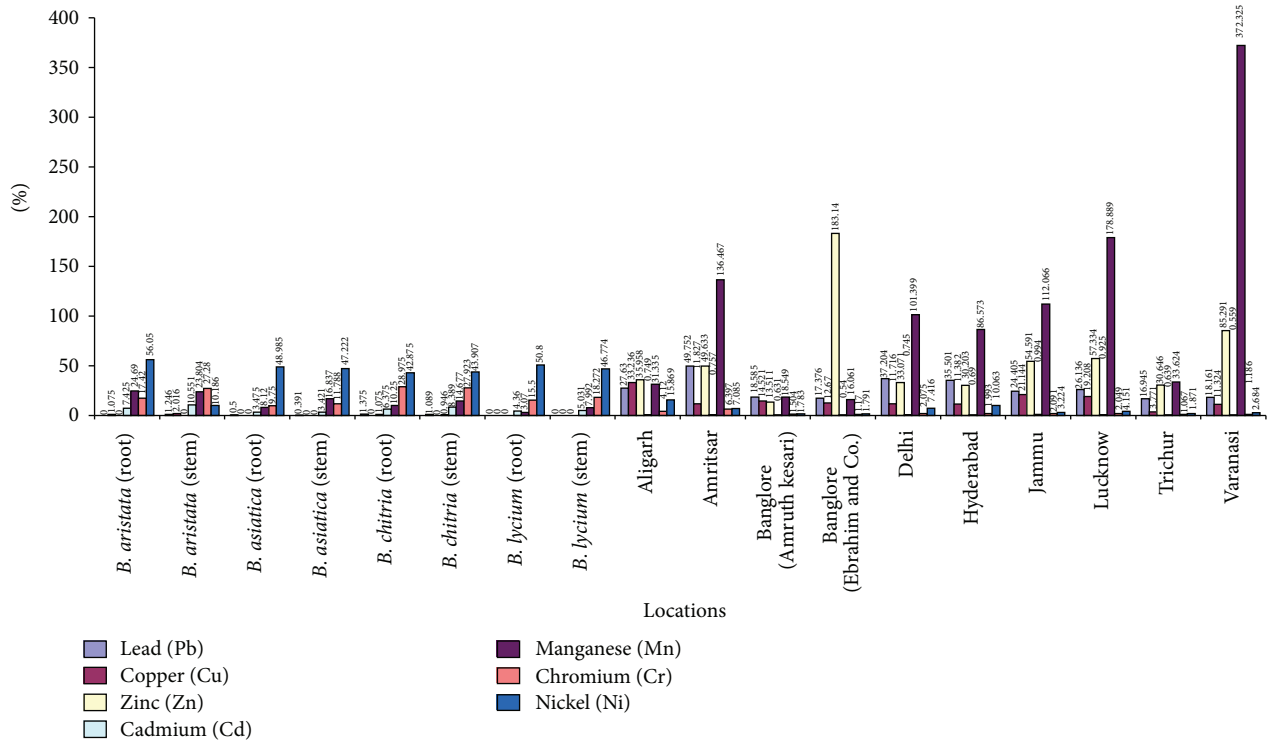


Figure 11: Comparative heavy metal studies of different *Berberis* species and market samples of *Daruharidra*.

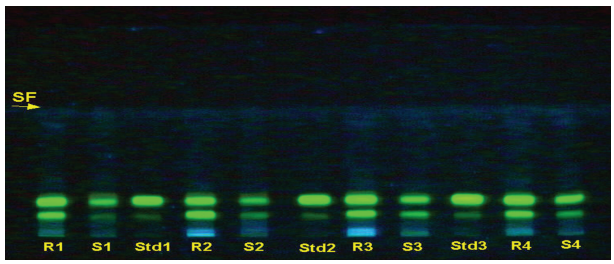


Figure 12: 8 HPTLC profile of different *Berberis* species (under UV 366) (solvent system: n-propanol: water: formic acid, 90 : 80 : 0.4).

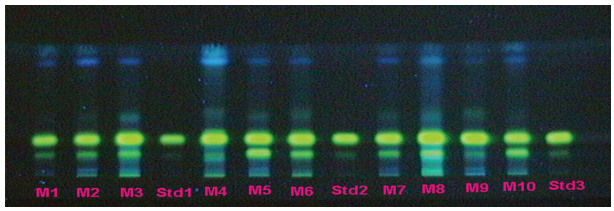


Figure 13: HPTLC profile of different market samples of *Daruharidra* (under UV 366); (Solvent system: n-propanol: water: formic acid, 90 : 80 : 0.4).

Table 5: Market samples of different regions from India.

Serial number	Markets	Findings
1	Amritsar	Mixture of root of *B. asiatica* and stem of *B. aristata*
2	Aligarh	Mixture of root and stem of *B. asiatica*
3	Banglore I	Root of *B. asiatica*
4	Banglore II	Stem of *Coscinium fenestratum*
5	Delhi	Stem *B. asiatica*
6	Hyderabad	Mixture of root *B. asiatica* Root *B. aristata* Root of *B. tinctoria*
7	Jammu	Mixture of root of *B. chitria* and stem of *B. asiatica*
8	Lucknow	Stem of *B. asiatica*
9	Trichur	Root of *Coscinium fenestratum*
10	Varanasi	Mixture of root and stem of *B. asiatica*

friable bark which was separated out immediately leaving muddy yellow surface of the wood; (ii) transverse cut surface lemon yellow (iii) sclerieds rarely solitary mostly in groups of 2–12 and comparatively more than other three species (iv) pericycle fibres interrupted by stone cells; (v) length of the vessel elements much more than the other species, that is, up to 500 μm (±102.0); (vi) presence of pith.

Similarly the morphological characters found in some pieces of Jammu samples showed close resemblance with those of stem of *B. asiatica*, as in Delhi and Lucknow markets. However, some other characters similar to roots of *B. chitria*. The different characters, this market samples showed are (i) outer surface light brown in colour with broad ridges and grooves and transverse marks; (ii)

Figure 14: Crude samples of *Daruharidra* fromdifferent market of India.

Table 6: Identity, purity, and strength as prescribed by Ayurvedic Pharmacopoeia of India.

Parameters	Values
Foreign matter	Not *more* than 2 percent
Total ash	Not *more* than 14 percent
Acid-insoluble ash	Not *more* than 5 percent
Alcohol-soluble extractive	Not *less* than 6 percent
Water-soluble extractive	Not *less* than 8 percent

bark up to 5 mm thick but not easily detachable as in other three species; (iii) transverse cut surface bright yellow (iv) sclerieds mostly in groups of 2–4; (v) pericycle fibres present but are much lesser in number than *B. aristata* and *B. asiatica*; tracheidal fibres up to 960 μm (±86.0) long.

The percentage of successive extractives (Soxhlet), sugar, starch, and berberine content also support the above finding (Figures 6–10).

On the contrary the Hyderabad commercial sample seems to be the mixture of three *Berberis* species. Some pieces showed close affinity with roots of *B. asiatica*; other pieces resembled roots of *B. aristata*. Besides some of the pieces have no resemblance with any of the four species studied. These may be the roots of *B. tinctoria* as it is a common species found in these areas.

The samples procured from Trichur and Bangalore II do not resemble any of the Berberis species studied. Presence of wedge shaped medullary rays and wheel shaped transverse section indicate that these pieces may belong to Menispermaceae. Hence an attempt was made to compare the characters of these samples with the root and stem of *Coscinium fenestratum*. After comparison it was found that samples procured from Trichur and Bangalore II were root and stem of *C. fenestratum* Gaertn, respectively.

From the heavy metal analysis of crude drug samples of different herbal market, it was observed that the concentration of majority of heavy metals, namely, Cd, Co, Mn, Zn, and Cu within the permissible limits of WHO in both the collected and commercial samples. However, the significant variations in Lead concentration were observed in both samples that is, in collected as well as in commercial ones. For example, in the stem of collected samples, the increase is quite low but in some of the commercial samples, namely, Amritsar, Delhi, and Hyderabad many fold increase in lead concentration was observed, this may be due to vehicular pollution or may be due to edaphic factors (Figure 11).

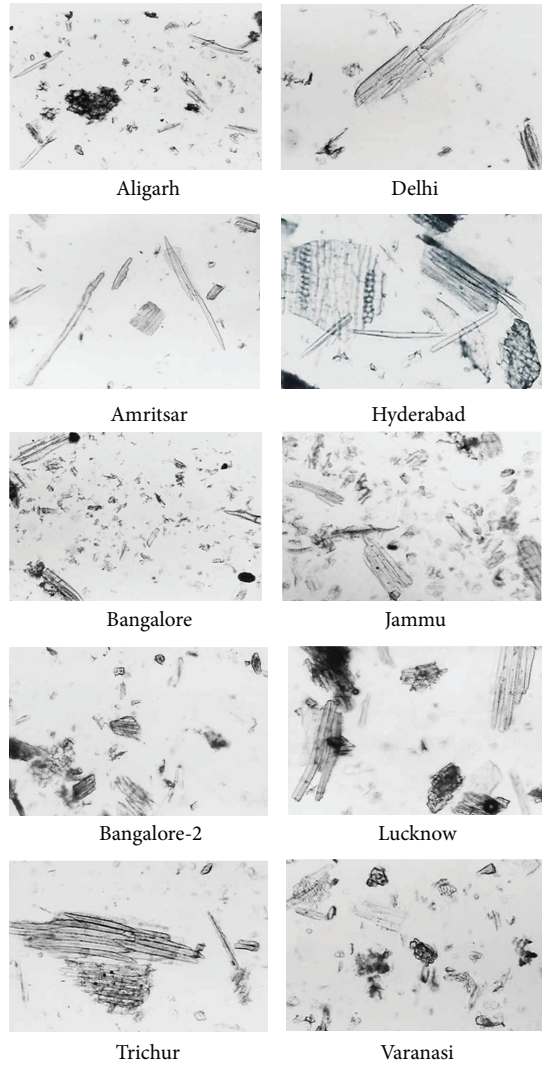


Figure 15: Powder study of different market samples of *Daruharidra*.

4. Conclusion

From the ongoing discussion it is quite clear that most of the commercial samples consist of mixture of roots or stems of *Berberis asiatica* while *B. aristata* is found only in the market samples of Amritsar and Hyderabad mixed with roots of *B. asiatica*.

On the basis of the above study it may be concluded that in India different *Berberis* species and their parts are being sold in the name of crude drug *Daruharidra* (Table 5).

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Research Article

The Effect of *Elephantopus scaber L.* on Liver Regeneration after Partial Hepatectomy

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Liver regeneration after partial hepatectomy (PHx) is a physiological response for maintaining homeostasis. The aim of this study is to investigate effects of *Elephantopus scaber L.*- (ESL-) induced liver regeneration on growth factors (HGF and IGF-1), cell cycle regulation, and apoptosis suppressed. In this study, we fed five Chinese medicinal herbs (1 g/kg/day), *Codonopsis pilosula* (CP, Dangshen), *Salvia miltiorrhiza Bunge* (SMB, Danshen,), *Bupleurum kasi* (BK, Chaihu), *Elephantopus scaber L.* (ESL, Teng-Khia-U), and Silymarin (Sm, 25 mg/kg) for 7 days to male Spraue-Dawley rats. Then surgical 2/3 PHx was conducted and liver regeneration mechanisms were estimated on the following 24 hrs and 72 hrs. The activities of growth factors (HGF and IGF-I) and cell cycle proteins were measured by Western blot and RT-PCR. Histological analysis and apoptosis were detected by H&E stain and TUNEL. The results showed that extraction of *Elephantopus scaber L.* (ESL) and Silymarin (Sm, positive control) were increased protein expression levels of HGF and IGF-1 which leads into cell cycle. These results suggest that the ESL plays a crucial role in cell cycle-induced liver regeneration and apoptosis. These results suggested that the ESL plays a crucial role in cell cycle-induced liver regeneration and suppressed hepatocytes apoptosis.

1. Introduction

The liver is an excellent tissue when it is underwent surgical resection for growth regulation. Hepatocytes are ability to regenerate by a process of compensatory growth and then return to quiescent state [1–3]. Much of the investigation on the mechanisms of hepatic growth has been done in partial hepatectomy (PHx). Most liver cancer patients have to section partial liver by surgery. After surgery, hepatocytes need to regeneration and increase cell numbers. The native hepatocyte function cannot maintain the integrated whole liver function. Therefore, we suggested that Chinese herbal

medicines may act as cell cycle progression agents. On the other hand, Silymarin has been used to protect the liver agent as a cytoprotection for treatment of liver disease. Several mechanisms of cytoprotection have been identified, but liver resection has not been reported. In vitro and animal studies have suggested that milk thistle's active ingredient, Silymarin, promoted hepatocyte regeneration and survival [4]. In this study, we suggested Silymarin as a positive control. Almost immediately after PHx, there are major changes in the complete mitogens expression for hepatocytes and in the expression of a relatively large number of genes. TGF beta1 is a potent antagonist to the mitogenic effects of terminating the proliferative response of hepatocytes during liver regeneration [5–7].

In this study, we detected traditional Chinese medicines, such as *Codonopsis pilosula* (CP), *Salvia miltiorrhiza Bunge* (SMB), *Bupleurum kasi* (BK), *Elephantopus scaber L.* (ESL), and *Silymarin* (Sm) effects on liver regeneration. *Codonopsis pilosula* is an widely edible traditional Chinese medicine (TCM) in China [8]. Some paper studies evaluate that CP would be even stimulated the survival signaling [9], control cell cycle [10], and antiscar formation. *Salvia miltorrhiza Bunge* root is also a traditional Chinese medicine, which is considered to promote blood flow and remove blood stasis. Some studies show that SMB has protective effects on human kidney possibly through inhibition of inflammatory cytokines and has long been used for treating liver and heart disease in China [11, 12]. Recent papers have indicated that SMB plays an adjuvant role in inhibited the proliferation and anticarcinogenesis. *Bupleurum kasi* is one of the most important traditional Chinese crude drug [13, 14] for treating hepatitis malaria and intermittent fever. It has the function of soothing the liver. BK was observed in resisting the level of cytokines and antifibrosis [15]. *Elephantopus scaber L.* is a folk medicine of Taiwan derived from the entire plants of *Elephantopus scaber L.* E mollis H.B.K and Pseudeelephantopus spicatus (Jass) Rohr. However, some studies elucidated Taiwan folk as a medicine. ESL has hepatoprotective effects [16]. There are some studies showed that ESL exerted anticancer effects on various cancer cells and induced cancer cells apoptosis from cell cycle arrest [17, 18].

As it is well known, partial patients with hepatocellular carcinoma need section partial liver. At the same time, liver needs proliferation to maintain original liver mass. We detected possible molecular mechanisms for these traditional Chinese medicines by examining the levels of external and intrinsic signal mechanisms. The liver can precisely regulate its growth and mass after surgical resection of hepatic lobes or hepatocytes loss caused. Hepatocyte replication while enlarged liver mass is corrected by apoptosis. Regeneration requires the cytokines TGF-beta1 to prevent cytotoxicity. In addition, extensive remodeling of the hepatic extracellular matrix occurs shortly after PHx. Several growth factors have been suggested to play a crucial role in liver regeneration after treatment TCM. HGF is believed to play a primary role in liver regeneration and promotes cell proliferation, survival, and morphogenesis through regulated DNA synthesis. Downstream of hepatocyte growth factor receptor activation is FAK (focal adhesion kinase), an important mediator of integrin signaling in the regulation of cell cycle, survival and regulates cell cycle progression [19, 20]. However, we also detected cell apoptosis expression by examining the levels of cytochrome c and bad from mitochondrial to find cell lost.

2. Materials and Methods

2.1. Animals. Male Sprague-Dawley rats weighing 180 to 220 g were obtained from the Animal House of National Science Council in Taiwan, and house five to a cage in a room with a controlled temperature of 22 ± 5°C, relative humidity of about 60% and free access to standard food in pellets and tapwater. Two or three cages were randomly assigned into the same group. All rats were acclimatised for 1 week prior to the beginning of all experiments.

2.2. Preparation of Hot-Water Extract from Chinese Medical Herbs. The hot-water extract was prepared by boiling the dried roots with distilled water for 1 h. The extract was filtered, freeze-dried, and kept at 4°C. The yield of extraction which *Codonopsis pilosula* (*CP,* Dangshen)was 21.34% [21], *Salvia miltiorrhizae Bunge* (*SMB,* Danshen) was 16.95% [22], *Buplearum kaoi* (*BK,* Chaihu) was 23.24% [23], *Elephantopus scaber L.* (*ESL,* Teng-Khia-U) was 11.84% [20], and *Silymarin* (Sm) was 16.73% [24]. The dried extract was dissolved in distilled water before use.

2.3. Experimental Partial Hepatectomy (PHx) and Sham (0 hr). Three randomly selected animals were used for each time point. After injecting ketamine subcutaneously at a dose of 30 mg/kg, liver resections consisting of 2/3 of the liver mass were performed in partial hepatectomy group. Animals underwent the same operative anesthesia with the partial hepatectomy (PHx) group [25]. All the surgical operations were done the same as PHx, except the liver lobes were not resected. All the operations were performed between 8:00 AM and 12:00 PM to minimize diurnal effects. After completion of the procedure, the animals were placed under a lamp to prevent hypotermy and then put into cages (five animals per cage) with continuous supply of food and water. The animals in the PHx and corresponding were sacrificed at 6 hrs, 24 hrs, 72 hrs, and 168 hrs after the operation. The group of animals in which no surgery was performed, was used as control liver group and mentioned time "0" in quantitated groups. After all animals were sacrificed by cervical dislocation, the remnant liver lobes were excised and washed in PBS, then immediately frozen in liquid nitrogen.

2.4. Histological Analysis. Rats of all groups from different parts of time at 0 hr, 6 hrs, 24 hrs, and 72 hrs were sacrificed. The liver sections were taken out and fixed in 10% formalin and embedded in paraffin. Paraffin blocks were cut into 5-mm sections and stained with Hematoxylin-eosin (H&E) solution stain [26]. Silymarin (Sm, 25 mg/kg) oral gavages after PHx at 0 hr, 6 hrs, 24 hrs, and 72 hrs were also sacrificed and fixed and stained with H&E solution stain.

2.5. Transferase-Mediated dUTP Nick End Labeling (TUNEL). Left ventricular sections were deparaffinized by immersing in xylene, rehydrated, and incubated in 2% H_2O_2 to inactivate endogenous peroxidases. The sections were then incubated with proteinase K (20 μg/mL), Protein K, working solution: [10-20 ug/ml in 10 mM Tris/HCl, pH 7.4-8]. Use Proteinase K from Roche Applied Science, because it is tested for absence of nucleases which might lead to false-positive results [27, 28]. Wash in phosphate-buffered saline, and incubated with terminal deoxynucleotidyl transferase for 90 min and fluorescein isothiocyanate-Dutp for 300 min at 37°C using an apoptosis detection kit [29]. Silymarin (S, 25 mg/kg) and *Elephantopus scaber L.* (ESL) oral gavages after PHx at 6 hrs, 24 hrs, and 72 hrs were also fixed and stained with kit. Samples were analyzed in a drop of PBS under a fluorescence and UV light microscope at this state by an excitation wavelength in the range of 450–500 nm.

2.6. Western Blot. Proteins were separated by 12% SDS-PAGE and then transferred to nitrocellulose. Membranes were blocked in 5% milk (diluted in Tris-buffered saline and 0.1% Tween 20) and incubated with the appropriate primary antibodies (TGFβ1, HGF, IGF-I, Cyclin D1, Cyclin E, pRb, cytochrome c, Bad, and E2F) at 4°C overnight and HRP anti-IgG was used as secondary reagent. After extensive washing, the targeted proteins were detected by enhanced chemiluminescence (ECL) [30].

2.7. Reverse Transcriptase PCR (RT-PCR). 0.5 μg of total RNA derived from liver plus primers by RT-PCR. The first-strand synthesis kit was applied according to the manufacturer's instructions of PCR. The primer pairs used for each gene were as follows.

(1) Cyclin D1: F:5′AGGAGACCATTCCCCTGACT3′

R:5′TTCTTCCTCCACTTCCCCTT3′

(2) pRb: F:5′AGGAGGACTGTTCTCTAAGG3′

R:5′GAGTGAGGTGTGTCTTCTGA3′

(3) E2F: F:5′AACATCCAGAACATCCAGTGGGTAGGCAG3′

R:5′GGCTGTCAGTAGCCTCCAAG3′

(4) Cyclin E:F: 5′CACCCCTGGCATCTTCTCCTT3′

R:5′AGCGTCTTCAGAGACAGCCAG3′

(5) Cytochrome c: F:5′ACAGCACGCTTGTGGAT3′

R:5′GTCTTCAAGCAAGAGGACCA3′

(6) Bad: F:5′TAAGACTCACCTGGGTACTG3

R:5′GCATGTAGTCACTCTTCACC3′

(7) GAPDH: F:5′GGGTGTGAACCACGAGAAAT3′

R:5′CCACAGTCTTCTGAGTGGCA3′

The RT-PCR results were analyzed based on the assessment of product sizes upon ethidium bromide agarose gel electrophoresis. For each gene, we determined the cycle number of PCR reactions in which the PCR reaction was not saturated [31]. Based on this, we used the following PCR conditions, The initial denaturation step was at 95°C, then at annealing temperature and extension at 72°C. The final extension at 72°C for 10 min was applied to all the reactions and the PCR products were electrophoresed on a 1.2% agarose gel.

2.8. Quantification of Western Blot and RT-PCR. The intensity (area × density) of the individual bands on western blots and RT-PCR were measured by densitometry [32]. The background was subtracted from the calculated area.

2.9. Statistical Analysis. All data examined were expressed as mean ± S.E. For Western blot and RT-PCR analysis, quantitation was carried out by scanning and analyzing the intensity of the hybridization signals using FUJIFILM Imagine program. Statistical analysis of the data was performed using SigmaStat software. Comparison between group was made using one way ANOVA test [32]. A P value of less than 0.05 and 0.01 was considered to be statistically significant.

3. Results

3.1. Establishment of Liver Regeneration Animal Model Partial Hepatectomy. During liver regeneration after 2/3 hepatectomy, hepatocytes divide once or twice and return to quiescence. We detected the role of Chinese medicinal herbs in the process of liver regenerating after PHx. We suggest that Chinese herbal medicines may act as cell progression agent to make cell progress. Several mechanisms of cytoprotection have been identified, but the mechanisms of liver resection have not been reported. Surgical resection to remove a tumor together with surrounding liver tissue while preserving enough liver remnant for normal body function. After PHx, we found liver regeneration was started at 24 hrs and increases liver mass (Figure 1(b)), until 72 hrs and 168 hrs. However, Liver regeneration (%) was increased at 24 hrs, 72 hrs and 168 hrs PHx (Figures 1(a), 1(b), and 1(c)). More commonly, during liver regeneration the liver is injured and it attempts to repair the injured site referred to as internal scar tissue as quickly as possible. Cytokine, TGFβ1, increased in the plasma very shortly time kinetics and then decreased, but increased at the long time (Figure 1(f)). TGFβ1 increased reaching plateau amounts at 72 hrs PHx. Hepatocyte proliferation and apoptosis are coordinately regulated by TGFβ1. TGFβ1 protein expression were increased by treatment of SMB, CP, ESL, and Sm at 24 hrs PHx. However, at 72 hrs TGFβ1 was increased only by CP, ESL, and Sm (Figure 1(d)). *Silymarin* induced TGFβ1 decreased at 6 hrs PHx ($^{*}P < 0.05$ versus Sham), but increased at 72 hrs ($^{**}P < 0.01$ versus Sham; $^{\#\#}P < 0.01$ versus PHx). *Silymarin* mitigated

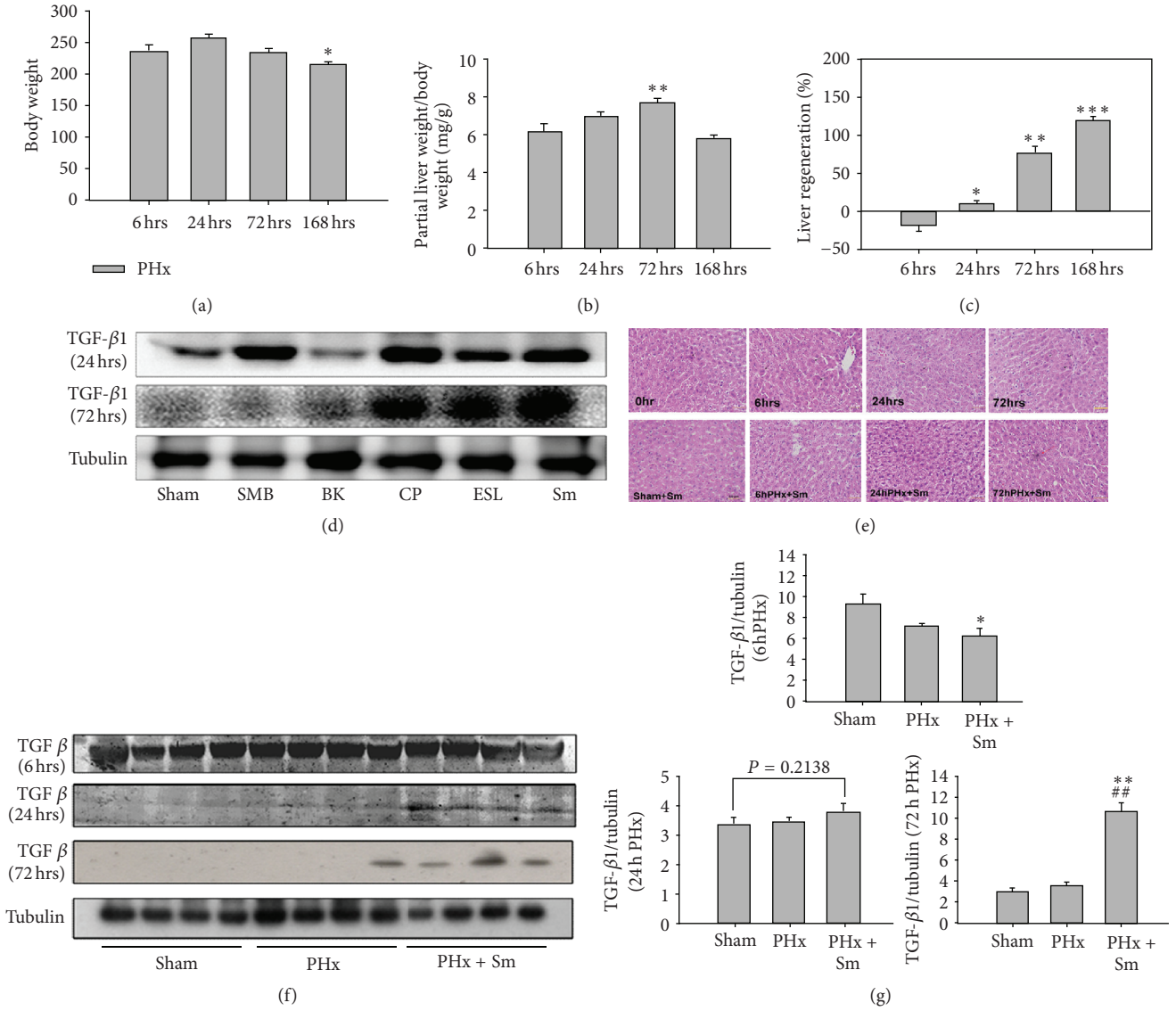

Figure 1: Traditional Chinese medicine improves liver regeneration after liver toxicity injury. (a) Body weight was decreased at 168 hrs PHx. (b) Partial liver weight was increased at 72 hrs PHx. (c) Liver regeneration (%) was increased at 24 hrs, 72 hrs, and 168 hrs PHx. (d) Cytokines, TGF-β1, was increased in SMB, CP, ESL, and Sm at 24 hrs PHx, but CP, ESL, and Sm was at 72 hrs PHx. (e) Histology of PHx section and after Sm section during liver regeneration. (f) TGF-β1 expression was decreased at 6 hrs PHx after Silymarin, but increased at 72 hrs PHx. (g) Quantification of densitometry analysis of protein levels. All data are presented as means ± SEM, $^{*}P < 0.05$ significant difference compared with Sham. $^{\#\#}P < 0.01$ significant difference compared with PHx.

regeneration and made cell normal. At long time, we did not find apoptotic body in regeneration liver (Figure 1(e)).

3.2. Elephantopus scaber L.-Induced Growth Factors Immediately Increased after 2/3 PHx. Growth factor signals (HGF and IGF-I) play a role in initiating regeneration of hepatocytes after 2/3 PHx. We suggested that Chinese herbal medicines may act as a cell cycle progression agents to make primed cells progress through the cycle and undergo DNA synthesis. However, progression through the cell cycle beyond the initiation phase requires growth factors. Starting with expression of a large number of immediate growth factors in the regenerating stage, hepatocytes can fully respond to the growth factors (HGF and IGF-I) to stimulate cell cycle from G1 phase to S phase to increase DNA synthesis and rebuild the lost hepatic tissue. ESL and Sm were increased HGF and IGF-I protein expression (Figure 2) ($^{*}P < 0.05$, $^{**}P < 0.01$ versusSham) at 24 hrs PHx and 72 hrs PHx. In addition, Silymarin (Sm) was induced HGF increased compared with Sham or PHx in spite of 6 hrs, 24 hrs, and 72 hrs PHx ($^{*}P < 0.01$ versus Sham; $^{\#}P < 0.01$ versus PHx) (Figure 3(a)).

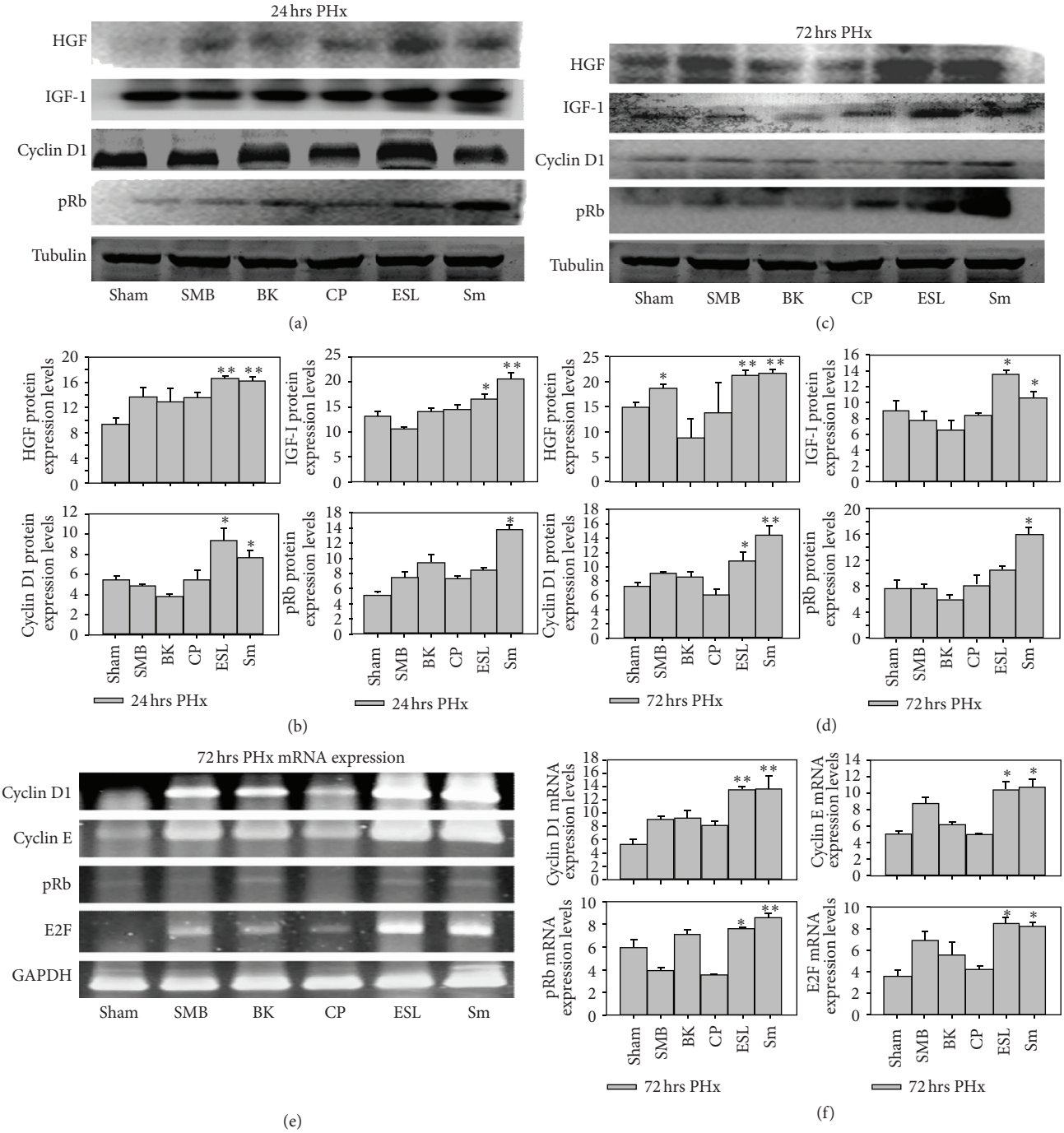

Figure 2: Effects of *Elephantopus scaber L.* (ESL) and *Silymarin* (Sm, positive control) on liver regeneration after PHx. (a), (c) Equal amounts of protein lysate were separated by 12% SDS-PAGE by western blotting with antibodies to HGF, IGF-I, cyclin D1, and pRb. Protein expression levels are increased in *Elephantopus scaber L.* (ESL) and *Silymarin* (Sm) at 24 hrs and 72 hrs PHx during liver regeneration. (b), (d). Quantification of densitometry analysis of protein levels. All data are presented as means ± SEM, $^{*}P < 0.05$, $^{**}P < 0.01$ significant difference compared with Sham group. (e). Expression mRNA of Cyclin D1, Cyclin E, pRb, and E2F were increased in ESL and Sm after 72 hrs PHx. (f). Quantification of densitometry analysis of mRNA levels. All data are presented as means ± SEM $^{*}P < 0.05$, $^{**}P < 0.01$ significant difference compared with Sham group.

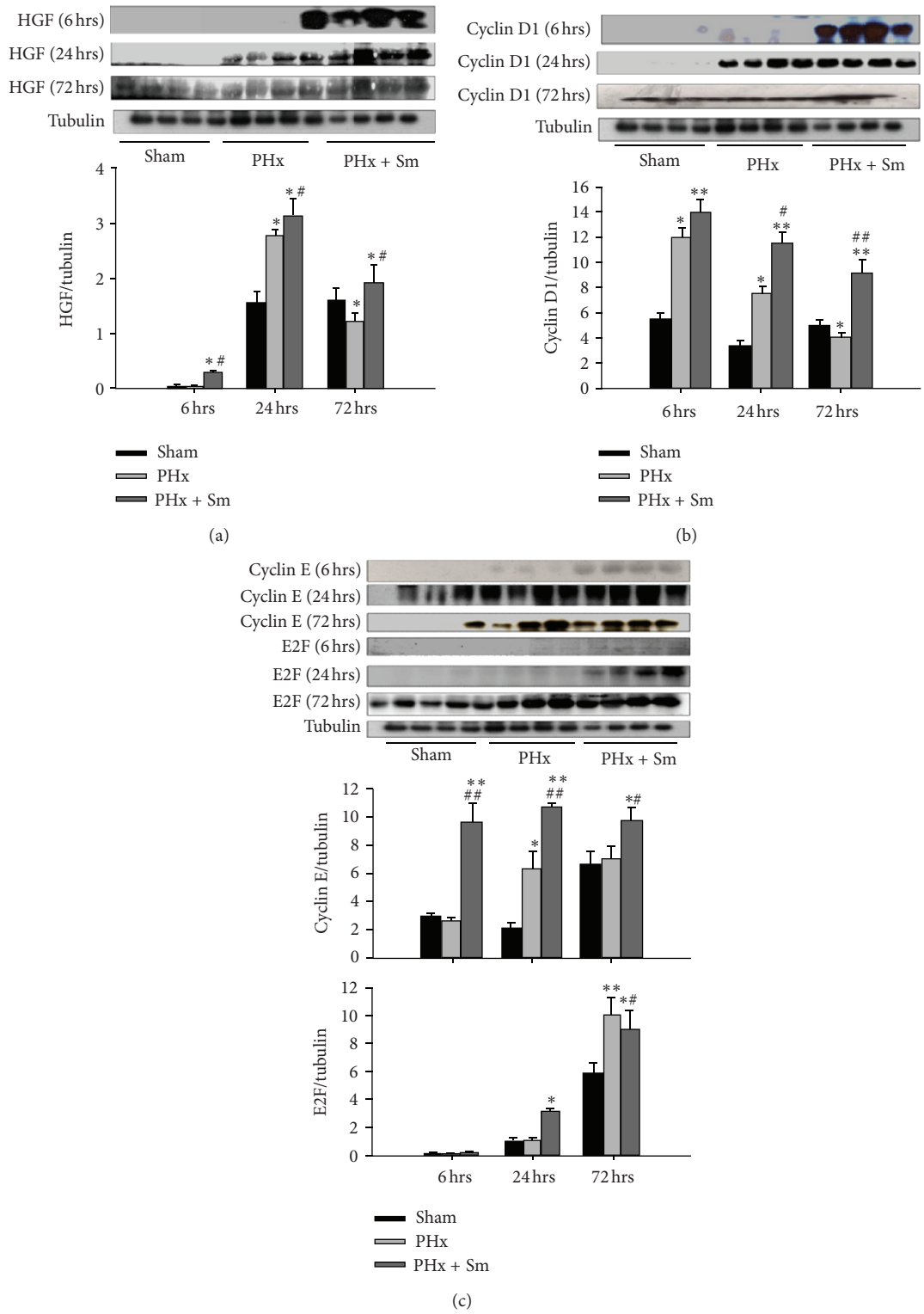

Figure 3: Expression of cell cycle proteins in G1 to S Phase. Western blot analysis of HGF, Cyclin D1, Cyclin E, and E2F expression were increased in *Elephantopus scaber L.* (ESL) and *Silymarin* (Sm) at 24 hrs and 72 hrs PHx. Tubulin was used as a loading control for western blotting. Quantification of densitometry analysis of protein expression levels. All data are presented as means ± SEM $^{*}P < 0.05$, $^{**}P < 0.01$ significant difference compared with Sham group. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ significant difference compared with PHx group.

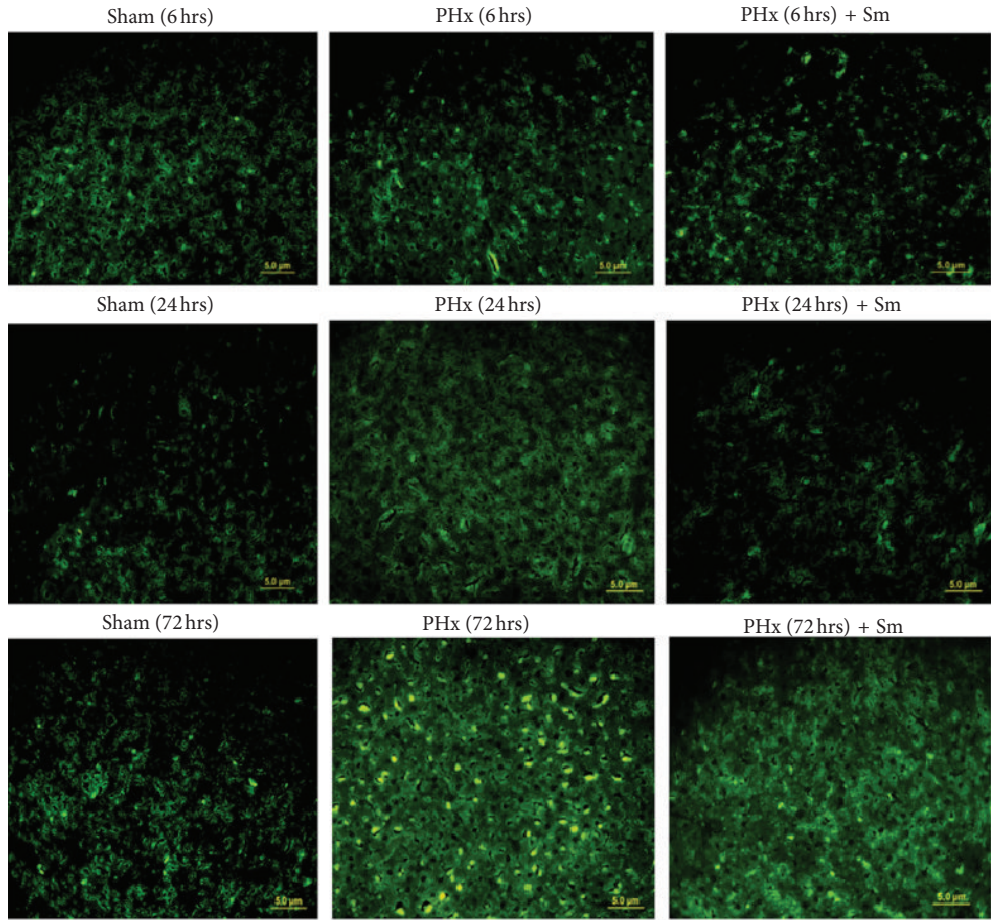

Figure 4: TUNEL assay in liver regeneration after PHx. To detect cell apoptosis during liver regeneration at different times. At 6 hrs PHx and 24 hrs PHx was not found, but at 72 hrs PHx appeared. Also not observed after silymarin treatment PHx 72 hrs.

3.3. Elephantopus scaber L. Accelerated Cell Cycle in Liver Regeneration. Cyclin D1/pRb and Cyclin E/E2F are key regulators of G1-to-S phase progression of the cell cycle. We found Cyclin D1 was increased at 24 hrs and 72 hrs PHx by ESL and Sm ($^{*}P < 0.05$, $^{**}P < 0.01$ versusSham); however, pRb was only increased in Sm treatment (Figures 2(a) and 2(c)). The positive control, *Silymarin*, was permission increased at 6 hrs, 24 hrs, and 72 hrs PHx compared with Sham ($^{*}P < 0.05$, $^{**}P < 0.01$ versus Sham) and PHx ($^{\#}P < 0.05$, $^{\#\#}P < 0.01$ versus PHx (Figure 3(b)). Moreover, Cyclin D1, Cyclin E, pRb, and E2F mRNA expression levels were increased at 72 hrs PHx by ESL or Sm treatment ($^{*}P < 0.05$, $^{**}P < 0.01$ versus Sham). The same result we found Sm also increased compared with Sham and PHx (Figure 3(c)).

3.4. Effects of Elephantopus scaber L. on Cell Death after PHx. During liver regeneration after liver injury, hepatocytes were lost. Cell death or apoptosis was a physiological process to regulate hepatocyte development and maintain liver mass. We detected apoptosis protein bad and cytochrome c at 24 hrs and 72 hrs (Figures 5(a) and 5(b)). Apoptosis occurs rapid cellular divisions after PHx, resulting in fine-tuning of the liver size and tissue remodeling. Therefore, the results showed us that *Elephantopus scaber L. (ESL) and* Silymarin (Sm) induced bad and cytochrome c protein and mRNA expression downregulated ($^{*}P < 0.05$, $^{**}P < 0.01$ versus Sham). Moreover, TUNEL assay showed apoptotic body only at long time 72 hrs PHx including *Silymarin* treatment (Figure 4). In contract, we also observed apoptotic body in traditional Chinese medicines. We did not find apoptotic body in ESL and Sm treatment at long time 72 hrs PHx. We did not found any apoptotic body in treatment TCM at 24 hrs PHx.

4. Discussion

The liver is one of the most complex organs, and the regeneration induced by surgical injury is an orchestrated response. In order to set in the optimal mass in relationship to its body size, the liver induced its compensatory hyperplasia mechanisms. Herbal medicines have been used to treat liver disorders for thousands of years in the East and have now become a promising therapy internationally for pathological liver conditions. Growth factors (HGF and IGF-I) and cytokine (TGFβ1) are triggering cell cycle progression from G0 phase to G1 phase. Hepatocyte growth factor, also known

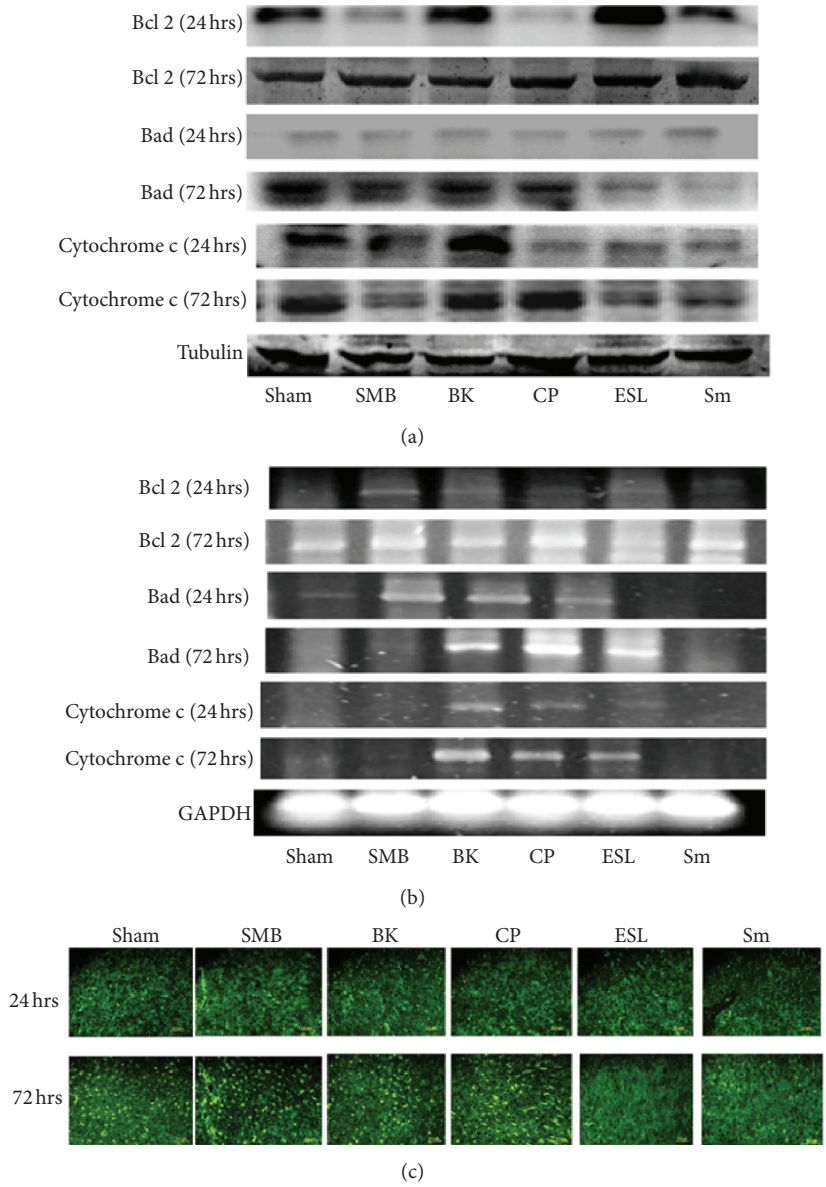

Figure 5: *Elephantopus scaber L.* suppressed apoptosis during liver regeneration. (a) Expression protein levels of Bad and cytochrome c were decreased protein expression in *Elephantopus scaber L. (ESL) and Silymarin (Sm)* after 24 hrs and 72 hrs PHx by western blot. However, antiapoptosis protein, Bcl 2, was increased by *Elephantopus scaber L. (ESL) and Silymarin (Sm)* at after 24 hrs PHx, but no changes at 72 hrs PHx. (b) mRNA expression levels of Bad and cytochrome c decreased apoptosis in ESL and Sm treatment at 24 hrs PHx; however, we can observed a little elevated expression at 72 hrs PHx. In contrast, antiapoptosis protein, Bcl 2, was increased by *Elephantopus scaber L. (ESL) and Silymarin (Sm)* at 24 hrs, but no changes at 72 hrs. (c) TUNEL assay after traditional Chinese medicines in liver regeneration at 24 hrs and 72 hrs PHx. Only ESL and Sm have suppressed apoptosis function.

as scatter factor, is believed to play a primary role in liver regeneration. Growth factors may play a role in initiating the proliferation of hepatocytes after PHx in the rat were investigated immediately after surgical resection of the liver. In this paper, we presumed that Chinese medicines including *Codonopsis pilosula* (CP, Dangshen), *Salvia miltiorrhiza Bunge* (SMB, Tanshinone), *Bupleurum Kasi* (BK, Chaihu), *Elephantopus scaber L.* (ESL, Teng-Khia-U), and *Silymarin* (Sm) may promote the function of liver regeneration after PHx.

We found that ESL (Teng-Khia-U) and *Silymarin* (Sm) have the best effects on liver regeneration. In the present study, ESL from the toxicity study they were observed that the root extract are nontoxic and caused no death up to a dose of 3.2 g/kg orally [24]. It is safe and was used in doses for the this study. Two known compounds, isodeoxyelephantopin and deoxyelephantopin [33, 34], were isolated from the whole plant of *Elephantopus scaber L.* (ESL, Teng-Khia-U) [35]. The whole plant of ESL is rich in novel antitumor substances-sesquiterpene lactones. The plant of

ESL extracts has the ability to influence programmed cell death or arrest proliferation of tumor cells. We find that ESL and Sm stimulated several growth factors to regulate cell cycle and DNA synthesis. Growth factors are paracrine-regulated hepatic regenerative response [36]. The active form of HGF is a powerful stimulator of DNA synthesis and cell motility [37, 38]. PHx triggers the entry of rat liver cells into the cell cycle. We found ESL induced growth-regulated genes (HGF and IGF-I) to express later and persist longer, paralleling the rapid growth phase of the liver after PHx [39, 40]. The maximal expression after 24 to 72 hrs when the maximal growth period ends and are thought to be involved in re-establishing quiescence. Therefore, we can find that ESL mediated growth factors (HGF and IGF-I) and cytokines (TGFβ1) to remodel hepatic at 24 hrs PHx, but fail to at 72 hrs PHx. However, the other TCMs are also enhanced cytokines expression during this time [41–45]. Thus, PHx is a cell cycle-dependent regulation and a potential physiological role in G1 progression. Liver growth after PHx does not involve cell death and is a purely proliferative event. In summary, our data suggest liver regeneration may regulate the kinetics of cell cycle progression at the G1 to S phase transition [46, 47]. However, we found ESL induced growth factors and cell cycle expression at 24 hrs, until 72 hrs. Because ESL maybe delay apoptosis [48, 49].

Overall, the information thus derived should enhance our knowledge on the liver regeneration functions of treatment of TCMs as well as the basic mechanisms of cell cycle and apoptosis [50, 51].

Abbreviations

HGF: Hepatocyte growth factor
PHx: Partial hepatectomy
IGF-I: Insulin-like growth factor I
CP: Codonopsis pilosula
BK: Bupleurum kasi
ESL: *Elephantopus scaber L.*
SMB: Salvia miltiorrhiza Bunge
Sm: Silymarin
UPA: Urokinase plasminogen activator
TCMs: Traditional Chinese medicines.

Authors' Contributions

C.-C. Tsai and J.-P. Wu equally contributed to this work.

Acknowledgments

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Research Article

Herbal Medicine in Primary Healthcare in Germany: The Patient's Perspective

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Herbal medicine (HM) is one of the most widely used Complementary and Alternative Medicine (CAM) therapies throughout the world. The WHO has recognized HM as an essential component of primary healthcare. The aim of this study was to explore patients' attitudes towards using HM, their sources of information and the role of costs. Within a qualitative research approach, semi-standardized interviews with 18 patients using HM were conducted and analyzed according to Mayring's content analysis. Patients highlighted their active role and perceived autonomy choosing HM. Most interviewees experienced HM as better, with more sustainable effects and fewer side effects compared to conventional medicine. All media, family, friends, and healthcare professionals were reported as sources of information. Some patients complained that doctors and pharmacists have insufficient knowledge of HM. Most patients expressed their regret that HM is not reimbursed by statutory health insurances but also their general willingness to pay extra for HM. The main challenge for German primary care, besides the reintroduction of reimbursement, is the promotion of knowledge and skill development in HM. This is to ensure patient safety and work in partnership with patients. Appropriate strategies for education must be tailored to the specific needs of health professional groups.

1. Introduction

Herbal medicine (HM) is one of the most widely used Complementary and Alternative Medicine (CAM) therapies used throughout the world. In many countries, HM has a long tradition and the knowledge about local medical plants is ingrained into cultural memory. The WHO estimates that 70–90% of the rural population in developing countries use HM to meet, in part or completely, their health needs [1]. Also, in many developed countries, HM as an element of CAM is highly popular. Therefore, HM is recognized as an essential component of primary healthcare by the WHO [1].

In most developed countries—also in Germany—patients have access to HM via physicians, nonmedical CAM practitioners and on a self-initiated basis. Consequently, diverse healthcare professionals mainly doctors, nurses, pharmacists, and nonmedical CAM practitioners are involved in HM. In Germany, HM is known as one of the five main elements of classic naturopathy (phytotherapy, hydrotherapy, exercise therapy, dietetic therapy, and "life style regulation" therapy) also known as Kneipp therapies. The overall percentage of Germans using HM increased from 52% in 1970 to 70% in 2010 [2].

In 2011, about one billion euro were spent on herbal medicine corresponding to approximately 20% of the total expenditure for over the counter (OTC) drugs in Germany. In addition, herbal medicines are sold in drugstores, via internet, and so forth. About 20% of herbal medicine was sold on a prescription-basis and about 80% were sold over the counter [3]. By far the highest sales are made for the indication of respiratory tract infections, followed by cardiovascular disease and gastrointestinal symptoms. However, sales have slightly decreased since the introduction in 2004 of the Statutory Health Insurance Modernization Act, which excluded several groups of drugs including phytotherapeutics from reimbursement.

German doctors can obtain a postgraduate qualification for "naturopathy," which includes HM. At the end of the year 2011, 15.949 German doctors had the qualification "naturopathy," with 70% thereof working in the outpatient sector [4]. However, recent data suggests that far more doctors prescribe or recommend HM to their patients. In a cross-sectional study, more than half of the responding general practitioners (GPs) recommended HM in their day-to-day practice, most of them having no additional qualification for "naturopathy" [5].

Furthermore, HM is often provided by nonmedical CAM practitioners called "Heilpraktiker." They have to pass an exam on basic medical knowledge to obtain a state license but have no formal training on CAM and HM specifically [6]. While the number of GPs in Germany is decreasing (41.642 GPs in 2011), the number of "Heilpraktiker" has increased over the last years to nearly 32.000 today [4]. Hence, it is clear that Heilpraktikers play a substantial role in Germany for providing patients with HM.

Altogether these developments suggest that, on the one hand, HM has reached mainstream medicine yet on the other hand, studies show that a substantial percentage of patients do not inform their doctors about their use of HM [7, 8]. Furthermore, international studies suggest that doctors and other health professionals are not well prepared to inform their patients about HM [9, 10]. Considering the interactions between HM and conventional drugs this deficit may affect patient safety and, therefore, should be a point for debate and further research.

In view of the above, this study is intended to explore the perspectives and experiences of patients using HM in a primary care context. Using a qualitative approach, we focus particular attention on central patient attitudes and motives for using HM, the methods and needs for information and communication and the role of the costs in choices made.

2. Methods

2.1. Design of the Study. A qualitative study consisting of guideline-based in-depth interviews was chosen to allow an intensive analysis of patients' perspectives and experiences.

2.2. Sample. In 2008, patients were recruited via newspaper advertisements and via a study call on the website of the University Hospital Heidelberg. Inclusion criteria were the use of HM for upper respiratory tract infection in the previous three months, age > 18 yrs and German speaking. We consecutively included all patients meeting the inclusion criteria and with interest to take part in the study. Individual appointments for the interviews were arranged. After 18 interviews, saturation point was reached and recruitment was stopped.

2.3. Data Collection. The in-depth interviews were conducted in the Department of General Practice and Health Service Research, University Hospital of Heidelberg, Germany. The interviews were semistructured based on a predefined interview guideline and conducted by a doctoral research student. Each interview lasted between 15 and 45 minutes. All interviews were recorded digitally and transcribed verbatim.

The results presented in this article are based on the following questions from our interview guideline.

(i) How did you come to use Herbal Medicine?

(ii) What were your expectations towards Herbal Medicine?

(iii) What are your main sources of information about Herbal Medicine?

(iv) What role does the cost of Herbal Medicine play regarding your decision to use it?

(v) Do you tell your doctor that you use Herbal Medicine?

The aims of the study were explained to each interviewee. The interviewer ensured that each aspect of the questions was explained sufficiently, so that no questions or misunderstandings remained.

2.4. Ethics Approval. The study was approved by the ethics committee of the Heidelberg Medical Faculty (approval number 394/2006).

2.5. Data Analysis. The interviews were carried out in 2008. Analysis was conducted using the software ATLAS.ti. Key issues were identified, summarized, labeled with codes and sorted into main and subcategories based on the qualitative content analysis technique from Mayring [11]. The aspects of interpretation and categories were developed based on the material. For each category, a typical quotation was selected. The interviews and analyses were conducted simultaneously, so that researchers could control for topic saturation. Disagreements during coding process were discussed until a consensus was reached. The quotations cited here were translated into English by Pia Weiss and cross-checked by SJ and BM.

3. Results

Eighteen patients participated in the study. The sample of the 18 interviewees is shown in Table 1. Among the interviewees 16 were female and two were men. Mean age was 46.5 years, nearly half of the participants were employed, four were students, and five were retired. The most common way of recruitment was via newspaper advertisement, followed by the internet and word of mouth.

The categorical framework developed in the course of the qualitative content analysis is displayed in Tables 2, 3, and 4. In the following, all categories are explained and examples of typical quotations are given for the subcategories.

3.1. Attitudes, Values, and Motives. Underlying attitudes, motives, and values for using HM were categorized in five main groups: "medical culture," "personal experience," "subjective theory of disease," "defensive attitude towards conventional medicine," and in "active role" (Table 2).

Table 1: Study sample of participating patients ($n = 18$).

Gender	
Female	16
Male	2
Age	
Mean	46.5 years
Range	Min. 23/max. 82 years
Occupation	
Employed	8
Retired	5
Students	4
Housewife	1
Way of recruitment	
Newspaper	8
Internet	6
Word of mouth	4

3.1.1. Medical Culture. Among the main category "medical culture" comments regarding patients' desire to maintain traditional knowledge can be found. The fact that herbal remedies have existed for hundreds of years is perceived as proof of evidence by some patients. One patient describes it as an "*implicit knowledge of society.*"

> "*Those are ancient natural remedies which are regaining their popularity since there's simply an abundance of those chemical things, all those chemical pills.*"

> "*They don't meddle around with your system quite like chemical drugs and knowledge about them has been around for a long time. Also, about that [. . .] society knows if they are of good use or not, doesn't it?*"

Some of the patients are reporting that their mothers or grandmothers have already been using herbal therapies and that it is "family tradition."

> "*Well, my mother didn't send us to the doctor right away then or fed us antibiotics, rather she tried herself, you know, with home remedies.*"

Not only family but also the social environment as a mediator of "medical culture" may play an important role in motivating patients to use herbals. One patient uses the expression "trend" in this context. For others, the use of herbals is an indicator of "higher education."

> "*Maybe if you studied it and lived in an environment where everyone has an idea, that this symptom could be remedied with this tea, and so forth, then it simply amplifies, I think, over the course of time.*"

> "*If I knew that only uneducated people or readers of the yellow press believed in natural healing then I wouldn't take it as seriously, too.*"

Table 2: Attitudes, values and motives (main and subcategories).

Main category	Subcategory
(A) Attitudes, values and motives	
(A1) Medical culture	• Maintenance of traditional knowledge • Long experience as proof of evidence • Family tradition • Social trend • Marker of higher education • Doctors as main representatives • Refusing exaggerated faith in progress
(A2) Personal experience (mainly expressed in comparison to conventional medicine)	• Better effects • More gentle effects • Fewer side effects • Sustained, not only symptomatic cure and • Slower onset of action/more time with herbals • Preventive effects • Herbals as primary treatment approach • Substitution of antibiotics • Herbals appeal to the senses/evocate positive memories
(A3) Subjective theory of disease	• Holistic approach • Herbals strengthen body's natural defence system • Herbals do not disturb the body's balance • Causal way of healing
(A4) Defensive attitude towards conventional medicine	• High risk of side effects (especially antibiotics) • Conventional doctors' lack of time • Patients do not feel taken seriously • Mistrust towards pharmaceutical companies
(A5) Active role	• Elevated health awareness • Active role • More autonomy • High self-responsibility • Curiosity/inquisitiveness • Looking for other sources of help

Moreover, doctors as main representatives of "medical culture" play a central role in influencing patients' attitudes towards herbal medicine.

> "*It does depend on my doctor. I just have trust in him now [. . .] if he would say we're trying that now [. . .] then I would take it and try it.*"

Table 3: Communication and Information (main and subcategories).

Main category	Subcategory
(B) Communication and information	
(B1) Doctors' approach	• Categorical refusal
	• Dogmatism
	• Unconcern
	• Sincerity
	• Positive conviction
	• Henchmen of pharmaceutical industry
(B2) Quality of communication	• Feeling accepted by their doctors
	• Shared decision-making
	• Patriarchal style
	• Miscommunication
	• Placebo-effects
	• Nocebo-effects (e.g., induction of fear)
(B3) Sources of information	• Family/social environment
	• Doctors
	• Pharmacists
	• CAM practitioner
	• Media (TV, newspapers, internet, brochures)
	• Literature (books, journals)
	• Advertising (pharmacy brochures)
	• Patient information leaflets
(B4) Relevant elements of information	• Indication
	• Side effects
	• Mechanism of action
	• Dosage recommendations
	• Safety recommendations for pregnancy
	• Quality differences in drugs and possible consequences regarding side effects.
	• Declaration of toxic remnants

A lot of patients using herbals refuse to have exaggerated faith in medical and scientific progress.

> *"I have been reading a lot of articles lately that were critical towards the current practice in medicine [...] there's such an overstated belief in progress. And if people resort to herbal medicine they say "We don't even want modern medicine, we want the time-tested.""*

3.1.2. Personal Experience. In general, interviewed patients expressed expectations based on their previous experiences regarding HM. Patients commented on their experiences with HM primarily in comparison with conventional drugs. Experiences are described as "better and with a more lasting effect," "a more gentle effect," "fewer side effects," "sustained, not only symptomatic cure."

Table 4: Cost (main and subcategories).

Main category	Subcategory
(C) Cost	
(C1) Individual cost	• Health worth the money
	• Additional cost only for evidence-based therapies
	• High cost of some herbals
	• Difference in cost
	• Internet order
	• Collecting plants in regional environment
	• Home-cultivated plants
(C2) Public cost	• Lack of reimbursement,
	• Herbals less expensive than conventional drugs
	• Access to herbals as a question of cost
	• Cost efficiency of HM,
	• Reduced frequency of doctor visits
	• Oversupply, undersupply, inappropriate healthcare

> *"I think their effects are gentler, that they are - that they don't have as many side effects, that you may get a longer-lasting effect if you are taking them on a regular basis."*

There were many comments on preventive effects of herbals. However, some patients bring into consideration that it is difficult to say whether the effect is caused by herbals or not.

> *"And as soon as I get that feeling, like I am outside for more than an hour, I prepare a cup of tea for myself right away when I get home, as a preventive measure."*

> *"Of course you can't prove it afterwards. As in I did not get a cold because I took that. And then if you get one you say 'Now I got sick anyway'."*

For some patients herbals are always the first course of action if feeling symptoms.

> *"Well, as first course of action I almost always prefer natural remedies. Especially for the gastro-intestinal tract, but also for cold-like illnesses, be it a head cold, a cough, hoarseness, a sore throat."*

Some patients mention that there is more time needed with HM in comparison to conventional medicine. Many patients report a longer duration of treatment but a continuing effect of herbals. A few of them think that this could also be an advantage because patients have to cure themselves properly.

> *"And with herbs and teas and all those alternative treatments it takes longer for it to go away sometimes but then it really is gone."*

> *"If I have the time for it and really recover from it then I do drink tea all day long or I try to stay warm, and well, keep to the rules that are known to help recovery."*

Some interviewees explicitly explain that they use HM as a substitute for antibiotics in the case of respiratory tract infections.

> *"Well to me it's about the question antiobiotics [sic] or no antibiotics. And that's actually my motivation there to get around the antibiotics [. . .] well, since I have been taking that early on I have, if I remember correctly, not taken any more antiobiotics [sic]. And before, I did that on a regular basis."*

Some patients describe that herbals appeal to the senses and evocate positive memories.

> *"Well what is pleasant is that it does have an immediate effect as you have a taste of eucalyptus [sic] in your whole mouth, well in your nose, in your mouth. So you instantly feel that it's working."*

> *"You do already feel better with a cup of tea in your hands. And it heats you up from the inside for sure. Though I don't know if it really does that. Yet, with a cold I don't really know if the tea really plays such a big part. Maybe it has a soothing effect on your throat or something like that, or it's simply relaxing."*

3.1.3. Subjective Theory of Disease. Most of the interviewed patients prefer a more "natural way of healing" embedded in a holistic approach. This opinion was often combined with the idea that disease is caused by an imbalance of the organism and HM would act as an aid for self-help, for example, strengthen the body's natural defense system. In this sense herbals provide a causal way of healing.

> *"So you don't just fight an illness, but treat the whole person as such. I have studied the topic quite thoroughly."*

> *"So they support the body's abilities but not interfere too heavily so that they support the body's healing power"*

HM as therapy which does not disturb the body's balance is seen as one of the main differences in comparison to conventional medicine.

> *"When the body gets out of balance I help it get back to its balance rather than I just take something and not restore its balance but fight a symptom instead."*

3.1.4. Defensive Attitude towards Conventional Medicine. Some of the interviewed patients have developed a defensive attitude toward conventional medicine, which is mainly based on side effects they have experienced taking antibiotics or other conventional drugs.

> *"Sure, everything you take regularily [sic] has side effects. I think, however, that you can downright poison yourself with conventional medicine."*

> *"I was prescribed so many antibiotics that I developed an allergic reaction to penicillin [sic]. Now, those agents are extremely harmful, actually, I am left with an infection of the intestines every time."*

Further, the lack of time of conventional doctors is given as an explanation for the preference of HM. A few patients report that they do not feel taken seriously by their conventional doctors.

> *"And one of the reasons is doctors not taking enough time, for sure."*

One patient expressed a great mistrust towards pharmaceutical companies as an explanation for his defensive attitude towards conventional medicine.

> *"For example, I made the decision not to go to any doctor meeting with medical representatives. And every doctor meets them. So you have to go to an alternative practitioner separately, though even they meet them [. . .] I would rather try charmstone therapy than have a medical representative tell me what drugs I to take because that's not objective information [. . .] what would be important to me were to improve the doctor-patient relationship, that for example there would be a law that they cannot advertise at doctors' offices."*

3.1.5. Active Role. In many interviews an elevated health awareness was expressed by the interviewees.

> *"No, you have to have a little patience, but I do take it from the start. I don't even let it get that far and then it's taken care of very soon too. You just have to have a little feel for it."*

An important reason stated by some patients to use HM was to take over an active role in the process of treatment combined with a feeling of high autonomy and self-responsibility. A few patients see HM as a possibility to reduce consultations with a doctor.

> *"Sometimes it doesn't work any other way, but essentially, you can take many matters into your own hands. Maybe that's one of those instances where you have to think for yourself and not have yourself get back on your feet with meds."*

"And I think I am the same as many others, that I decide to just treat myself, if that harms me I will bear responsibility for it and won't hand it over."

Additionally, feelings of curiosity, inquisitiveness, fascination, or just the search for something else were reported by some of the patients as reasons to use HM.

"When I hear of a plant that's new to me. "Stevia" I heard of for the first time recently. I look it up after. And find out if someone has done any research about it or what effects it has. If I do find someone who knows something about it then I interrogate him thoroughly."

3.2. Communication and Information. Statements on communication and information were categorized in four categories "doctors' approach," "quality of communication," "relevant elements of information," and "sources of information" (Table 3).

3.2.1. Doctors' Approach. According to the patients' experiences doctors' attitudes concerning HM range from categorical refusal, dogmatism, unconcern to sincerity, and a positive conviction.

"My doctor at home was fairly conservative."

"Yes, he does know about that [that I am taking herbal medicine]. I think he doesn't care about it, pretty much."

"My current GP does have a good attitude but he also has further training in herbal medicine. But I am very happy now there is a response."

One patient expresses a deep mistrust towards conventional doctors because he thinks that they are henchmen of the pharmaceutical industry.

"I think of doctors as the henchmen of pharmaceutical companies, too. Politicians rather like to gather their belongings. They also profit from it."

3.2.2. Quality of Communication. Some patients report their doctors being very open or even enthusiastic towards trying out new approaches to treatment with HM. They feel accepted by their doctors in the sense of a shared decision.

"He is always thrilled whenever I get him something like that [information about HM]."

"I know from acquaintaces [sic] who can afford a real homeopath, partly they don't go there because of the medicine, but because he is taking his time and there's a real conversation, you can talk to him about everything, and your problems and fears can be treated with drugs, too. It just felt better somehow…"

Other doctors seem to have a rather patriarchal style communicating with their patients about HM.

"I used to have a different doctor too: 'I am the doctor, you can't tell me what to do.' And so on."

Some patients describe a miscommunication between diverse providers (doctor, CAM practitioners, pharmacists, and pharma companies) and between doctors and patients.

"I've noticed many doctors being very self-opinionated. They will say 'I am prescribing this to you and you don't have a say in it'. And when I inquire 'But what kind of side effects does this have, which alternatives are there?'. It's like that […] they don't like that at all, to be questioned."

Placebo and nocebo-effects (especially induction of fear) play an important role in communication about HM.

"I can't say I have certain expectations towards a specific drug. Additionally, I can imagine that many herbal drugs are simply ineffective or just have a placebo-effect. Well actually […] I do have expectations. When I am taking something like that I always have a sense of having taking something without resorting to a chemical cocktail and I also think that's the reason why many others do that too."

"…But then you have a look on the patient information leaflet and see how many side effects there are."

3.2.3. Sources of Information. The sources of information of our interviewed patients were widespread across all media and healthcare professionals, family, friends and colleagues, advertising as well as patient information leaflets.

"Yes, I sometimes read what's in the paper or in magazines, or I watch TV and listen to what my mother says, or my grandmother, what kind of experiences they've had in the family circle. […] basically, that's where I get my information from"

"Ok sure, but if I asked about it I would ask a doctor. Or whenever I have a colleague who knows about that stuff […] I have the internet and I read medical journals, of course."

"Well I have to say those magazines in pharmacies are pretty valuable nowadays."

"While at the pharmacy and with friends, you just talk about that, because you […] I am a scientist and work with quite a lot of doctors, so mostly acquaintances or apothecaries."

"Well, I get it from the patient information leaflet or afterwards, during the talk with the doctor."

"I was in a self-help group. I was there when there was a meeting for people affected by fibromyalgia or rheumatism somewhere […]"

"I am a member of a society for alternative medicine. And if I don't know something myself then I will ask the alternative practitioner."

3.2.4. Relevant Elements of Information. Some patients wish to have more information about indications, side effects and mechanism of action of some herbal preparations. They would like to have specific recommendations about dosage with regard to side effects and specific conditions such as pregnancy.

> *"You don't find a lot about herbals in the patient information leaflet. They always write 'no side effects' or 'allergy' and that was it."*

> *"It is pretty difficult with herbal medicine because there is so little about the side effects in the patient information leaflet. Sometimes, I can't imagine there being so little side effects if it's an herbal drug."*

> *"I do know that [name of a herbal preparation] is highly problematic for pregnant women because there is some kind of active ingredient in there which is just problematic. I just think in this respect I would not think of this as any safer than the meds of conventional medicine."*

Furthermore, uncertainty exists about possible quality differences in drugs and possible consequences regarding side effects.

> *"I still prefer them [plants] from the pharmacy, however, because they have certain quality standards they have to adhere to. The prepackaged thyme tea, for example, is sometimes offered in the drugstore but I have made the experience that it wasn't so good."*

Patients want safe herbals with a lack of toxic remnants.

> *"That they're pure, that they have a certain quality, are free of pesticides and other harmful substances [...] basically what you would expect of other drugs, too."*

3.3. Cost. Patients' comments on costs were categorized in two main categories: "cost for the individual" and "cost for the public" (Table 3).

3.3.1. Cost for the Individual. Most patients seem to be willing to pay extra for HM, because health is worth the money, although some admitted that they would only pay for proven therapies. A few patients complained that prices for some herbals are far too high.

> *"With things like health or well-being, I don't care. If I know it will help me I will gladly spend the money."*

> *"I think it's okay for it to be more expensive than regular painkillers because I think that it has a higher value. But it can't cost like double. Under ten euro is manageable."*

> *"For example with gingko [...] I had been interested in it, for studying it's quite good. But it was too expensive [...] and I think that it is a kind of trend [...] it simply was too pricey for me."*

A few patients are looking for alternative ways to get herbals. One patient said that he orders herbals on the internet because it is less expensive. Another patient collects plants in the surroundings and cultivates medicinal plants in his own garden.

> *"The pharmacy is too expensive. I found a way of getting it directly from the factory."*

> *"Getting the herbs yourself, I did that too, but it's a bit more difficult and you have to be in the right area. In Heidelberg, it's somewhat difficult, but generally, I get them from the pharmacy."*

> *"And like I said, one always has a shrub of peppermint tea in one's garden."*

3.3.2. Cost for the Public. Some of the patients expressed their anger about the lack of reimbursement of HM although herbals are less expensive compared to most of the conventional drugs. They argue that access to HM should not be a question of cost.

> *"When they were saying you had to pay for your own medicine I was definitely upset because I thought it costs so little compared to the other drugs that are being prescribed. It's a shame that it's not being paid for anymore [...]"*

> *"I would do that [visit the alternative practitioner] if I could afford it [...] policy is always aiming at the patient paying for these things himself [...] but it's just a question of cost [...] I don't have a lot of money and healthy food is important, too. I mean I can't spend money on the alternative practitioner if I don't have money to pay for my food."*

In the patients' opinion HM is a cost efficient therapy.

> *"I can say something about the healthcare system. What I find a bit odd is that this herbal drug and the homeopathic one for your throat was recommended to me by an ENT specialist ... and since I have been taking it I have neither had tonsilitis nor bronchitis, which I had had regularily [sic] before and that was with antiobiotics [sic] and all that crap. I got sick right after. That ENT specialist hasn't seen me since and she doesn't make any money, since she has advised me so well, she doesn't make any money off me, because I am done [...] And that I find considerable. Because there's an error in the system somehow."*

Patients express in their own words general problems of modern health systems based on competition and profit maximization leading to oversupply, undersupply, and inappropriate healthcare.

> *"Truthfully, I don't find it acceptable for them to be allowed to influence doctor so much because health isn't a product that can be advertised for like for a car or something, if you know what I mean. And I think that it's not only me, but also the other patients that are becoming pretty suspicious and are just looking for alternatives. Well, that's simply something that is so noticeable and negatively so. And I think to myself, that's why phytotherapy is booming."*

4. Discussion

This study reveals some new and specific aspects for HM from the patients' perspective which may supplement the results of preceding studies. The interviewed patients feel a traditional link to HM nourished by experiences in the family, by friends or persons in their social environment. A major part of the patients highlight the active role and the feeling of more autonomy using HM fitting well with a higher health awareness, curiosity, and motivation to try out something else. Most interviewees have the experience that HM has softer and slower effects, but in a more causal and, therefore, more sustainable way. HM is regarded to have fewer side effects compared to conventional medicine. However, some of the interviewed patients do not feel sufficiently and reliably informed about indications of HM, dosage and possible side effects, or interactions with conventional drugs. Furthermore, our results show that patients draw their information from multiple sources: the media, family and friends, healthcare professionals including health insurances and self-help groups. Patients' experiences with doctors regarding HM are varied, ranging from active recommendations of specific preparations to categorical refusal of HM. Patients' opinions regarding costs all point in the same direction: a lack of understanding that HM is not being reimbursed by health insurances, but a readiness to pay extra for HM.

Our results are in good agreement with preceding qualitative studies exploring patients' views on HM, revealing autonomy and patient-practitioner collaboration as main themes in this context [12–14]. In a UK interview study with 19 adults regularly consulting medical herbalists participants reported that, in comparison to conventional medicine, HM satisfied their expectations of healthcare because it has greater consistency with their own understanding about health, illness, and healthcare [12, 13]. Also, in our study the "subjective theory of disease" was identified as a main category, being of great importance for patients. Another qualitative study from the UK with female patients demonstrates severe information deficits regarding herb-drug interactions [14]. From our study it can be demonstrated that patients draw on a number of information sources with the risk of wrong or missing information. Partly they are complaining about their doctors' lack of information about HM. A lack of knowledge may be also suggested considering results of international studies. According to a survey among GPs in the United States the overwhelming majority (84%) thought they needed to learn more about CAM to adequately address patient concerns [9]. A cross-sectional study among doctors, pharmacists, nurses, and dietitians has shown that physicians assess their levels of knowledge about HM as moderate and their communication skills as poor [10]. A systematic review about HM and pharmacists points in the same direction. Pharmacists do not perceive their knowledge about herbals to be adequate [15]. Comparable studies for Germany have not yet been conducted.

In some countries attempts have been made to overcome this problem with educational programs. Fact sheets about herbals were developed and tested among 92 GPs within a research project in Australia. The results of this study show that fact sheets increased knowledge and improved communication with their patients about those specific herbals [16]. In Canada, core competencies in the education of pharmacists about HM have been developed and agreed among pharmacy educators [17]. In a US study an E-curriculum about herbs led to significant and sustained improvements in clinicians' expertise about herbs regardless of the delivering strategy [18]. Another way could be the use of the internet as a scientifically qualified source of information about HM for patients. In Germany, first projects have already put into practice, for example, www.phytodoc.de. However, the impact of those platforms needs to be assessed in the future.

Educating health professionals about HM should include the concept of "meaning effects" [19]. According to our main categories "medical culture," "subjective theory of disease," and "active role" HM is more than just taking a pill. Also, clinical studies point in the same direction and show that—similar to other CAM therapies—"meaning effects" (or so-called placebo effects) play a major role in the therapeutic success of HM. A recently published study investigating placebo effects in patients with common cold receiving placebo or *Echinacea* supports the general idea, that beliefs and feelings about treatments may be important and should be taken into consideration when making medical decisions [20]. The findings from our qualitative study may provide a deeper understanding of this idea. For example, some comments within our interviews show that herbals appeal to the senses and evocate positive memories—a potentially potent way of encouraging the healing process and promoting health.

Furthermore, our findings show that the social environment in this context is highly important. This is also supported by the findings of an Australian study among older women showing that CAM use was reflective of the personal beliefs of the women and members of their close social networks. The differences between women living in rural versus urban regions observed in this study have been attributed to the difficulties inherent in accessing certain types of CAM in rural areas [21].

According to the WHO medicines Strategy 2008–2013, the integration of HM into the national health systems should be facilitated with a focus on better regulation of products and providers [22]. In Germany, the time has come to educate health professionals working in primary care

about the basics of HM. Special emphasis should be placed on frequent indications such as respiratory tract infections and conditions where herb-drug interactions are probable, such as depression. It has to be ensured that information provided by the different professionals is consistent and in line with evidence—as far as evidence exists. This process should be promoted by universities in cooperation with the professional bodies of health professions in order to maintain independence of the pharmaceutical industry. Ideally, learning objectives regarding HM should be integrated into residency training curricula of GPs to ensure each GP having a basic knowledge in HM. A challenge for doctors will be to understand the individual needs of each patient regarding HM. This may include clarification of and communication about possible side effects and interactions of herbal drugs. This is particularly important in multimorbid patients with multimedication, furthermore, in patients with specific indications and while supporting them in their active role and self-management. All that is only possible if reimbursement of herbal medicine by the statutory health insurance will be reintroduced. Indeed, there is some evidence by health economic studies making this an interesting option also for health policy. As indicated by a Dutch study, patients whose GP had additional CAM training generate up to 30% less healthcare costs [23]. This may be caused by the more active role patients have in CAM. As our results show, HM can increase self-responsibility of patients and, hence, doctor visits may become less frequent. Furthermore, HM may reduce overuse and misuse of antibiotics, for example, in treatment of viral respiratory tract infection and therefore prevent negative consequences of antibiotics' overuse. However, this should be subject of future studies.

Our study may have some limitations because we mainly interviewed female patients having had positive experiences with HM. However, our aim was not to draw conclusions on a representative basis but to explore the experiences of patients with HM and to understand their motives for using HM.

5. Conclusion

The use of HM meets patients' personal understanding about health and illness and their need for autonomy and self-care. However, there is a demand for better and consistent information on HM. The main challenges in the context of primary healthcare are to promote and upgrade the knowledge and skills of the providers about HM to ensure patient safety and to support patients in their self-management. Appropriate strategies for education have to be developed and tailored to the specific needs of health professional groups. At the same time, further research under inclusion of patients' views is much-needed in order to improve the evidence base for HM.

Conflict of Interests

The authors declare that they have no conflict of interests.

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Research Article

Analgesic and Anti-Inflammatory Activities of the Ethanolic Extract of *Artemisia morrisonensis* Hayata in Mice

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The aim of this study was to investigate the possible analgesic and anti-inflammatory mechanisms of the ethanolic extract of *A. morrisonensis* Hayata (AM_{EtOH}). Two models were employed for evaluation of the analgesic effects: acetic acid-induced writhing response and formalin-induced paw licking. The results demonstrated that AM_{EtOH} decreased writhing response for both the acetic acid assay and the licking time in the formalin test. The anti-inflammatory effect was evaluated by paw edema of mice induced by λ-carrageenan. AM_{EtOH} significantly decreased induced paw edema three to four hours after λ-carrageenan injection. Additionally, the results indicated that the anti-inflammatory mechanism of AM_{EtOH} may be due to the declined levels of nitric oxide (NO) and malondialdehyde (MDA) in the edematous paw. Furthermore, AM_{EtOH} decreased the tumor necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6) levels, leading to the reduction of prostaglandins and subsequently alleviated edema. Isolation and purification of the AM_{EtOH} extract determined *p*-hydroxyacetophenone to be a major component at 130 mg/g of extract. No mortality was observed in the acute toxicity test given at the dose of 10 g/kg. This study demonstrated the possible mechanisms for the analgesic and anti-inflammatory effects of AM_{EtOH} for mice and provided evidence for the ethnobotanical uses of *A. morrisonensis* in treating inflammatory diseases.

1. Introduction

Rheumatoid arthritis (RA) is a chronic, systemic, inflammatory, and immunological disorder causing joint destruction and disability, typically observed in people between the ages of 30 and 50 [1]. This disease affects 0.5–1% of adults and its incidence and prevalence are higher in industrialized countries [2]. Drug treatment for RA includes nonsteroidal anti-inflammatory drugs (NSAIDs), low-dose oral or intra-articular glucocorticoids, disease-modifying anti-rheumatic drugs, biologic response modifiers, and daily calcium/vitamin D [3]. Inflammation is the result of host defense to tissue injuries and/or against pathogenic stimuli; however, persistent or hyperinflammation can lead

to tissue damage and eventually to organ failure if not properly controlled. In response to infectious agents or pro-inflammatory stimuli, activated macrophages/monocytes secrete cytokines, growth factors, and inflammatory mediators including interleukin-1 (IL-1), interleukin-6 (IL-6), tumor necrosis factor-alpha (TNF-α), nitric oxide (NO), prostaglandin E_2, and reactive oxygen species (ROS) which will cause inflammation injury [4, 5]. Excessive NO will rapidly combine with superoxide anion (O_2^-) to generate peroxynitrite ($ONOO^-$), which can lead to pathogenesis by promoting oxidative stress, tissue injury, and cancer. Peroxynitrite is a potent oxidant that reacts with protein, lipids, and DNA, leading to subsequent multistage carcinogenesis [6, 7]. ROS can cause oxidative damage, which in turn can initiate and promote the progression of a variety of chronic diseases, such as cardiovascular diseases, Alzheimer's disease, and diabetes [8]. Therefore, superoxide dismutase (SOD), glutathione (GSH), glutathione peroxidase (GPx), and glutathione reductase (GRd) play crucial roles in ameliorating inflammatory reactions via decreasing oxidative stress and damage through reducing the production of free radicals [9, 10]. Malondialdehyde (MDA), a low-molecular-weight end product formed from decomposition of cell membrane, provides an indicator in the evaluation of the inflammatory process [11]. Also, lipid peroxidation in many disease states is a reliable assessment in oxidative injury. Besides inhibition to PG and NO by suppression of its corresponding synthases, cyclooxygenase-2 (COX-2) and inducible nitric oxide synthases (i-NOS) have demonstrated to be beneficial in the treatment of inflammatory diseases [12]. Natural products from herbal medicine or medicinal plants provide a vast pool of COX-2 and/or NO inhibitors that can possibly be developed into new anti-inflammatory drug leads. The motivation of this research is trying to seek for novel substances of natural origin that will demonstrate effectiveness in anti-inflammatory and analgesic activities with low toxicity.

Artemisia genus (Compositae) is used for treating flu, rheumatoid arthritis, back pain, and headaches among American Indians [13, 14]. *A. morrisonensis* Hayata is an endemic and perennial herb/subshrub found along roadsides or slopes above 2700 m mountains in Taiwan [15]. Traditionally, this plant has been used to treat rheumatoid arthritis, allergic rhinitis, headache, and edema in aboriginals. However, the therapeutic potential, and underlying mechanisms still remain elusive and the active component(s) have not been systematically identified. Phytochemical studies to *Artemisia* genus revealed that monoterpenes, sesquiterpenes, coumarins, and flavonoids with great structural diversity were isolated and identified [16]. The previous studies have indicated that phenolics in medicinal plants possessed anti-inflammatory activities via scavenging ROS and reduced pro-inflammatory cytokines (e.g., TNF-α, IL-1β, and IL-6). Some research indicated that the content of phenolics increases as the altitude becomes higher due to the protective role against UV [17]. Thus, quantitative analysis by HPLC interfaced to UV-Vis and/or MS is imperative to certain target constituents claimed to be responsible for the treatment of specific diseases.

In this study, the analgesic and anti-inflammatory activities of the ethanolic extract of *A. morrisonensis* Hayata (AM_{EtOH}) were evaluated in mice. Analgesic activity was undertaken by acetic acid-induced writhing response and formalin test while the anti-inflammatory activity of AM_{EtOH} was determined by using λ-carrageenan-induced mice paw edema model. In order to disclose the mechanisms of anti-inflammatory effect, the activities of TNF-α, IL-6, and COX-2 and the levels of NO and MDA in the edematous tissues were assayed, and the activities of antioxidant enzymes (SOD, GPx, and GRd) in the liver were measured.

2. Materials and Methods

2.1. Chemicals and Reagents. λ-Carrageenan, indomethacin, formic acid (FA), and Griess reagent were purchased from Sigma-Aldrich Chemical Co. (Taipei, Taiwan). Formalin was obtained from Nihon Shiyaku Industries Ltd. (Taipei, Taiwan). SOD, GPx, GRd, and MDA assay kits were purchased from Randox Laboratory Ltd. (Crumlin, UK). IL-6 and TNF-α were obtained from Assay Designs Inc. (Michigan, USA). COX-2 antibody was purchased from R&D System. NO assay kit was obtained from Cayman Chemical Co. HPLC-grade methanol and acetonitrile were purchased from Merck (Darmstadt, Germany). All other reagents used were of analytical grade.

2.2. Plant Material. *Artemisia morrisonensis* Hayata was collected along the highway 14 A at 2718 m (N24°06′57.8″ E121°13′52.0″), Nantou, Taiwan, in June 2011. The plant was identified by Dr. Yen-Hsueh Tseng, Department of Forestry, National Chung Hsing University. A voucher specimen (AM-1-06032011) has been deposited at the Graduate Institute of Ecology and Evolutionary Biology, China Medical University. Dried stems and leaves (260 g) of this plant were sliced into small pieces and extracted with 70% ethanol for four times and passed through filter paper. The combined filtrate was concentrated under reduced pressure to give a dried extract (8.6 g, yield ratio 3.3%). Prior to pharmacological tests, this extract was dissolved in dd H_2O.

2.3. Chromatographic Separation and Analyses. AM_{EtOH} was dissolved in 250 mL water and partitioned thrice against ethyl acetate with the same volume to give ethyl acetate layer (2.75 g). This layer was chromatographed through silica gel column to give 30 subfractions from which the 22th (222.8 mg) was further purified via Sephadex LH-20 eluted with acetone to yield 122.0 mg of *p*-hydroxyacetophenone. ^{1}H-NMR (500 MHz, $CDCl_3$): 2.58(s, 3H), 6.95(d, J = 8.5 Hz, 2H), 7.90(d, J = 8.5 Hz, 2H) and ^{13}C-NMR: 26.2(q), 115.6(d), 115.6(d), 129.3(s), 131.2(d), 131.2(d), 161.7(s), 198.9(s). The ESI-MS (negative mode) showed molecular ion at *m/z*: 135 and at 120 and 93 corresponding to the deletion of methyl group and further removal of CO, respectively. The above data was completely consistent with those of *p*-hydroxyacetophenone reported and confirmed by its structure. The HPLC system consisted of a Hitachi L-2130 detected by UV-Vis detector L-2420. Chromatographic

separation was performed on NUCLEODUR C-18 HTec (4.6 mm × 250 mm ID, 5 μm) with an auto-sampler L-2200. The mobile phase consisted of a mixture of water (A) and acetonitrile (B) using a gradient program set up as follows: 0 min: 5% B, 5 min: 30% B, 30 min: 95% B, and 50 min: 5% B. The flow rate was 1.0 mL/min and the detection wavelength was at 315 nm. The LC-MS was performed on Phenomenex Prodigy ODS(2), 5 m, 532610-1, 2.0 mm × 150 mm with flow rate of 250 μL/min coupled to Bruker HCT Ultra Ion Trap MS spectrometer. The mobile phase A was H_2O (+0.1% FA) + 5% ACN (+0.1% FA) and mobile phase B was ACN (+0.1% FA). The gradient program was set as follows: 0 min: 10% B, 15 min: 60% B, 17–20 min: 90% B, and 21–25 min: 10% B. The identity of *p*-hydroxyacetophenone was determined by retention time and molecular and fragment ions found in LC-MS. Quantitative analysis was made by the establishment of calibration curve with peak areas under curves in the standard addition method. The concentration of AM_{EtOH} was prepared as 5 mg/mL and spiked with 0, 0.5, 1, 2, and 5 mg/mL of *p*-hydroxyacetophenone and the amount of this component was determined as 0.13 ± 0.15 g/g extract (mean ± RSD) with $R^2 > 0.99$.

2.4. Experimental Animals. Male ICR mice (20–23 g each for analgesic test and 32–35 g each for anti-inflammatory test) were provided by BioLASCO Taiwan Co., Ltd. The mice were kept in the animal center of the China Medical University at a controlled temperature of 22 ± 1°C, relative humidity 55 ± 5%, and with 12 h light/12 h dark cycles for 1 week before the experiment. Animals were given with rodent diet and clean water *ad libitum*. All studies were conducted in accordance with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals. All tests were conducted under the guidelines of the International Association for the Study of Pain [18]. The experimental protocol was approved by the Committee on Animal Research, China Medical University. Ether was used to anesthetize the animals before sacrificing them.

2.5. Acute Toxicity Study. The acute toxicity test in mice was performed according to the method of Liao et al. [19]. Male ICR mice (22–25 g) were randomly divided into three groups with 10 mice in each and the mice were administered orally with AM_{EtOH} (10 g/kg). The experimental mice were given with forage and water *ad libitum* and were kept under regular observation for any mortality or behavioral changes within 14 days.

2.6. Acetic Acid-Induced Writhing Response. The writhing test in mice was conducted by the method of Koster et al. [20]. Male ICR mice ($n = 10$) were fasted for 24 h before the experiment with free access to water. Acetic acid (1.0%) was injected intraperitoneally (i.p., *v/v*, 0.1 mL/10 g body weight) to induce writhing. AM_{EtOH} (20, 100, and 500 mg/kg) was orally administered (p.o.) to each group of mice 60 min before acetic acid injection. Indomethacin (10 mg/kg) was administered intraperitoneally as a positive control 30 min before the injection of acetic acid. The number of muscular contractions was counted 5 min after the injection of acetic acid and lasted for over 10 min. The recorded data represented the total number of writhes observed.

2.7. Formalin Test. The formalin test was conducted based on the method of Tjølsen et al. [21]. Male ICR mice ($n = 10$) were fasted for 24 h before the experiment with free access to water. Twenty microliter of 5% formalin in saline was injected subcutaneously into the right hind paw of each mouse. AM_{EtOH} (20, 100, and 500 mg/kg) were orally administered to the animals 60 min prior to the formalin injection. The same volume of distilled water was orally administered as the vehicle control. Indomethacin (10 mg/kg, i.p.) was administered 30 min before formalin treatment. These mice were individually placed in a transparent Plexiglas cage (25 × 15 × 15 cm). The time spent in seconds on licking and biting the injected paw was recorded in both of the early phase (0–5 min) and late phase (20–30 min) after the formalin injection as neurogenic and inflammatory pain, respectively.

2.8. λ-Carrageenan-Induced Mice Paw Edema. The test was conducted according to the method of Vinegar et al. with some modification [22]. Male ICR mice ($n = 10$) were fasted for 24 h before the experiment with free access to water. Fifty microliter of 1% λ-carrageenan suspended in normal saline solution (0.9% w/v NaCl) was injected into the plantar side of right hind paw and the paw volume was measured by a plethysmometer at the 1st, 2nd, 3rd, and 4th hour after the injection. The degree of swelling was evaluated by the delta volume ($a - b$) where "a" stands for the volume of the right hind paw after the chemical treatment and "b" being the volume before the treatment. AM_{EtOH} (20, 100, and 500 mg/kg) and normal saline solution were administered orally after 120 min for experimental and vehicle control after λ-carrageenan injection, respectively. Indomethacin (10 mg/kg, i.p.) was administered 150 min after the λ-carrageenan injection for positive control. In the secondary experiment, another set of mice were orally administered with normal saline, AM_{EtOH}, and indomethacin with the same condition described previously. The right hind paws and liver tissues of the mice were taken at the fourth hour after λ-carrageenan injection. The paw tissue was rinsed and immediately placed in ice-cold normal saline four times its volume before homogenization at 4°C. Subsequently, the homogenate was centrifuged at 12,000 rpm for 5 min and the supernatant was obtained and stored at −20°C for MDA, COX-2, NO, TNF-α, and IL-6 analyses. The whole liver tissue was rinsed and placed in ice-cold normal saline with equal volume before homogenization at 4°C. The homogenate was centrifuged at 12,000 rpm for 5 min and the supernatant was obtained and stored at −20°C for antioxidant enzymes (superoxide dismutase, glutathione peroxidase, and glutathione reductase) activity analysis.

2.9. COX-2, TNF-α, and IL-6 Assay. COX-2, TNF-α, and IL-6 were measured by a quantitative sandwich enzyme

immunoassay technique [23]. The capture antibodies of COX-2, TNF-α, and IL-6 were seeded to each well of a 96-well plate overnight. In the next day, a second set of the biotinylated antibody was incubated with sample tissues or standard antigens in the plate before streptavidin-HRP was finally added. COX-2 and TNF-α were measured at 450 nm to determine their amount. The COX-2 activity was expressed as U/mL per protein (U/mL/mg protein) whereas TNF-α was represented as picogram per milligram protein (pg/mg). The absorption of IL-6 was measured at 405 nm and was represented as pg/mg.

2.10. NO Assay. NO was measured based on the method of Moshage and Green et al. [24, 25]. Nitrate was converted to nitrite utilizing nitrate reductase. Nitrite subsequently reacted with sulfanilic acid to produce diazonium ion and reacted with N-(1-naphthyl)ethylenediamine to give chromophoric azo derivative (purplish red), which could be recorded at 540 nm. Values obtained by this procedure represented the sum of nitrite and nitrate and represented as M.

2.11. MDA Assay. The injection of λ-carrageenan will induce the production of MDA, which is evaluated by the 2-thiobarbituric acid reacting substance (TBARS) method [26]. MDA can react with thiobarbituric acid (TBA) under the acidic and high-temperature conditions. MDA and TBA then formed a red-complex TBARS, which can be measured colorimetrically. The absorbance of TBARS was determined by measurement of 532 nm. MDA levels were expressed as nmole MDA/mg protein.

2.12. Measurement of Antioxidant Enzymes. SOD was measured following the method of Woolliams et al. [27]. Xanthine and xanthine oxidase generated superoxide radicals reacting with 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride (INT) to form a red formazan dye whose color was recorded at 540 nm to determine its amount. previous supernatant from liver tissue (20 μL) was added to 120 μL of 0.1 M phosphate buffer (CAPS 40 mmol/L, EDTA 0.94 mmol/L, pH 7.0) and mixed well. An aliquot of 5 μL mixture was added to 340 μL of mixed substrate (xanthine 0.05 mmol/L, INT 0.025 mmol/L) followed by adding xanthine oxidase to record at 505 nm, 37°C at intervals of 30 sec for six times on a Hitachi U 2000 spectrophotometer. The enzyme activity was represented as the amount that inhibited the oxidation of INT by 50% from which is equal to 1 unit (U) and expressed as U/mg protein. GPx was measured according to the method of Ceballos-Picot et al. by detecting the contents of GRd and NADPH [28]. Oxidation of NADPH into $NADP^+$ is accompanied by a decrease in absorbance recorded at 340 nm. GRd was measured while detecting the decrease of glutathione (GSSG) in the presence of NADPH [29].

2.13. Statistical Analysis. All data represented the mean ± SE ($n = 10$). Statistical analyses were performed with SPSS software and were calculated using one-way ANOVA followed by Scheffe's multiple range tests. The criterion for statistical significance was determined as $P < 0.05$.

3. Results

3.1. Chromatographic Analyses of AM_{EtOH}. LC-MS fingerprint profile was established as base peak chromatogram (BPC) for AM_{EtOH} (Figure 1(a)) and *p*-hydroxyacetophenone was identified with retention time (RT) at 6.6 min. Extracted ion chromatogram (EIC) was shown in Figure 1(b) as *m/z* at 134.9 and was selected in negative ion mode. BPC for authentic *p*-hydroxyacetophenone was shown in Figure 1(c) and was consistent with RT, molecular and fragment ions. The chromatogram detected at 315 nm was shown in Figure 2 with RT at 15.99 min and was used for quantitative analysis.

3.2. Acute Toxicity Study. Acute toxicity of AM_{EtOH} was evaluated in mice at the doses of 10 g/kg. After 14 days of oral administration, AM_{EtOH} did not cause any behavioral changes and no mortality was observed. Therefore, the LD_{50} of AM_{EtOH} was concluded to be greater than 10 g/kg in mice, indicating that it was practically nonacute toxic.

3.3. Acetic Acid-Induced Writhing Response. Figure 3 shows that acetic acid-induced writhing responses in mice serve as an indication of analgesic activities exerted by AM_{EtOH}. Intraperitoneal injection of acetic acid produced 47.0 ± 1.4 writhes in the control group. The writhing response was significantly reduced by treatment with indomethacin (10 mg/kg) or AM_{EtOH} at 100 and 500 mg/kg with *P* being less than 0.05 and 0.001, respectively.

3.4. Formalin Test. In the early phase, AM_{EtOH} (20, 100, and 500 mg/kg) and indomethacin (10 mg/kg) did not show any significant changes compared with the control group (Figure 4(a)). In the late phase, licking and biting responses induced by subcutaneous injection of formalin have lasted for 146.8 ± 3.7 s in the control group. As shown in Figure 4(b), the time decreased significantly with indomethacin (10 mg/kg) or by treatment with AM_{EtOH} at 100 and 500 mg/kg with *P* being less than 0.05 and 0.001, respectively.

3.5. Effect of AM_{EtOH} on λ-Carrageenan-Induced Mice Paw Edema. As shown in Figure 5, the volume of mouse paw increased as edema developed and served as an indication of inflammatory activity after injection of λ-carrageenan. AM_{EtOH} (100 and 500 mg/kg) and indomethacin (10 mg/kg) significantly decreased paw edema at the 3rd and 4th h after the injection. AM_{EtOH} at the concentration of 100 and 500 mg/kg demonstrated almost as equal activity with inhibition as indomethacin (10 mg/kg) with *P* being less than 0.001.

3.6. Effect of AM_{EtOH} on COX-2 Concentration. In Figure 6, COX-2 concentration dramatically increased in the λ-carrageenan group (U/mL/mg protein). After the treatment

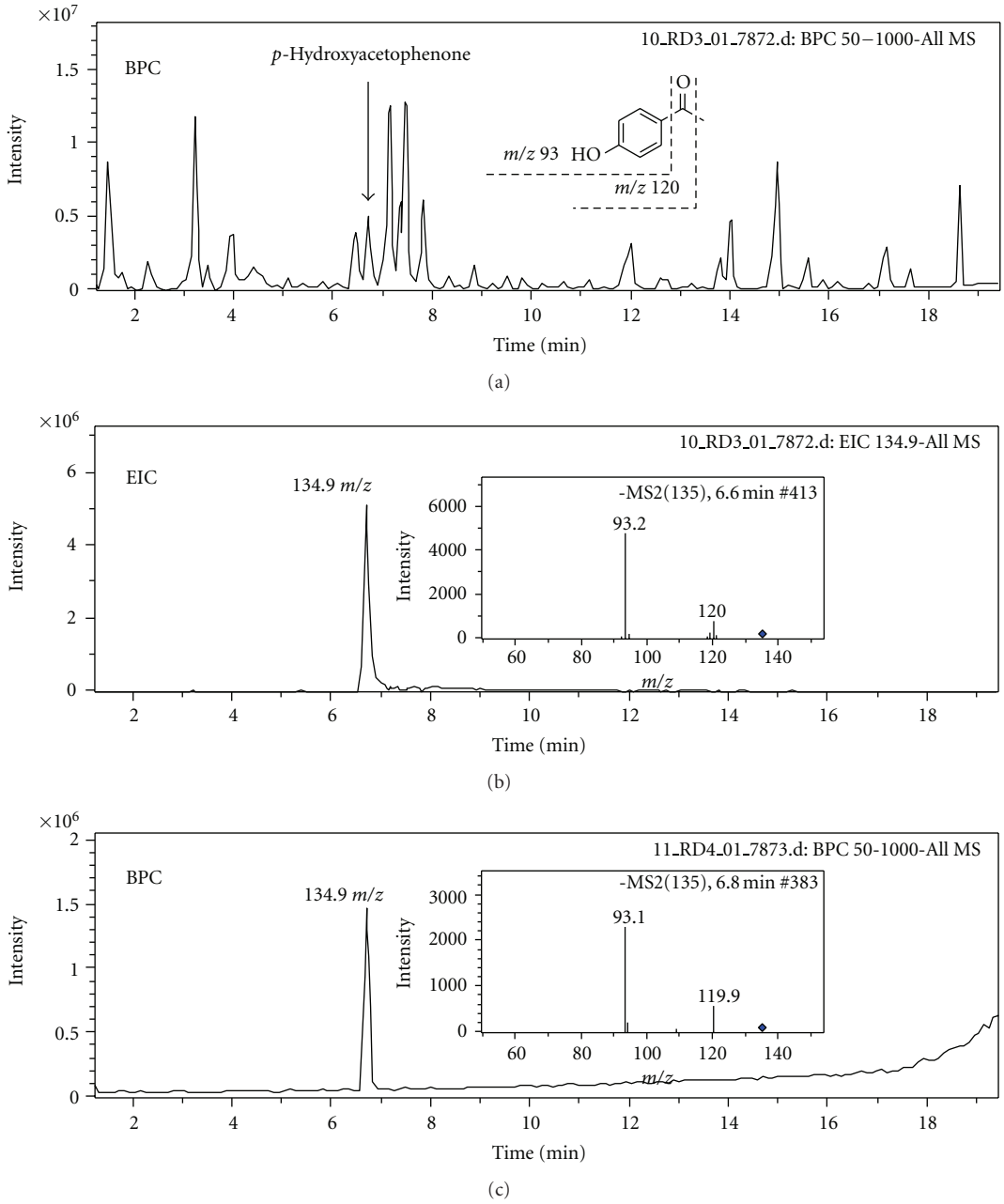

Figure 1: (a) Base peak chromatogram (BPC) from LC-MS of AM_{EtOH}. (b) Extracted ion chromatogram (EIC) from m/z 134.9 of AM_{EtOH}; fragment ions were shown as insert. (c) BPC of authentic p-hydroxyacetophenone; fragment ions were shown as insert. All chromatograms were detected in the negative ion mode.

with indomethacin (10 mg/kg) or AM_{EtOH} at 100 and 500 mg/kg, significant inhibition was observed in the COX-2 concentration with P being less than 0.01 and 0.001, respectively.

3.7. Effects of AM_{EtOH} on TNF-α and IL-6. As shown in Figures 7 and 8, TNF-α and IL-6 levels in λ-carrageenan-induced edema paws remarkably raised. Treatment with indomethacin (10 mg/kg) or AM_{EtOH} at 100 and 500 mg/kg significantly suppressed the concentration of TNF-α with P being less than 0.05 and 0.001, respectively (Figure 7). Similarly, IL-6 levels were significantly lowered by AM_{EtOH} (100 and 500 mg/kg) 3rd h after injection or indomethacin (10 mg/kg) with P being less than 0.05 (Figure 8).

3.8. Effect of AM_{EtOH} on NO Concentration. In Figure 9, NO concentration dramatically increased in the λ-carrageenan group (μM). After the treatment with indomethacin (10 mg/kg) or AM_{EtOH} at 100 and 500 mg/kg, significant

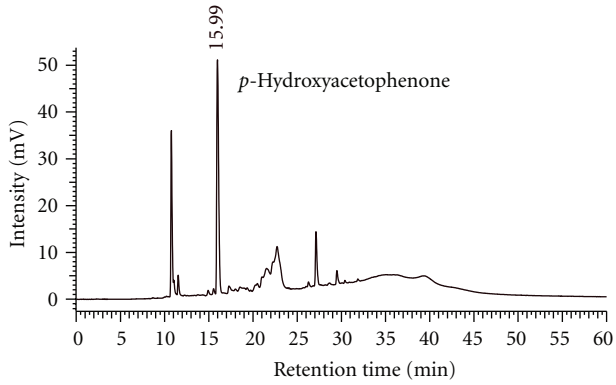

Figure 2: HPLC chromatogram of AM_{EtOH} detected at 315 nm; *p*-hydroxyacetophenone is identified at RT = 15.99 min.

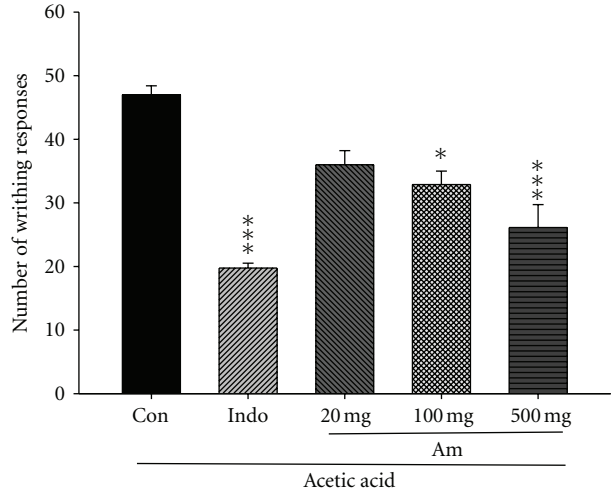

Figure 3: Analgesic effects of ethanol extract of AM_{EtOH} and indomethacin (Indo) on acetic acid-induced writhing response in mice. Each value represents as mean ± SEM ($n = 10$), $^{*}P < 0.05$, $^{***}P < 0.001$ as compared with the control (Con) group. (One-way ANOVA followed by Scheffe's multiple range test.)

inhibition was observed in the NO concentration with P being less than 0.01 and 0.001, respectively.

3.9. Effect of AM_{EtOH} on MDA Level. In Figure 10, MDA level dramatically increased in the λ-carrageenan group (nmole/mg protein). After the treatment with indomethacin (10 mg/kg) or AM_{EtOH} at 100 and 500 mg/kg, significant inhibition was observed in the concentration of MDA with P being less than 0.01 and 0.001, respectively.

3.10. Effect of AM_{EtOH} on the Activity of Superoxide Dismutase (SOD). In Figure 11, SOD activity increased by treatment with indomethacin (10 mg/kg) or AM_{EtOH} at 100 and 500 mg/kg compared to that of λ-carrageenan group with P being less than 0.01 and 0.001, respectively.

3.11. Effect of AM_{EtOH} on the Activity of Glutathione Peroxidase (GPx). In Figure 12, GPx activity increased by treatment with indomethacin (10 mg/kg) or AM_{EtOH} at 500 mg/kg compared to that of λ-carrageenan group with P being less than 0.01.

3.12. Effect of AM_{EtOH} on the Activity of Glutathione Reductase (GRd). In Figure 13, GRd activity increased by treatment with indomethacin (10 mg/kg) or AM_{EtOH} at 100 and 500 mg/kg compared to that of λ-carrageenan group with P being less than 0.01 and 0.001, respectively.

4. Discussion

Compositaceous plants are well known for their anti-inflammatory and hepatoprotective activities as well as dietary supplements for chemofpreventive purposes in a certain cancer based on ethnopharmacological evidences [30]. *Artemisia* is a genus of about 400 species found in temperate regions worldwide. The chemical constituents from the well-known *A. indica* are mainly volatile oils, coumarins, and flavonoids; however, the phytochemicals in *A. morrisonensis* Hayata are still unclear [16, 31]. We, therefore, embarked on the investigation into active ingredients and possible mechanisms of analgesic and anti-inflammatory effects of *A. morrisonensis* Hayata.

Two animal models including acetic acid-induced writhing response and formalin test were employed to evaluate the analgesic effects of AM_{EtOH}. The nociceptive responses induced by acetic acid had been demonstrated to be the involvement of eicosanoids and sympathomimetic amines. The analgesic activity of acetic acid-induced writhing model might attribute to the release of TNF-α, interleukin-1 (IL-1), and interleukin-8 (IL-8) by resident peritoneal macrophages and mast cell in mice [32]. In this study, AM_{EtOH} (100 and 500 mg/kg) possessed anti-nociceptive effects to relieve abdominal writhes induced by acetic acid in mice. Formalin test involved a biphasic pain response including a transient early phase followed by a tonic late phase. The early phase was mediated by peripheral nociceptive stimulation caused by formalin while the tonic late phase was due to inflammatory response. However, recent research indicated that the pain response in the late phase depended on the prolonged presence of the early phase, therefore, being interdependent [33]. The present study showed that the treatment of AM_{EtOH} (100 and 500 mg/kg) diminished the nociceptive response in the second phase induced in the formalin test. The results indicated that the analgesic effect exerted by AM_{EtOH} might result from its anti-inflammatory activity. λ-Carrageenan-induced paw edema in mice is a biphasic development as well in the evaluation of anti-inflammatory agents *in vivo* [22]. Histamine, bradykinin, 5-hydroxytryptamine (5-HT), and platelet activating factor (PAF) were released in the first phase occurred from 1 to 4 h after injection. Agents derived from the injured tissues stimulated the release of TNF-α which further stimulated the release of IL-1, IL-6, and IL-8. IL-6 and IL-8 in turn increased the cyclooxygenase which catalyzed the conversion of arachidonic acid to prostaglandins and thromboxanes. The mRNA expression of inducible COX-2

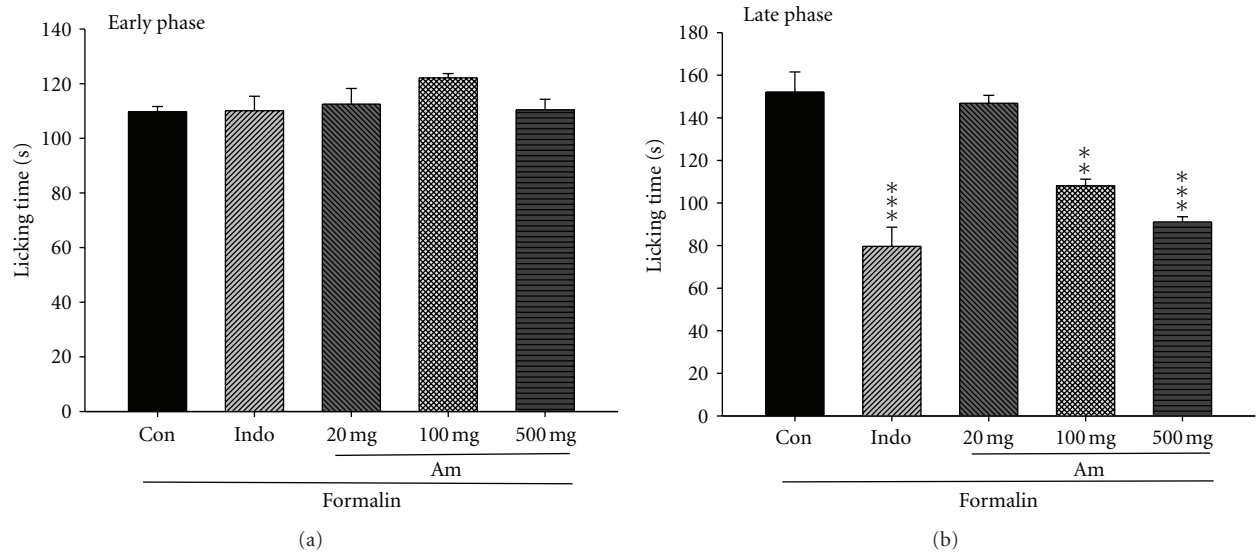

FIGURE 4: Analgesic effects of AM_{EtOH} and indomethacin (Indo) on the (a) early phase and (b) late phase in formalin test in mice. Each value represents as mean ± S.E.M. ($n = 10$), $^{**}P < 0.01$, $^{***}P < 0.001$ as compared with the control (Con) group. (One-way ANOVA followed by Scheffe's multiple range test.)

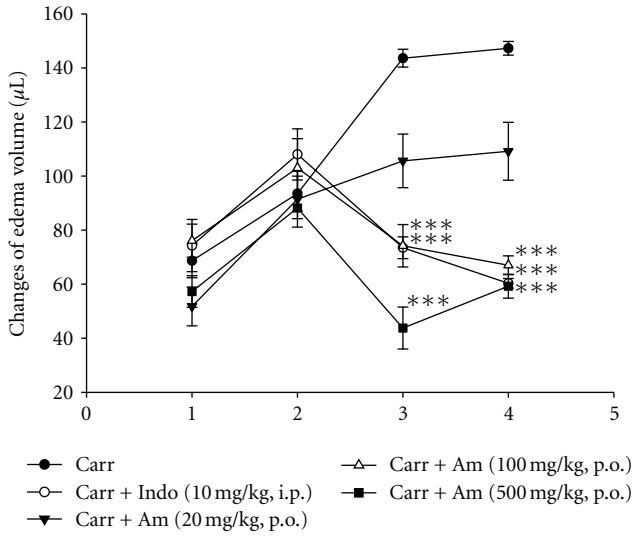

FIGURE 5: Effects of AM_{EtOH} and indomethacin (Indo) on hind paw edema induced by λ-carrageenan in mice. Each value represents as mean ± S.E.M. ($n = 10$), $^{***}P < 0.001$ as compared with the λ-carrageenan (Carr) group. (One-way ANOVA followed by Scheffe's multiple range test.)

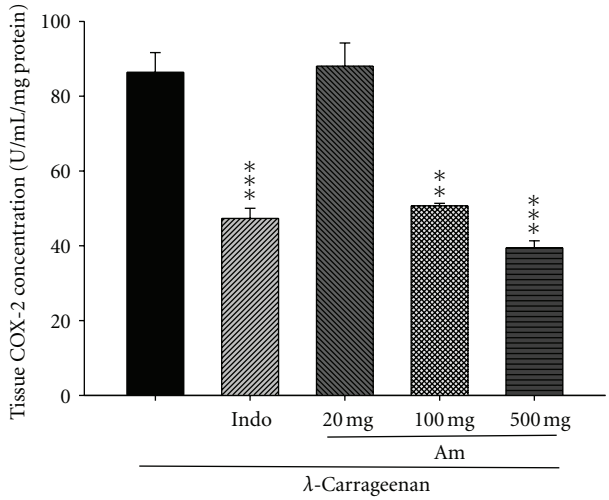

FIGURE 6: Effects of AM_{EtOH} and indomethacin (Indo) on the tissue COX-2 concentration of edema paw in mice. Each value represents as mean ± S.E.M. ($n = 10$), $^{**}P < 0.01$, $^{***}P < 0.001$ as compared with the Carr group. (One-way ANOVA followed by Scheffe's multiple range test.)

was markedly increased at the site of λ-carrageenan-induced paw edema [34]. TNF-α stimulated the release of cytokine-induced neutrophil chemoattractant 1 (CINC-1) associated with IL-8 to mediate the sympathetic pain by stimulating the release of sympathetic amines [35]. Neutrophil infiltration and activation also contributed to this inflammatory response by producing oxygen-derived free radicals, that is, superoxide anion (O_2^-) and hydroxyl radicals [36]. Four to 10 hours after λ-carrageenan injection, NO produced from i-NOS was involved in the maintenance of inflammatory responses, including the increase in vascular permeability and edema through changes in local blood flow. Nitric oxide reacted with superoxide anion to become peroxynitrite ($ONOO^-$), which is proposed to play a major pathogenic role in the inflammatory process especially in macrophage-like cells. In this study, AM_{EtOH} showed significant anti-inflammatory effect in λ-carrageenan-induced mice paw edema from the 3rd to the 4th hour in a dose-dependent manner. Furthermore, the levels of TNF-α and IL-6 were also decreased by treating with AM_{EtOH} and indomethacin. Therefore, the plausible anti-inflammatory mechanisms of

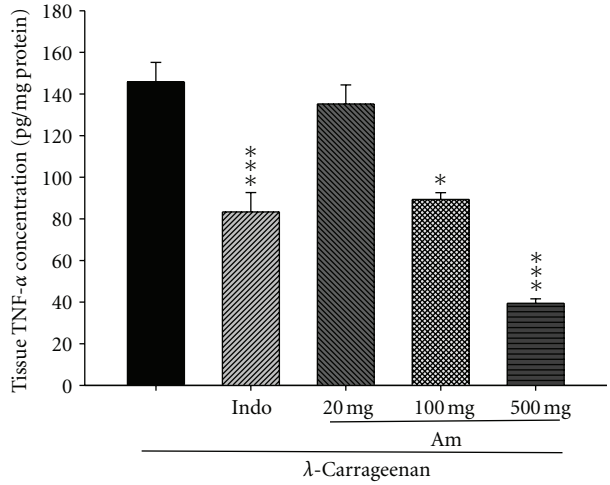

Figure 7: Effects of AM_{EtOH} and indomethacin (Indo) on the tissue TNF-α concentration of edema paw in mice. Each value represents as means ± SEM ($n = 10$), $^{*}P < 0.05$, $^{***}P < 0.001$ as compared with the Carr group. (One-way ANOVA followed by Scheffe's multiple range test.)

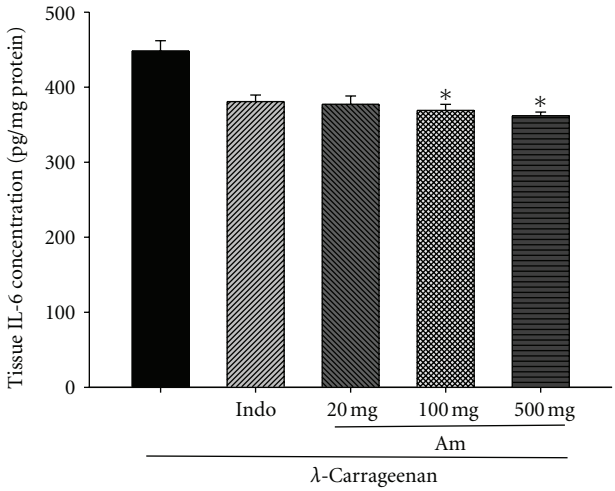

Figure 8: Effects of AM_{EtOH} and indomethacin (Indo) on the tissue IL-6 concentration of edema paw in mice. Each value represents as means ± SEM ($n = 10$), $^{*}P < 0.05$ as compared with the Carr group. (One-way ANOVA followed by Scheffe's multiple range test.)

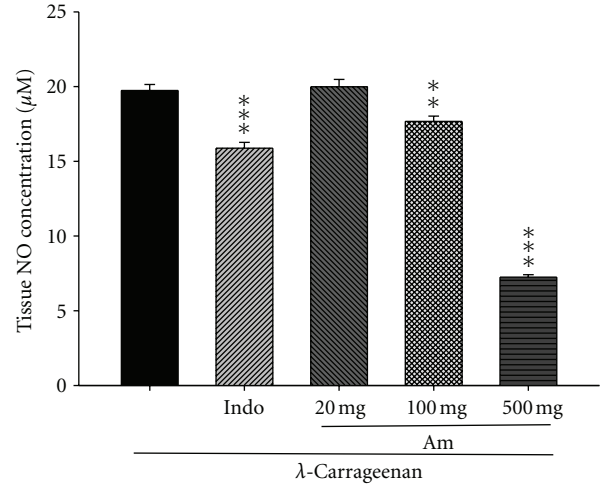

Figure 9: Effects of AM_{EtOH} and indomethacin (Indo) on the tissue NO concentration of edema paw in mice. Each value represents as mean ± S.E.M. ($n = 10$), $^{**}P < 0.01$, $^{***}P < 0.001$ as compared with the Carr group. (One-way ANOVA followed by Scheffe's multiple range test.)

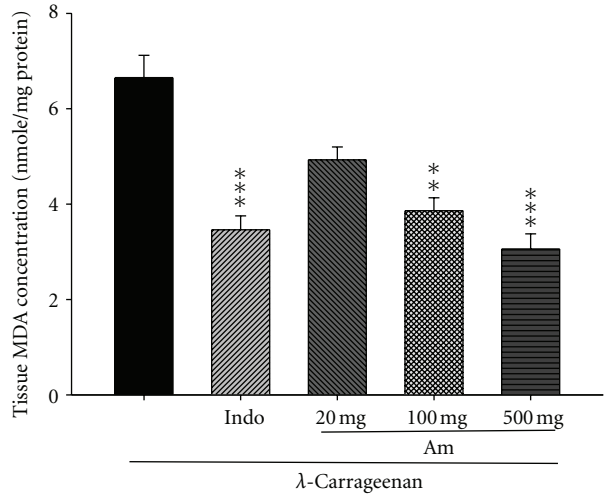

Figure 10: Effects of AM_{EtOH} and indomethacin (Indo) on the tissue MDA concentration of edema paw in mice. Each value represents as mean ± S.E.M. ($n = 10$), $^{**}P < 0.01$, $^{***}P < 0.001$ as compared with the Carr group. (One-way ANOVA followed by Scheffe's multiple range test.)

AM_{EtOH} might be associated with the inhibition of TNF-α and IL-6. In acute inflammatory responses, reactive oxygen species (ROS), including hydrogen peroxide, hydroxyl radical, and superoxide anion in neutrophil all play pivotal roles, hence leading to DNA, lipid, and protein damage [37]. MDA is a reactive aldehyde that serves as a biomarker in the evaluation of oxidative stress and inflammatory process. Antioxidant defense systems scavenge and minimize the formation of ROS through enzymes, such as SOD, GPx, and GRd [38]. SOD catalyzes the conversion of superoxide anion to hydrogen peroxide followed by catalases to water and oxygen. GPx removes hydrogen peroxide to water and oxygen, in the meantime, giving oxidized glutathione (GSSH), which subsequently regenerates via GRd with NADPH as a source of hydrogen. Therefore, the increment of SOD, GPx, and GRd will diminish ROS and serves as the assessment of the degree of inflammation. Cytokine is a group of polypeptides synthesized by the host in response to immunity and inflammatory responses. For instance, IL-1, TNF-α, and IL-6 have been reported in implication in the inflammatory response. IL-1 and TNF-α also regulate the expression of i-NOS and COX-2 and subsequently modulate NO and PGE_2 [39]. Efforts to attenuate these pleiotropic inflammatory mediators have been attracting much attention in the development of new anti-inflammatory drugs. The major component

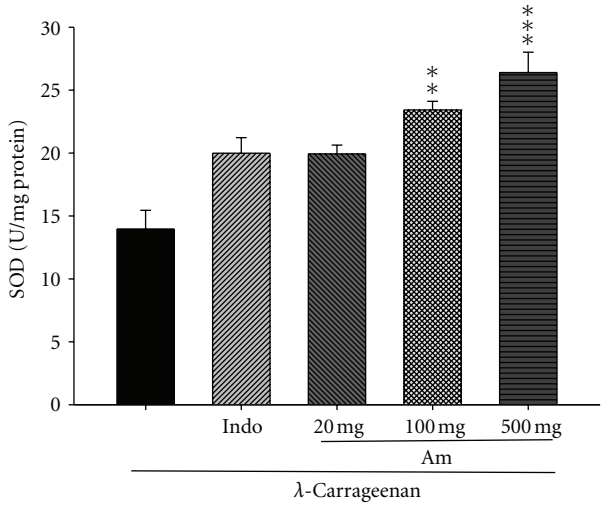

Figure 11: Effects of AM_{EtOH} and indomethacin (Indo) on the liver SOD activities in mice. Each value represents as mean ± SEM ($n = 10$), $^{**}P < 0.01$, $^{***}P < 0.001$ as compared with the Carr group. (One-way ANOVA followed by Scheffe's multiple range test.)

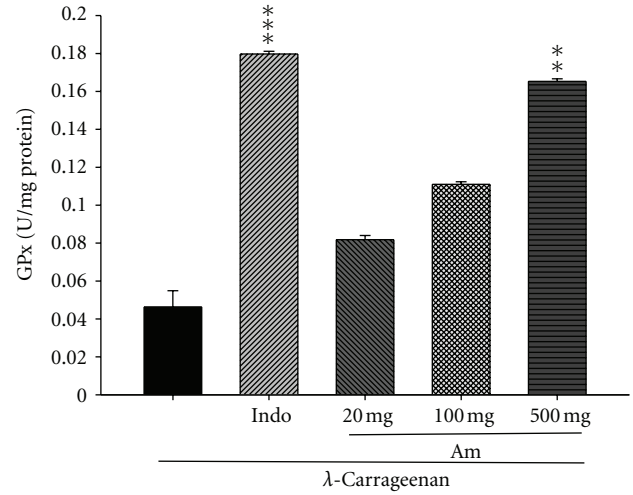

Figure 12: Effects of AM_{EtOH} and indomethacin (Indo) on the liver GPx activities in mice. Each value represents as mean ± SEM ($n = 10$), $^{**}P < 0.01$, $^{***}P < 0.001$ as compared with the Carr group. (One-way ANOVA followed by Scheffe's multiple range test.)

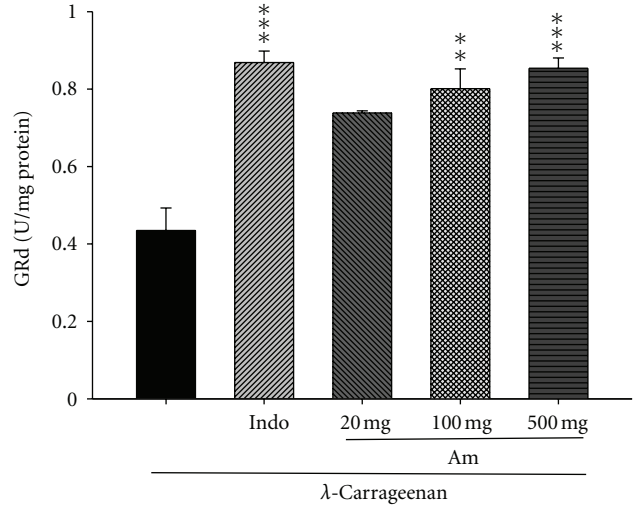

Figure 13: Effects of AM_{EtOH} and indomethacin (Indo) on the liver GRd activities in mice. Each value represents as mean ± S.E.M. ($n = 10$), $^{**}P < 0.01$, $^{***}P < 0.001$ as compared with the Carr group. (One-way ANOVA followed by Scheffe's multiple range test.)

from AM_{EtOH} is *p*-hydroxyacetophenone whose structure was totally consistent with the literature data in NMR spectroscopy [40]. Quantitative analysis was performed by HPLC detected at 315 nm with standard addition method [41]. The major ingredient in *Lonicera japonica* is chlorogenic acid whose anti-inflammatory activity was confirmed [42, 43]. Chromatogram showed that chlorogenic acid was identified at RT of 3.9 min. However, the amount was tiny in our observation though it had been found from *A. scopariae* [44]. One study showed that *p*-hydroxyacetophenone produced protective effects in λ-carrageenan-induced paw edema in mouse at 1–3 h after the injection [45]. Quantitative study to *p*-hydroxyacetophenone had been reported from *A. scopariae* by capillary electrophoresis and determined to be 6.732–8.795 mg/g [44]. In this study, the major component in AM_{EtOH} was *p*-hydroxyacetophenone and the amount was determined to be 130 mg/g.

This study demonstrated that AM_{EtOH} possessed analgesic activity against nociceptive responses induced by the intraperitoneal injection of acetic acid at concentration of 100 and 500 mg/kg. The paw licking time was significantly reduced in the late phase by intraplantar injection of formalin at 100 and 500 mg/kg. AM_{EtOH} declined the paw edema volume at dose of 100 and 500 mg/kg induced by λ-carrageenan. Two possible pathways were proposed for the anti-inflammatory effect exerted by AM_{EtOH}. The first was the reduction of the concentration of TNF-α and IL-6, not only for the suppression of COX-2 leading to a decrease in PG released but also for the decrease of NO concentration to prevent edema. The second was by increasing the activity of anti-oxidant enzymes (SOD, GPx, and GRd) in the liver, in which the free radicals were scavenged and the level of MDA was declined. The analgesic effect was found to be related to its anti-inflammatory activity and served as a possible rationale on AM_{EtOH} in traditional medicine.

Conflict of Interests

The authors have no conflict of interests to declare.

Author's Contribution

S.-C. Chou and Y.-J. Chiu contributed equally to this work.

Acknowledgments

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Research Article

Effects of Tai Chi versus Proprioception Exercise Program on Neuromuscular Function of the Ankle in Elderly People: A Randomized Controlled Trial

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Background. Tai Chi is a traditional Chinese medicine exercise used for improving neuromuscular function. This study aimed to investigate the effects of Tai Chi versus proprioception exercise program on neuromuscular function of the ankle in elderly people. *Methods*. Sixty elderly subjects were randomly allocated into three groups of 20 subjects per group. For 16 consecutive weeks, subjects participated in Tai Chi, proprioception exercise, or no structured exercise. Primary outcome measures included joint position sense and muscle strength of ankle. Subjects completed a satisfaction questionnaire upon study completion in Tai Chi and proprioception groups. *Results*. (1) Both Tai Chi group and proprioception exercise group were significantly better than control group in joint position sense of ankle, and there were no significant differences in joint position sense of ankle between TC group and PE group. (2) There were no significant differences in muscle strength of ankle among groups. (3) Subjects expressed more satisfaction with Tai Chi than with proprioception exercise program. *Conclusions*. None of the outcome measures on neuromuscular function at the ankle showed significant change posttraining in the two structured exercise groups. However, the subjects expressed more interest in and satisfaction with Tai Chi than proprioception exercise.

1. Introduction

An increasingly aging population presents a global challenge to human society. Such a population shift arises from two demographic effects: increasing longevity and declining fertility [1, 2]. Based on World Health Organization projections, the proportion of the global population that is 60+ years old is expected to increase from 10.0% in 2000 to 21.8% in 2050, and then to rise to 32.2% in 2100 [3]. Due to decreased fertility, China and many other developing countries are going through more rapid fertility transitions than these projections predict. These countries will experience even faster population aging in future years than currently developed countries [3, 4].

Impaired motor performance in elderly people is often characterized by a slowing of movement, a decrease in muscle strength, and a loss of fine motor coordination [5]. These impairments can increase the likelihood of fall in elderly people, as well as decrease these individuals' ability to participate in standard activities of daily living [6, 7]. Thus, realizing an effective method of improving the neuromuscular function of elderly people could be expected to improve quality of life and to reduce social medical costs [8].

Physical activity is an effective strategy for improving neuromuscular function, particularly among the elderly population [5, 8–10]. However, many forms of physical activity are either too intense or too monotonous for older adults to maintain over an extended period of time.

Tai Chi (TC) is a popular form of exercise among older adults, especially in Asia. This activity involves a series of slow, smooth, and graceful movements, with an emphasis on smooth coordination of the eyes, head, body, and upper and lower extremities; for these reasons, TC is assigned special significance in the daily routine in many older adults [11]. Elderly TC learners may chat and learn from each other as they practice these skills, which can increase learning motivation and maintain a steady exercise habit. In addition, TC learners can practice by themselves at any time and in any place. An added benefit is that TC programs do not require any special equipment, which further adds to this activity's convenience and accessibility.

Numerous studies have investigated TC as an intervention for a wide variety of health problems, especially balance and musculoskeletal disease [12–14]. Li and his colleagues conduct a rigorous, multicenter randomized controlled trial with a large sample size in the New England Journal of Medicine, and they found that TC was more effective in improving postural stability in limits-of-stability tasks than a resistance-based exercise or low-impact stretching [15]. However, it is not yet known whether a proprioception exercise (PE) program has better long-term effects on the neuromuscular function of the ankle than a TC program. Ankle function is of special concern to older adults, as failure of this joint can lead to balance problems and increased risk of fall. Hence, we designed the present study to examine the effects of a 16-week TC training program versus 16 weeks of PE on the proprioceptive function and muscle strength of the ankle in elderly subjects. Here, we hypothesized that a TC program would lead to greater improvements in proprioceptive function and muscle strength outcomes than an alternative PE program.

2. Methods

2.1. Subjects. Subjects ($n = 60$) were recruited from several community elderly centers in Shanghai, China. Subjects were required to have had no previous experience in TC, as well as no regular physical exercise habits. A computer-generated random-number sequence randomly assigned all subjects to either a TC ($n = 20$; mean age ± SD, 70.5 ± 2.1 y), PE ($n = 20$; mean age ± SD, 72.8 ± 2.3 y), or control group ($n = 20$; mean age ± SD, 68.6 ± 1.6 y). The TC group and the PE group both performed the assigned exercise twice a week (45 min/session) for 16 weeks. The control group had no regular physical exercise habits.

Inclusion criteria are the following: (1) be aged 60–85 years old; (2) score at least 24 on the Mini-Mental State Examination (MMSE) to show that they had no cognitive impairment [16], using the Chinese version validated by Chiu et al. (1994) [17]; (3) demonstrate a sufficient active range of motion in their upper limbs to perform the requisite finger-pointing tasks, which required subjects to flex and extend their shoulder, elbow, wrist, and fingers; (4) demonstrate through the Independent Activity of Daily Living test that they could be considered independent in activities of daily living [18].

Exclusion criteria are the following: (1) cardiovascular pathologies such as symptomatic cardiovascular disease or uncontrolled hypertension; (2) previous experience in TC; (3) any musculoskeletal disease referred to the lower limbs such as low back pain, serious arthritis, and ankylosing spondylitis; (4) any pathology affecting lower extremity function such as stroke, Parkinson's disease, or any other disabling neurologic illness.

2.2. Procedure. Prior to initiation of the study, all subjects completed a questionnaire that asked for such details as the subjects' past and present job status and their medical history. They also completed the MMSE and the Activity of Daily Living test and described their exercise habits (frequency and time/session). TC and PE subjects were asked to exercise twice a week (45 min/session) for 16 weeks at the Shanghai University of Sport. Joint position matching and muscle strength tests were conducted at baseline and after the 16-week intervention. Subjects completed a satisfaction survey after completing the 16-week intervention.

2.3. Testing Protocol. The assessment test was divided into two sections. The first section assessed subjects' joint position matching ability of the ankle in different degrees. Then, after 30 min of relaxation, the strength of the subjects' ankle dorsiflexors and plantar flexors was evaluated. All tests were performed on both legs of each subject.

Subjects performed a submaximal warm-up exercise (50–60 W) on a bicycle ergometer (MOTOmed viva2, Reck, Germany) for 5 min prior to the muscle tests. A Biodex System 3 isokinetic dynamometer (Biodex Medical Systems, Shirley, New York, USA) was used to measure peak torque, peak torque/weight, and ankle joint position sense (JPS).

2.3.1. Ankle Joint Position Passive Matching Test [19]. Each subject was positioned semirecumbent on the associated special testing chair, with the calf of the tested leg resting on a 40 cm high platform. The hip and knee were positioned at a 45° flexion, and the talocrural joint was in neutral position. The bare foot of the subject was aligned with the axis of the dynamometer and attached to the footplate by a very small wrap to reduce cutaneous receptor input. During testing, subjects kept their eyes closed and wore headphones with music playing to eliminate visual and auditory stimuli from the testing apparatus. There were two reference degrees: (1) ankle at 10° inversion and (2) ankle at 20° inversion.

The subject's foot was first passively moved by the investigator to the maximal inversion or eversion position. The investigator then moved the foot to the two reference positions. This test position was maintained for 10 s, with each subject instructed to concentrate on the position of the foot. The foot was then passively brought to maximal inversion or eversion and moved passively back toward eversion or inversion with a speed of 1°/s. The subject was instructed to push on a stop button when he or she thought that the test position had been reached. This trial was repeated three times, and the error with which the subject reproduced the initial position was subsequently

calculated. The three absolute error values were averaged, and the average value was termed the absolute angle error.

2.3.2. Muscle Strength Test [20]. We used a Biodex System 3 dynamometer to determine isokinetic peak torque and peak torque/weight values for reciprocal concentric plantar flexion to dorsiflexion movements of the ankle. Subjects were tested in a semi-recumbent position with 30° of seat-back tilt. The ankle was in 10° plantar flexion. The knee of the tested ankle was in extension to minimize substitution from the hamstrings and other tibial rotators. Dynamometer and chair adjustments were made to align the midline of the foot with the midline of the patella. Two straps were wrapped around the extremity proximal to the patella and the pelvis to minimize movements of the hip and knee during testing. Isokinetic contractions were performed at an angular velocity of 30°/s.

Prior to testing, each subject performed a warm-up exercise of three submaximal repetitions to familiarize themselves with the equipment. For the isokinetic test, the subjects were instructed to push the foot away from them and pull it toward them at maximum velocity through the full available range of motion for each repetition. Peak torque was determined as the highest torque generated from the three trials. Peak torque/body weight is an important consideration for improved comparison among subjects of varied body types. None of the subjects noted any discomfort while testing.

2.4. Training Program

2.4.1. The TC Intervention. Subjects participated in 45 min TC sessions twice weekly for 16 weeks. Classes were taught by a Tai Chi master with more than 10 years of teaching experience. In the first session, we explained TC theory and procedures and provided subjects with printed teaching materials, including TC principles, practicing techniques, and safety precautions for the elderly. For the remaining sessions, each subject practiced TC under the instruction of the Tai Chi master. Each session included (1) 5 min of warm-up and a review of TC principles, (2) 30 min of TC movement, (3) 5 min of breathing techniques, and (4) 5 min of cooldown. The program consisted of 24 forms from classic Yang-style Tai Chi, with minor modifications that were suitable for older adults. We encouraged subjects to participate in their usual sports activities, but to not engage in extra strength training.

2.4.2. The Proprioceptive Exercise Intervention. Proprioceptive exercise classes were led by a registered physical therapist for 45 min, twice per week, over 16 weeks. The exercise protocol emphasized static and dynamic balance exercises, including transitions between differing sensory conditions and functional everyday movements. Each lesson incorporated a similar general plan as follows: (1) 5 min warm-up; (2) 20 min of static balance exercises such as squats (two-leg stance) and one-leg stance; (3) 15 min of dynamic balance exercises such as jogging, sideways walking or running with crossovers, forward walking or running in a zigzag line, or backward walking or running in a zigzag line; (4) 5 min of cool-down. Exercises gradually increased in difficulty and training load over the 16-week training period.

2.5. Statistical Analyses. Statistical analyses were performed using SPSS 17.0 and Microsoft Excel 2003 software. Data are expressed as mean ± SD. Changes in variables between pre- and posttraining and between groups were analyzed. A one-way analysis of variance (ANOVA) was used to examine the differences among the characteristics at baseline of the TC, PE, and control groups. Changes in the JPS, peak torque, and peak torque/weight between baseline and followup among the TC, PE, and control groups were compared using two-way (3 groups × 2 repetition assessments) repeated measures ANOVA. When ANOVA analysis revealed significant time and time-by-group interaction effects, paired t-tests were used to compare the changes in measures within groups. Statistical significance was assumed at P less than 0.05.

We used five-point Likert scales to contrast the differences between the TC and PE groups with regard to health satisfaction and recommending others to participate in the project after 16 weeks of intervention [21]. With five-point scales, the points could be labeled as (1) 1: very satisfied; 2: satisfied; 3: neither satisfied nor dissatisfied; 4: dissatisfied; 5: very dissatisfied for health satisfaction; and (2) 1: strongly agree; 2: somewhat agree; 3: neutral/no opinion; 4: somewhat disagree; 5: strongly disagree about recommending that other people participate in the program. The Mann-Whitney Test was used to compare the differences between groups. Statistical significance was assumed at P less than 0.05.

3. Results

The study design is outlined in Figure 1. Twelve of the 72 individuals initially recruited were deemed ineligible for study participation; eight subjects did not meet the inclusion criteria, two subjects had previous Tai Chi experience, one subject's work schedule was incompatible, and one subject withdrew consent. A total of 42 subjects completed the 16-week study program, and 18 subjects (TC $n = 5$, PE $n = 10$, control $n = 3$) were lost to followup. Reasons for dropout included illness, withdrawal, serious family problems, and not attending the final evaluation session. Hence, follow-up data were available for 15 of the 20 subjects in the TC group, 10 of the 20 subjects in the PE group, and 17 of the 20 subjects in the control group. Table 1 lists baseline characteristics of the three groups. The groups were well matched at the baseline assessment, with no differences in key outcome variables apparent.

3.1. Ankle Joint Position Sense. For the absolute error after the 16-week program (see Figure 2), (1) subjects in the TC group and PE group could be matching significantly more accurate than those in the control group ($P = 0.014$ for TC, $P = 0.039$ for PE) in the left ankle, (2) subjects in the TC group and PE group had a smaller amount of absolute error in the right ankle than the control group, but there were no significant differences ($P = 0.184$ for TC, $P = 0.883$ for PE),

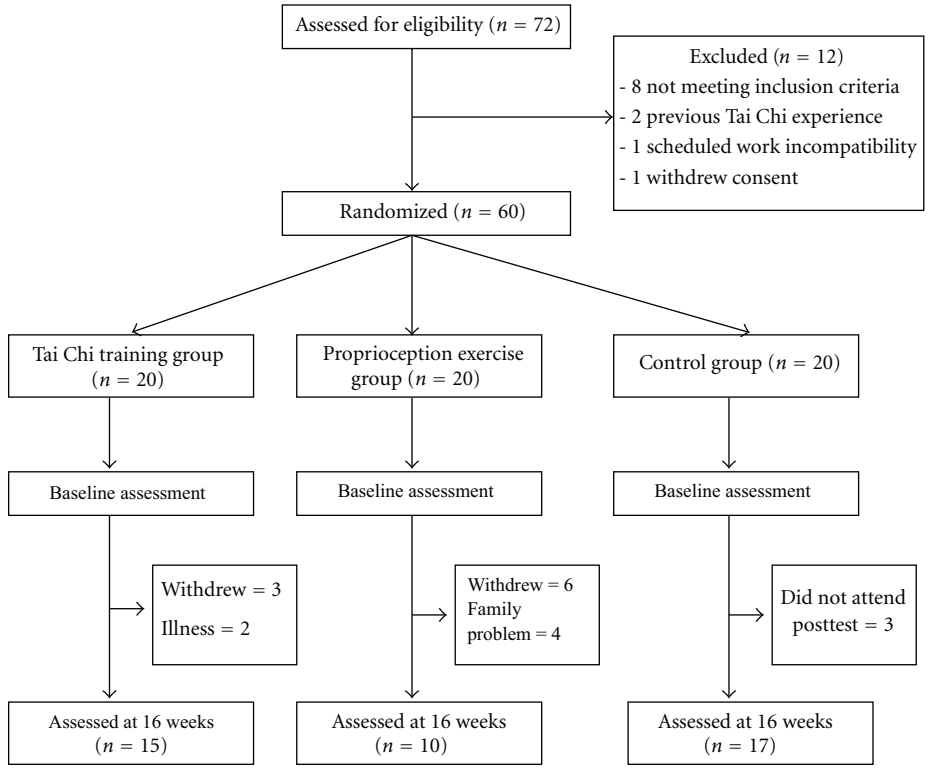

Figure 1: Flow diagram of eligibility assessment, exclusion, inclusion, and analysis.

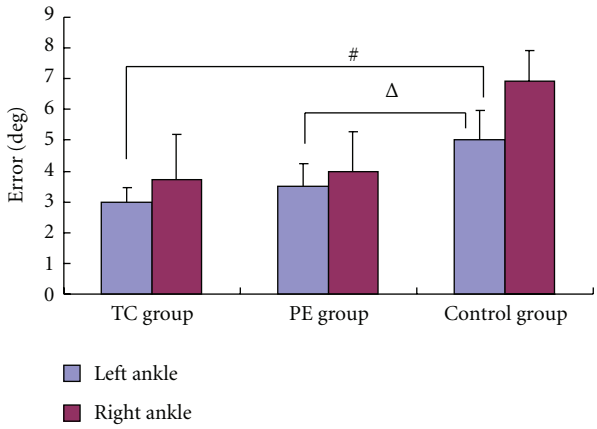

Figure 2: Joint position sense for 3 groups. #There were significant differences between TC group and control group, $P = 0.011$. ΔThere were significant differences between PE group and control group, $P = 0.045$. TC: Tai Chi; PE: proprioception exercise.

and (3) there were no significant differences in joint position matching of both ankles for the TC group and PE group ($P = 0.979$ for left, $P = 0.184$ for right).

3.2. Muscle Strength. We found no significant differences in the strength and endurance of the bilateral ankle dorsiflexors and plantar flexors at a speed of 30°/s for concentric test conditions among groups (Table 2).

3.3. Satisfaction Survey. Elderly subjects expressed significantly higher health satisfaction in the TC group than in the PE group after 16 weeks ($P = 0.036$), and more subjects in the TC group recommended the program than did those in the PE group (Figures 3 and 4).

4. Discussion

4.1. Joint Position Sense. The TC and PE interventions evoked improvements in the joint position sense of bilateral ankles in this study. However, we were unable to show a difference after a 16-week exercise intervention between the TC group and PE group on a joint position sense. Leung et al. [22] performed a systematic and meta-analytical review of the effects of TC on balance function in elderly people. The systematic review based on 13 randomized controlled trials (RCTs) indicated that TC was effective in improving the balance function of elderly people. However, a TC program may not necessarily be superior to other interventions. Lelard et al. [10] assessed the effects of a TC program versus a balance training program on postural control and walking ability in elderly people. The authors measured static postural control and walking speed. They also did not find any significant modifications of postural parameters and walking speed between TC and balance training. Indeed, studies under more challenging postural conditions should be performed to verify this specific effect of TC training on proprioception function of the ankle in the daily living activities of older adults.

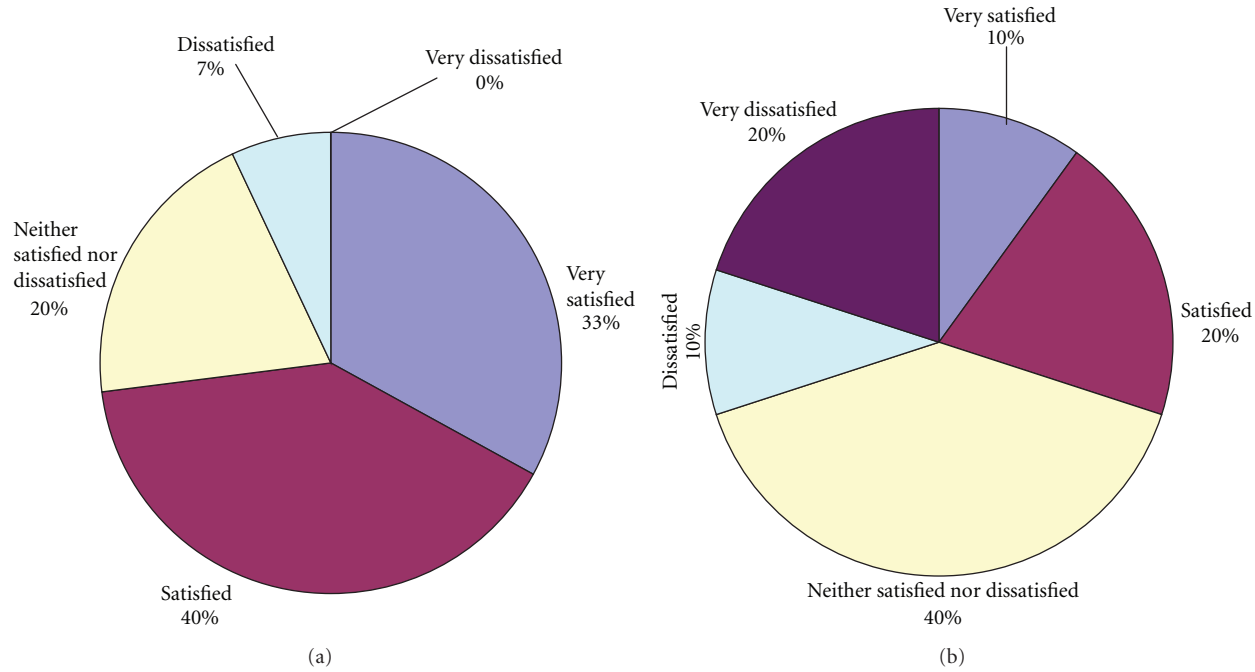

Figure 3: (a) Health satisfaction for Tai Chi group. (b) Health satisfaction for proprioception exercise group. The Mann-Whitney Test was used to compare the differences between groups. There were significant differences between PE group and control group, $P = 0.036$.

Table 1: Baseline values for the 3 groups.

Variable	TC ($n = 15$)	PE ($n = 10$)	Control group ($n = 17$)	P
Men/female	7/8	4/6	7/10	0.932*
Age (y)	68.0 ± 1.41	68.8 ± 1.03	69.8 ± 0.73	0.663#
Body weight (kg)	60.73 ± 2.14	55.60 ± 2.73	59.59 ± 1.45	0.252#
Height (cm)	164.33 ± 2.09	167.30 ± 1.51	165.28 ± 0.95	0.528#
JPS test (°)				
Left ankle	4.20 ± 0.75	6.13 ± 1.77	4.16 ± 0.72	0.431#
Right ankle	8.93 ± 1.62	7.40 ± 1.43	8.77 ± 1.28	0.785#
PT (Nm): concentric, 30°/s				
Left plantar flexion	49.88 ± 6.81	46.68 ± 6.97	49.62 ± 4.59	0.934#
Right plantar flexion	49.08 ± 7.13	54.64 ± 8.03	55.55 ± 4.86	0.729#
Left dorsiflexion	14.4 ± 1.32	14.98 ± 2.36	14.60 ± 1.41	0.975#
Right dorsiflexion	14.75 ± 1.45	17.10 ± 2.63	16.95 ± 1.72	0.630#
PT/weight (Nm/kg): concentric, 30°/s				
Left plantar flexion	0.82 ± 0.09	0.85 ± 0.13	0.84 ± 0.082	0.978#
Right plantar flexion	0.82 ± 0.11	0.99 ± 0.14	0.95 ± 0.91	0.534#
Left dorsiflexion	0.24 ± 0.02	0.27 ± 0.04	0.24 ± 0.02	0.782#
Right dorsiflexion	0.25 ± 0.02	0.31 ± 0.04	0.29 ± 0.03	0.467#

Data reported as mean ± SD.
*Chi-square test.
#One-way analysis of variance.
TC: tai chi; PE: proprioception exercise; JPS: joint position sense; PT: peak torque.

In addition, we found that the joint position sense of the left ankle was better than the right ankle in baseline values for the three groups. Significant matching of subjects in the TC group and PE group could be achieved more accurately than those in the control group ($P = 0.014$ for TC, $P = 0.039$ for PE) in the left ankle after 16 weeks of exercise. The result was similar to those reported previously in several studies [23–25]. Goble and Brown [23] found that for the proprioceptive matching task, errors were smaller for the nonpreferred left arm, whereas during the visual matching task, smaller errors were found for the preferred right arm. These results suggest a left-arm/right-hemisphere

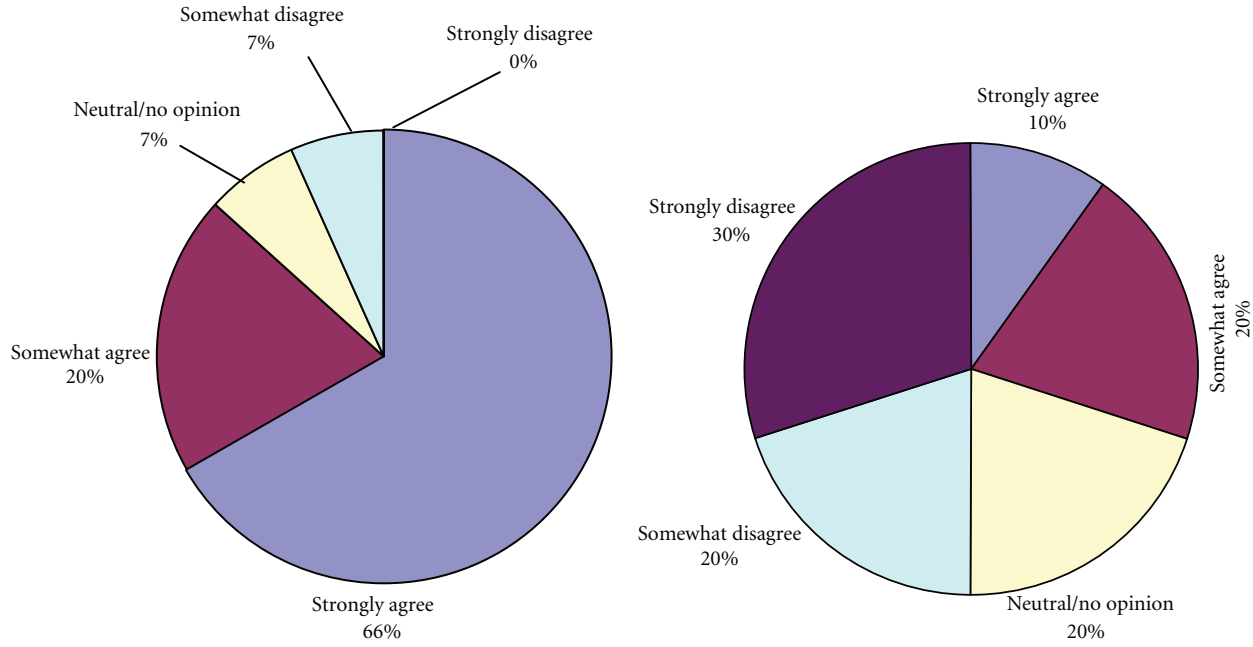

Figure 4: (a) Recommending other people learn the program for tai chi group. (b) Recommending other people learn the program for proprioception exercise group. The Mann-Whitney Test was used to compare the differences between groups. There were significant differences between PE group and control group, $P = 0.002$.

Table 2: Muscle strength for the 3 groups.

Variable	TC	PE	Control group	P^*
JPS test (°)				
Left ankle	$3.0 \pm 0.48^{\#}$	$3.5 \pm 0.72^{\Delta}$	5.03 ± 0.95	0.026
Right ankle	3.7 ± 1.49	4.0 ± 1.29	6.91 ± 0.99	0.083
PT (Nm): concentric, 30/°s				
Left plantar flexion	59.16 ± 6.71	61.4 ± 6.09	47.76 ± 3.69	0.138
Right plantar flexion	61.52 ± 7.72	69.53 ± 9.25	52.73 ± 4.37	0.221
Left dorsiflexion	17.84 ± 2.04	16.93 ± 2.71	13.11 ± 6.15	0.128
Right dorsiflexion	14.87 ± 1.37	18.02 ± 3.31	14.88 ± 1.37	0.079
PT/weight (Nm/kg): concentric, 30/°s				
Left plantar flexion	0.98 ± 0.1	1.13 ± 0.1	0.82 ± 0.04	0.11
Right plantar flexion	1.01 ± 0.1	1.25 ± 0.1	0.92 ± 0.1	0.19
Left dorsiflexion	0.31 ± 0.04	0.31 ± 0.05	0.22 ± 0.03	0.197
Right dorsiflexion	0.34 ± 0.03	0.32 ± 0.05	0.26 ± 0.03	0.150

Data reported as mean ± SD.
*A 2-way analysis of variance (group × time). #There was significant differences between TC group and control group, $P = 0.011$. ΔThere was significant differences between PE group and control group, $P = 0.045$.
TC: tai chi; PE: proprioception exercise; PT: peak torque.

advantage for proprioceptive feedback processing and a right-arm/left-hemisphere advantage for visual information processing. Such asymmetries may reflect fundamental differences between the two-arm/hemisphere systems during the performance of bimanual tasks in which the preferred arm requires visual guidance to manipulate an object, whereas the nonpreferred limb stabilizes that object on the basis of proprioceptive feedback. The result of our study also showed that joint position sense of the left ankle was more sensitive to TC training and to the PE program than the right ankle.

4.2. Muscle Strength. Many researchers reported only mean peak torque values rather than values normalized by body weight. Normalizing by body weight is, thus, an important consideration for better comparison among subjects of

varied body types. Additionally, as ankle sprains most usually occur in the closed kinetic chain, body weight also has an influence on the sprain moment generated at the ankle. Therefore, we consider peak torque/body weight a more relevant value compared with peak torque.

Our results affirm that subjects in the TC and PE groups did not have a larger amount of peak torque and peak torque/weight in both ankles than the control group, and there were no significant differences between groups. Perhaps, in order to improve muscle strength of the ankle joint, a 16-week TC intervention might not last long enough. For the lower limb muscle test, our results were consistent with previous studies [26–28], which showed that muscle strength of a TC program group for training lasting less than a year was not significantly higher than that of the control group. Wolf et al. [29] found that TC and elderly balance training participants with four months of training had no differences in ankle muscle strength. Another study confirmed that long-term TC exercisers with more than four years of experience showed significantly better muscle strength of the ankle joint compared with long-term regular joggers/swimmers and sedentary elderly people [30]. The results of these papers suggest that improving biomechanical characteristics of lower-extremity muscles may require a longer-lasting TC intervention for elderly people.

4.3. Satisfaction Survey. We did not find that the PE program has an improved effect than the TC program on proprioceptive function or muscle strength. However, the TC and PE groups showed a significant difference in health satisfaction and recommendation of the treatment to others after 16 weeks of practice. There may be several reasons for this result. First, a TC program is distinctive because it includes a structured cognitive component, also referred to as meditation [31]. In Tai Chi, a practitioner is required to choreograph slow movements according to visual imagery. In short, the mind directs the body in performing these movements. This makes TC different from other types of balance exercise.

Brown et al. [32] provided the hypothesis that exercise plus cognitive strategy training programs are more effective than exercise programs lacking a structured cognitive component in promoting psychological benefits. But in proprioception exercise without a structured cognitive component, the practitioner's cognitive processing is random and unstructured. And Silsupadol et al. [33] suggested that cognitive dual-task training programs (balance exercise + cognitive training) were superior to single-task training (balance exercise) in improving balance function. Second, a PE program is not usually sufficiently interesting that the majority of elderly participants will maintain a long-term and regular habit of exercise. Lastly, subjects felt comfortable with the intensity of the program, and none reported discomfort while practicing Tai Chi. Participants were enthusiastic and made every effort to attend sessions. Our TC program proved to be effective, interesting, and convenient as a form of physical activity for older adults.

4.4. Limitations. Although our study elicited important observations regarding the usefulness of TC on the ankle in elderly people, there are some limitations to our research. First, because subjects had not learned the movements of tai chi previously, it sometimes proved difficult for subjects to correctly perform this exercise. We note that the present findings cannot be generalized to elderly people living in nursing homes or hospital settings, as these individuals are more likely to have limited mobility and/or a preestablished exercise program that does not permit physical interventions such as those assessed here. Finally, the sample size was too small to draw any firm conclusions. Further rigorous, multicenter RCTs with a large sample size are warranted.

5. Conclusion

Results of our study demonstrated similar effects of 16-week TC and proprioception exercise programs on joint position sense or muscle strength of ankle joint in elderly people. None of the outcome measures showed significant change posttraining in the TC or proprioception exercise groups. However, the elderly felt significantly more interested in TC program than PE program and also significantly more satisfactory to their health in the TC group than the PE group. Further study with long-term followup is needed to substantiate the role of Tai Chi exercise in the physical and psychological benefits.

Ethical Approval

This project was approved by the ethics committee of the Shanghai University of Sport.

Conflict of Interests

The authors report no conflict of interests.

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Review Article

The Relationship between Complementary and Alternative Medicine Use and Breast Cancer Early Detection: A Critical Review

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Objective. Complementary and alternative medicine (CAM) use is prevalent. Concurrently, breast cancer is the most common cancer in women worldwide, with early detection techniques widely available. This paper examined the overlap between participation in allopathic breast cancer early detection activities and CAM use. *Methods*. A systematic review examined the association between breast screening behaviors and CAM use. Searches were conducted on the PubMed, Embase, CINAHL, and NCCAM databases and gray literature between 1990 and 2011. STROBE criteria were used to assess study quality. *Results*. Nine studies met the search criteria. Four focused on CAM use in women at high breast cancer risk and five on average risk women. CAM use in women ranged from 22% to 82% and was high regardless of breast cancer risk. Correlations between CAM use and breast cancer early detection were not strong or consistent but significant relationships that did emerge were positive. *Conclusions*. Populations surveyed, and measures used to assess CAM, breast cancer screening, and correlates, varied widely. Many women who obtained allopathic screening also sought out CAM. This provides a foundation for future interventions and research to build on women's motivation to enhance health and develop ways to increase the connections between CAM and allopathic care.

1. Introduction

Complementary and alternative medicine (CAM) is defined as medical practices infrequently taught in medical schools nor widely available in hospitals, the latter being defined as "allopathic medicine" [1]. According to the National Center for Complementary and Alternative Medicine (NCCAM), CAM can be described using broad categories: that is, natural products, mind body practices, manipulative and body-based practices, and other approaches [2]. Over time, CAM practices may become accepted and integrated into allopathic medicine [2].

In a recent national survey of Americans, most people using CAM did so in complement with allopathic medicine [3]. CAM usage was positively associated with the number of personal health conditions and the number of doctor visits in the past 12 months. Only a small percentage used CAM to replace allopathic medicine and such "alternative medicine users" may have poorer health than complementary users [4]. In addition to many cultural factors contributing to variations in CAM use, it is important to better understand how CAM use and health practices influence and inform each other.

A nationally representative study found that women in better health reported higher CAM use [5]. CAM users tend to have better health behaviors, with more physical activity, limited alcohol consumption, not smoking [6], and following a healthy diet [7], all of which are independently associated with CAM use. A survey of Medicare supplement plan enrollees found that 42% used CAM specifically for health improvements [8]. Since CAM users are highly involved in health practices, we hypothesized that they may also be more inclined to adhere to preventive strategies based in allopathic medicine, such as cancer screening.

Breast cancer is the most commonly diagnosed cancer in women worldwide, with 1.38 million new cases and 458,000 deaths in 2008 [9]. Using early detection interventions, breast cancer can be diagnosed at an early stage when successful treatment is more likely. Multiple agencies and organizations around the world support mammography as the most reliable way to find breast cancer early, particularly in women 50 and older [10, 11]. A professional clinical breast exam (CBE) and "knowing one's breasts" or breast self-examination (BSE) are also recommended by some organizations [12, 13].

We were interested in learning whether participation in allopathic breast cancer early detection activities is associated with CAM use for women at both high and average risk of breast cancer. This paper provides a critical review of the literature to identify CAMs used, correlates of use, methodological strengths and weaknesses of the literature, and suggestions for future research and practice.

2. Methods

References were identified through PubMed and Embase database searches for 1990–2011. For PubMed, Mesh terms included "complementary therapies/utilization" AND "health behaviors," and "breast neoplasms/prevention and control" AND "complementary therapies/utilization." In Embase, similar search criteria were used with keywords including the explosion of "breast neoplasms" to include the "prevention" subheading AND the explosion of "complementary therapies" to include all subheadings. We also searched major Canadian government documents and other gray literature sources including Cumulated Index to Nursing and Allied Health Literature (CINAHL), the NCCAM website research database, and Google, and identified no additional papers.

The Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) statement was used as a guideline to ensure a high quality of research in this review [22]. A checklist of 22 items included indicators of study design, participant eligibility, variable assessment, potential bias, statistical methods, outcome data, and generalizability. Each paper was assessed according to these indicators and is fully available upon request. The STROBE statement was not used as tool to assess the methodological quality of the studies.

We included all forms of CAM as described by the authors and excluded papers examining women currently or previously diagnosed with breast cancer as breast cancer screening recommendations differ vastly for these women. Use of self-reported or medical record-based mammography, BSE, and CBE were the indications of allopathic breast cancer early detection used here.

Results for CAM use and its association with breast screening behaviors are summarized separately for women at high and normal genetic risk for breast cancer. We support this separation as it has been suggested that many women at high-risk for breast cancer display signs of extreme cancer anxiety, leading to increased breast screening tendencies [23].

All studies included in the review were summarized using their description of the defined study population, participant response rate, data collection methods and analytic procedures.

3. Results

Table 1 summarizes nine studies (comprising 10 papers) that met the search criteria. Four studies focused on CAM use in women at high breast cancer risk (based on participation in a genetic testing or familial risk clinic or study), and five on average risk women. Most studies were based in the US, and two in Australia. Sample sizes ranged from a clinic-based sample of 104 [14, 15] to a large insurance claim database study with over 71,000 women [4]. A majority collected data through mailed questionnaires with response rates ranging 59%–86%. Eight of nine studies measured CAM through dichotomous responses regarding use of a series of CAM modalities. The exception was a records-based study where CAM use was extracted from claims data, which reported four CAM therapies paid for by the health insurance plan [18]. Numbers and types of CAMs queried varied considerably from one study to another, ranging from eight to 35. CAM content varied as well, with healthy diet considered a CAM in one study but not others. The period in which CAM use was queried ranged from "ever use" to "past year" with only one study assessing CAM use over two years [21]. Regarding breast screening, there was considerable variability in what was asked and the time frame used; mammography, CBE, and BSE were all frequently assessed.

3.1. Prevalence and Types of CAM Use. Table 1 indicates a wide range of prevalence of CAM use, ranging from 8.3% [16] to 82% [18]. The lowest figure listed is difficult to interpret since it is based on the overall study sample, which included both men and women aged 18 and older [16]; no information was provided regarding CAM use in subpopulations comparable to most papers reported here: that is, middle aged women. CAM use in the four studies of women at increased breast cancer risk was 42% [14], 50% [20], 55% [17], 58% [15], and 69% (without prayer included) [19]. In studies that reported data specific to women, rates went from 22% [4] to 46% [7], 50% [20], and 82% [18]. The data indicate that CAM is used in a majority or a large minority of women regardless of risk status. Many women, in fact most, used more than one kind of CAM. Field et al. [17] reported that one woman used 26 different kinds of CAM.

Table 2 provides a summary of the kinds of CAMs reported. The range is broad and includes the full range of approaches delineated by NCCAM. Each study, however, assessed only a subset of the total therapies possible. The most commonly queried therapies were acupuncture, massage therapy, and meditation. CAM definitions used varied, with most studies omitting healthy eating and nutrition or specific diets. The controversial role of prayer as a CAM was highlighted in one study [19], which computed CAM rates

Table 1: Summary of Studies.

Study[a] (Year)	Study design	Sample size	Selected participant characteristics	Data collection methods and CAM assessment	Period of CAM use assessed	Proportion of cancer-free participants using CAM	Breast screening measures[b]	Relevant findings	Correlates of more CAM use
DiGianni, (2003 and 2006), [14, 15]	Cross-sectional (2003) Prospective cohort (2006)	104 without cancer history	>18 yrs, F; Enrolled in a breast/ovarian genetic testing clinic; USA	Mailed questionnaire 83% response rate at 1 year Y/N 8 CAMs[c]	Ever use	*Baseline* 42%; 33% used 1-2 CAMs *1 yr followup* 58%	*Baseline* BSE (rarely/often) *1 year followup* CBE (# in past year) Mam BSE	*Baseline*—no associations *1yr followup* CBE negatively correlated with # of CAMs used at 1 year ($P < 0.004$); No association for Mam or BSE	*Baseline* Perceived cancer risk, sunscreen use, fruit/vegetable consumption *1 yr followup* Higher anxiety, lower perceived breast cancer risk
Downey, (2009), [4]	Cross-sectional	71,083	52–64 yrs, F; enrolled in two washington state insurance companies; USA	Insurance claims data 4 kinds of insurance-paid CAM	Past year	Approximately 22–26% used CAM (depending on year); average 8 visits/yr <1% used only CAM therapies	Mam (past 2 years)	Complementary CAM use more likely to have Mam (OR 1.044; $P = 0.031$); alternative CAM less likely to have Mam (OR 0.006; $P = 0.000$); naturopathy negatively associated with Mam (OR 0.736; $P = 0.000$); massage positively associated with Mam (OR 1.196; $P = 0.000$)	Younger age, higher disease burden, enrolled in fee-for-service products; over the 3 measurement years; areas with lower education, income, and percentage of minority residents
Druss, (1999), [16]	Cross-sectional	10,675 overall (# answering breast screening items NR[f])	>18 yrs, M/F, age and sex-appropriate subset answered breast questions; national probability sample (medical expenditure panel survey); USA	Interview 77% response rate Y/N 13 CAMs that are practitioner-based	Past year	8.3% of overall sample; NR[f] for women answering breast screening items	CBE Mam (past year)	More CBE in CAM users (58.7%–95% CI: 57%–60%) than non-users (69.7%–95% CI; 65%–74%) ($P < 0.001$)[d] No association with Mam	Female, caucasian, higher education, and residing in the west (USA) (only reported for overall sample)
Field, (2009), [17]	Cross-sectional	892	62% 40 yrs+, F; enrolled in the high breast cancer risk cohort; Australia and New Zealand	Mailed questionnaire—73% response rate Y/N 35 CAMs	Ever use	*General Use* 55%; 80% >1 CAM therapy, 30% >4 CAM therapies; *Intention to prevent cancer* 6% of participants	Mam (past 3 yrs)	No association	More education and physical activity, clinical anxiety, being a former smoker and lower perceived BC risk

Table 1: Continued.

Study[a] (Year)	Study design	Sample size	Selected participant characteristics	Data collection methods and CAM assessment	Period of CAM use assessed	Proportion of cancer-free participants using CAM	Breast screening measures[b]	Relevant findings	Correlates of more CAM use
Gollsche-wski, (2005), [18]	Cross-sectional	886	48–67 yrs, F; 61%, <55 yrs; random sample south-east Queensland, Australia	Mailed questionnaire—59% response rate Y/N questions on herbal, phytoestrogen, nutrition and supplement CAMs	Ever use	82% 67% used nutritional approaches, 56% used phytoestrogens, 41% used herbal therapies	CBE, BSE (past 2 yrs)	More BSE in herbal therapy users (OR 1.69, 95% CI 1.34–2.52; $P = 0.01$); and nutritional users (OR 1.68, 95% CI 1.13–2.50; $P = 0.01$); no association with CBE	Younger, higher education, middle income, lower smoking, previous hormone therapy, good physical/general health
Gray, (2002), [7]	Cross-sectional	4404	>40 yrs, M/F; stratified sample (by chronic conditions) from health plan; Minnesota, USA	Mailed questionnaire—86% response rate Y/N 17 CAMs	Past year	42% overall; 46% F	Mam (past yr)	CAM users significantly more likely to have had Mam (67% versus 62%)	Female, younger, higher education, single, employed, health limitations, improved health over past year. More exercise, vegetable intake, fast food consumption; less dietary fat and alcohol (only reported for overall sample)
Mueller, (2008), [19]	Cross-sectional	135 without cancer history, knew BRCA1/2 status	25–56 years of age, F; Enrolled in high genetic breast cancer risk clinic; USA	Telephone interview Y/N 13 CAMs	Past year	78% overall; 69% if spiritual healing/prayer are excluded[e]; average 2.3 CAM therapies; 34% ≥3 CAM therapies (overall sample)	Mam (annual) BSE (Monthly)	BSE and CAM use inversely related (OR 0.3, 95% CI 0.1–0.8; $P = 0.017$); no association with Mam	Older, higher education, ovarian cancer worry
Myers, (2008), [20]	Cross-sectional	2,198, varying risk based on family history	Average 63 yrs, F; family members of women enrolled in breast cancer family study; USA	Mailed questionnaire—70% response rate Y/N 8 CAMs	Ever use	*Intention: Preventing Cancer* 50%; 42% used 1 CAM, 32% used 2 CAMs, 15% used 3, and 12% used >3 CAM therapies.	BSE, CBE, Mam (ever)	In the univariate analysis, all 3 breast behaviors were associated with CAM use (OR 1.33, 95% CI 1.15–1.54; $P = 0.0002$); In the multivariate analysis, associations did not remain significant.	Higher education, general health behaviors, optimism (multivariate analyses)

Table 1: Continued.

Study[a] (Year)	Study design	Sample size	Selected participant characteristics	Data collection methods and CAM assessment	Period of CAM use assessed	Proportion of cancer-free participants using CAM	Breast screening measures[b]	Relevant findings	Correlates of more CAM use
Robinson, (2002), [21]	Cross-sectional	1,593	>18 yrs, M/F, attendees at health fair USA	Questionnaire Y/N 8 CAMs, 13 herbs	Past 2 years	68%; 63% used herbs/supplements	CBE Mam (Past 2 years)	No association	Younger, female, higher education (high school completion), lower levels of health insurance (only reported for overall sample)

[a] Studies listed by first author.
[b] CBE: clinical breast examination; Mam: mammography; BSE: breast self-examination.
[c] Y/N refers to dichotomous responses to use of each CAM treatment.
[d] These data reflect the authors' abstract, data section, and conclusions; the table in the paper presents opposite numbers and is assumed to be a typesetting error.
[e] Participants with cancer were included in this calculation because the authors state that overall patterns of the CAM therapies used didn't differ between cancer survivors and women without cancer and data were not presented separately for each group.
[f] NR: No response.

Table 2: Complementary and Alternative Therapies as Reported by Selected Studies.

	Study								
	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)
Natural products and diet									
General vitamins/supplements	x	x		x					
Chondroitin							x		
coQ10				x					
Creatin							x		
Glucosamine							x		
Omega-3 folic acid		x							
High dose megavitamins							x		x
Selenium		x							
Vitamin E					x				
Other vitamins/supplements		x							
General herbal remedies	x		x						x
Bee Pollen							x		
Black Cohosh					x				
Dong Quai					x				
Echinacea							x		
Essiac				x					
Evening Primrose oil					x				
Flaxseed		x							
Ginko							x		
Ginseng					x		x		
Green tea		x							
Kava Kava							x		
Milk thistle							x		
Red clover					x				
Saw Palmetto		x					x		
Shark cartilage		x		x					
Saint John's Wort				x			x		
Soy		x		x					
Valerian							x		
Wild Yam					x				
Other herbs		x							
Hormones									
Herbal Rx for menopause		x							
Phytoestrogen supplements					x				
Dietary phytoestrogens					x				
Melatonin		x					x		
Tropical progesterone cream					x				
Special diet	x		x						
Commercial weight loss programs									x
Healthy eating					x				
Low fat diet		x							
Macrobiotic		x							
Soy rich diet		x							
Vegan		x							
Vegetarian		x							
Organic products				x					

Table 2: Continued.

	Study								
	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)
Lifestyle diets									x
Nutritional supplements					x				
Other diets		x							
Manipulative and body-based practices									
Massage therapy	x	x	x	x		x	x	x	
Reflexology		x							
Mind and body medicine									
Acupuncture	x	x	x			x	x	x	x
Acupressure	x								
Hypnosis			x				x	x	x
Imagery/visualization		x	x					x	x
Meditation	x	x	x	x				x	
Relaxation techniques			x	x				x	x
Tai chi/Chi gong		x							
Yoga	x	x	x	x					
Other mind body		x							
Consultations									
Counselor/psychologist		x							
Chiropractic therapy				x		x	x	x	x
Dietician		x							
Herbalist								x	
Homeopathy		x	x				x	x	x
Lifestyle advice								x	
Naturopath		x				x	x		
Nutritional advice								x	
Osteopathy		x				x			
Energy therapies	x		x						x
Reiki		x							
Biofeedback/Energy healing		x	x				x	x	x
Whole medical systems									
Traditional Chinese medicine		x						x	
Ayurveda								x	
Folk remedies									x
American Indian								x	
Others			x	x				x	
Exercise	x								
Prayer/spiritual practices		x	x	x				x	x
Support groups				x					x
Other physical therapies		x							

Studies: (1) DiGianni et al., (2003 and 2006), [14, 15]; (2) Field et al., (2009), [17]; (3) Mueller et al., (2008), [19]; (4) Myers et al., (2008), [20]; (5) Gollschewski et al., (2005), [18]; (6) Downey et al., (2009), [4]; (7) Robinson et al., (2002), [21]; (8) Druss and Rosenheck (1999), [16]; (9) Gray et al., (2002), [7].

with and without prayer, given its high endorsement as part of everyday life.

CAM use was not assessed in a consistent way. The time frame for CAM use varied, from over the past month, to within the past year, to the past two years. Often CAM use was assessed through recall or prompting, with respondents asked to select those that they have used in the given time frame. In contrast, DiGianni's studies [14, 15] required participants to recall the types of CAM used from memory, with memory recalls aided by suggestions of major CAM categories. It is unclear how the assessment approach may have affected responses.

3.2. Is CAM Use Associated with Screening Behaviors for Breast Cancer?

3.2.1. Women at High Risk of Breast Cancer. Of the four studies in women at increased breast cancer risk, two found no statistically significant association between CAM use and BSE [14] or mammograms [17]. Myers et al. [20] found statistically significant positive relationships between CBE, BSE, and mammography in univariate analyses, which disappeared after taking account of covariates in multivariate analysis. A fourth paper found a weak but statistically significant inverse relationship between BSE frequency and

CAM use, such that women performing self exams less than once a month were more likely to use CAM [19].

3.2.2. Women at Average Risk of Breast Cancer. Five studies examined women at non-increased breast cancer risk, and four of these assessed mammography. Two studies reported positive associations between CAM and mammography [4, 7], whereas two studies [16, 18] found no relationship. Downey et al. found that naturopathy had a significant negative association with mammography, while massage had a significant positive association. This study also looked at alternative therapy use—that is, using CAM rather than allopathic medicine during the period of observation. The researchers found that women who used CAM as well as biomedical care (i.e., complementary therapy users) were more likely to have a mammogram, whereas those who used CAM as an alternative and did not see a physician, were less likely to obtain mammographic screening [4].

One study found that herbal therapies and nutritional approaches but not phytoestrogens, were significantly correlated with BSE over the previous two years [18].

Two studies focused on CBEs. Gollschewski et al. [18] found no relationship between CAM and CBE, whereas Druss and Rosenheck [16] found that CAM users were more likely to receive CBEs.

3.3. Correlates of CAM Use. Assessing correlates of CAM use was limited since some studies reported only relationships for the overall study population, whereas others focused on a specific target group. Nonetheless, certain trends stand out. Eight of nine studies found that higher education was linked with more CAM use [4, 7, 16–21] and most found that higher CAM use was linked with being younger [4, 7, 18, 21] and having better health behaviors [7, 15, 17, 18, 20]. These findings are consistent with other CAM literature [24]. Higher CAM use was linked with higher anxiety or worry in several studies [15, 17, 19] and with a lower perceived breast cancer risk [15, 17].

4. Discussion

This paper reviewed the literature on CAM use in women participating in early detection for breast cancer. The studies were high quality in terms of defining their study populations, response rates (all reported response rates of 59% or greater), well-defined data collection methods, and analytic procedures that used both univariate and multivariate strategies.

We identified nine studies that reflected a range of populations and assessment techniques. While the heterogeneity of the research makes drawing firm conclusions difficult, some findings are of particular interest. CAM use is common among women, regardless of risk status. Congruent with previous research, those who relied solely on CAM therapies as an alternative to conventional medicine were less likely to obtain mammograms whereas women who used CAM as a complement to allopathic medicine were more likely to be screened. Of particular interest is the positive association found for women who used massage therapy. Many barriers have been identified, including feelings of embarrassment or modesty, which prevent women from receiving breast exams [25]. Massage therapy could be a positive way to decrease these barriers, as individuals receiving massage had a higher body image perception, possibly due to positive effects of being physically touched [26]. This demonstrates the possible ways that CAM and allopathic medicine could complement one another and increase the odds that a woman will feel comfortable receiving a mammogram and will seek one out.

Literature in the area of soy consumption and breast cancer is controversial and to our surprise, seven studies did not directly assess soy intake. In higher soy-consuming cultures, mammographic densities have been positively related to soy intake [27] and dense breast tissue poses difficulties for effective mammographic breast cancer screening. It would have thus been important to have had additional data on the soy-breast screening relationship as high soy consuming women may have added benefits from increased breast screening. It is recognized that soy consumption, alongside other CAM practices, could have been captured in some questionnaires through "long answer" or "other" questions. It is positive that the findings from this review suggest that women with higher CAM use also have higher rates of breast screening procedures. Much more research in the area of breast screening and CAM soy use is needed to verify these associations.

Several methodological concerns need to be considered. These studies were almost all cross-sectional, making it impossible to determine a causal relationship between CAM use and breast screening. Although not all studies found CAM use and breast early detection use were correlated, significant relationships that did emerge were positive. However, this leaves the question of whether higher rates of breast screening result from the holistic and preventive focus of CAM; or whether the individuals who are interested in prevention and early cancer detection are more likely to use CAM; or whether both use of CAM and breast early detection modalities are due to another causal factor, such as self-efficacy for health. Developing and testing conceptual models in this area is a key research priority.

Assessing CAM accurately and consistently is challenging. As seen in Table 2, there was considerable variability among the studies in terms of types of CAM use assessed. In all but one study, CAM use was based on self-reports. The largest study used a sample of over 71,000 women had the advantage of drawing on objective claims reports of services billed to the health insurer which are not subject to recall or social desirability bias; however, only a limited number of CAMs were listed which precluded comparisons with other reports [4].

Another challenge when comparing CAM research papers is failure to ascertain CAM duration, frequency and dose. For example, a woman who partakes in a yoga session every month for an hour is likely to exhibit different health qualities than another who partakes in a 90-minute session each morning. Frequency and intensity of CAM use may be important to assess.

Researchers who assess nutrition and physical activity are familiar with the difficulties associated with measurement of lifestyle variables. Major advances have been made in these research areas through the introduction of standardized assessment tools that include food frequency and physical activity questionnaires. CAM research would benefit significantly from the introduction of standardized and validated questionnaires that would allow comparisons over time and across studies.

5. Conclusions

To our knowledge, this is the first review paper examining CAM use and its association with breast cancer screening. Although a majority of women use CAM therapies, and most women also participate in breast cancer early detection, there has been little attention to the overlap between these two phenomena, and the potential for one set of health behaviors to inform the other.

We found a wide variety of findings in populations assessed, and measures used to assess CAM, breast cancer screening, and correlates thereof. Some findings stand out such as the high use of CAM in general and the fact that when there is a significant relationship between CAM use and breast cancer early detection, it tends to be a positive relationship; in other words, women who are motivated to obtain allopathic screening are also motivated to seek out other ways to care for themselves. They tend to be more educated and in better health, and to exhibit better health behaviors, and as such, are availing themselves of a wide range of preventive care. This provides a foundation for future interventions to increase the connection between CAM and allopathic providers, to build on the strengths of what each can offer, and to maximize on patient motivation and preferences to increase breast health and reduce breast cancer risk. Research is needed to make this potential a reality.

Acknowledgment

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Research Article

Effects of Somatothermal Far-Infrared Ray on Primary Dysmenorrhea: A Pilot Study

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The purpose of this study was to assess the beneficial effects of using a far-infrared (FIR) belt on the management of patients with primary dysmenorrhea. This is the first study to determine the efficacy of somatothermal FIR using a parallel-arm randomized sham-controlled and double-blinded design with objective physical evidence and psychometric self-reports. Fifty-one Taiwanese women with primary dysmenorrhea were enrolled in the study. Results indicate that there was an increased abdominal temperature of 0.6°C and a 3.27% increase in abdominal blood flow in the FIR group (wearing FIR belt) compared to those in the control group (wearing sham belt). Verbal rating scale and numeric rating scale scores in the FIR group were both lower than those in the control group. Compared to the blank group (wearing no belt), the average dysmenorrhea pain duration of the FIR group was significantly reduced from 2.5 to 1.8 days, but there was no significant difference in the control group. These results demonstrate that the use of a belt made of far-infrared ceramic materials can reduce primary dysmenorrhea.

1. Introduction

Dysmenorrhea is pain with menstruation, which may be accompanied by headache, dizziness, nausea, vomiting, diarrhea, cold sweats, or other symptoms [1, 2]. For clinical purposes, dysmenorrhea is classified into primary and secondary dysmenorrhea. Previous studies showed that 50% to 80% of women worldwide have experienced dysmenorrhea, and the majority of these women are teenagers and have primary dysmenorrhea [2, 3]. Primary dysmenorrhea, a condition associated with ovulatory cycles, is the occurrence of menstrual cramps in the absence of demonstrable disease that could account for symptoms [4]. After the onset of menstruation, pain typically starts within hours peaking between 12 and 24 hours and persisting for 12–72 hours.

Clinical research has shown that the main cause of primary dysmenorrhea may be associated with increased production of endometrial prostaglandin, resulting in a high concentration of prostaglandins in blood. The pain-related biomolecular induction of cyclooxygenase (COX-2) and prostaglandin is strongly associated with the severity of primary dysmenorrhea [4]. Excessive prostaglandin causes uterine contractions, ischemia, cramping, and pelvic pain. Nonsteroidal anti-inflammatory drugs (NSAIDs) are commonly used in women with dysmenorrhea, because they can inhibit prostaglandin synthesis and effectively alleviate the symptoms of dysmenorrhea. However, NSAIDs may lead to many adverse effects, including indigestion, headache, and drowsiness, and their rate of failure to reduce dysmenorrhea may reach 20% [5]. Therefore, complementary and alternative medicine (CAM) is becoming an increasingly popular choice for alleviation of dysmenorrhea [6–13].

For women with dysmenorrhea, the application of local heat can reduce muscle tension and relax abdominal muscles to reduce pain caused by muscle spasms. Heat can increase pelvic blood circulation to eliminate local blood and body fluid retention and diminish congestion and swelling, thereby enabling a reduction in pain caused by nerve compression. Previous studies reported that local heat inhibits pain signals and increases proprioception [14, 15].

Heating pads and hot water have long been used as a folk remedy for the treatment of dysmenorrhea [14]. Akin et al. [14] reported that a heating patch on the abdomen can be as effective as ibuprofen to treat dysmenorrhea. However, external energy is needed to provide the heat, and there is a risk of burn accident with improper temperature control. In addition, there are a number of disadvantages using complementary and alternative medicine therapies, such as Chinese herbal medicine [12], acupuncture [16], acupressure [7, 17], aromatherapy [8], and transcutaneous electrical nerve stimulation [11], which may include inconvenience of application and energy consumption, among others.

Far-infrared (FIR) rays are invisible electromagnetic waves, which can produce many biological effects including thermal and nonthermal effects [18–23]. When FIR materials are used to cover the skin, it can reduce body heat loss owing to their thermal insulation properties [24–26]. FIR can promote microcirculation, accelerate wound healing, modulate sleep, and treat depression [27–29]. In general, as an alternative nonpharmacologic therapy, FIR has been shown to be an effective and safe modality for promoting health in patients with various medical conditions.

The use of electrothermal FIR belts at a temperature of 50°C was recently reported to be an effective treatment for primary dysmenorrhea [30, 31]. The most common adverse event was burn caused by the incorrect use of the hot pack [30]. While a number of studies have investigated the therapeutic effects of electrothermal FIR belts, to our knowledge the health benefits of somatothermal FIR belts have not been previously reported in the literature. Thus, the aim of the present study was to test the effectiveness of a somatothermal FIR belt as a novel alternative therapy for the relief of primary dysmenorrhea.

2. Materials and Methods

2.1. Participants. The clinical trial was approved by the institutional review board (IRB) at HungKuang University, (Project number 98-B-002). The participants were screened by a gynecologist. Inclusion criteria were age 18 years or older which can legally give consent to participate in this clinical trial and history of painful menstruation for at least one year. Physical examination and ultrasonography were used to support diagnosis of primary dysmenorrhea. Patients who had gynecological diseases or who used other FIR products within one month before the study were excluded. Patients were instructed not to use any type of medication, such as NSAIDs or oral pills, to relieve painful menstruation during the study period. In the last analysis, a total of 51 subjects aged 19 to 35 years were enrolled in this study. The investigation was conducted between January 1, 2010 and December 31, 2010.

2.2. Belts. Two kinds of belts each measuring 15 cm × 70 cm were used in this study. The FIR belt and the control belt were embedded with and without 10 wt% FIR ceramic powders, respectively. As previously reported [19–23], the FIR ceramic powders were composed of numerous mineral oxides, including aluminum oxide, ferric oxide, magnesium oxide, and calcium carbonate. The FIR energy, at wavelengths between 3 and 16 μm, was 10.16 mW/cm^2 as determined using an SR5000 spectroradiometer (CI, Ltd., Migdal HaEmek, Israel) at the Industrial Technology Research Institute, Taiwan.

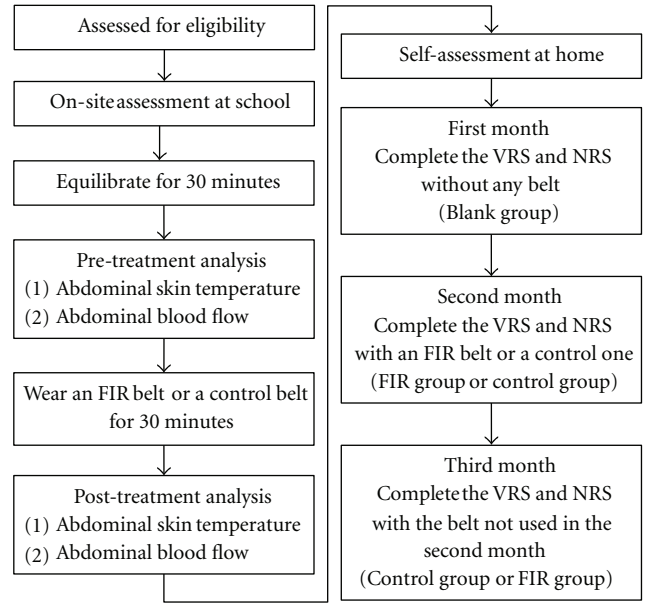
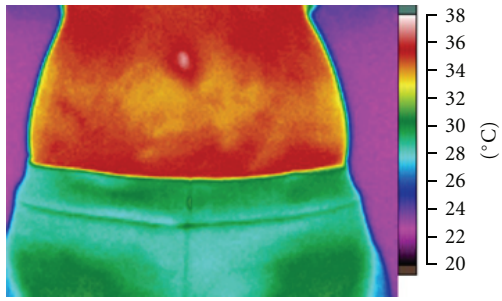


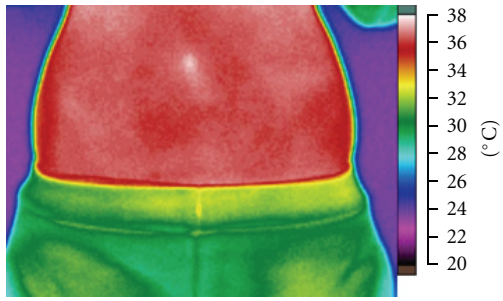
Figure 1: The flow chart of participants through the trial.

2.3. Abdominal Temperature and Blood Flow. All measurements and procedures in this study were performed in a climate-controlled room at a constant temperature and humidity (23°C and 60% relative humidity) [32]. Before assessment, participants were required to lie down in the aforementioned room for 30 minutes to acclimatize to the indoor climatic conditions. Participants were thermographed to measure abdominal temperatures using a Fluke Ti25 thermal imager (Fluke Corporation, Everett, WA, USA), and their abdominal blood flows were detected using a MoorLDI2-IR Laser Doppler Imager (Moor Instruments Ltd., Devon, UK) as described in the previous report [31]. Subsequently, a double-blind method was used to randomly assign subjects to the FIR belt ($n = 26$) group or control belt ($n = 25$) group. Each subject then wore the belt around the abdominal region for 30 minutes. After removing the belt, posttreatment measurements of abdominal temperatures and blood flows were performed.

2.4. Menstrual Pain Assessment. All participants ($n = 51$) completed two questionnaires, the verbal rating scale (VRS) and the numeric rating scale (NRS) [33, 34], to assess the extent of pain on days 1 to 3 of three menstrual cycles. As shown in Figure 1, the pain assessment for the first menstrual cycle was conducted without any belt (blank group). For the second and third menstrual cycles, each participant was instructed to wear the belt on the abdominal region for



(a)



(b)

Figure 2: The abdominal skin temperature distribution of pretest (a) and posttest (b) of applying an FIR belt.

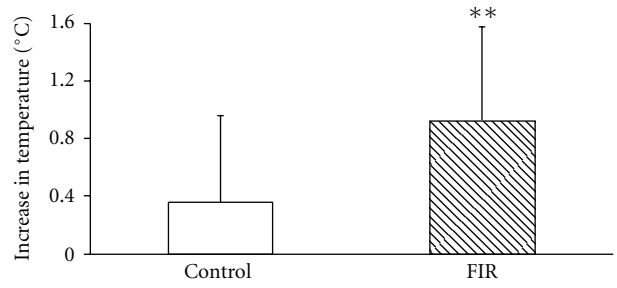


Figure 3: The average increases in temperature after wearing the control belt and FIR belt. The symbol ** indicates a highly significant difference between groups using the Wilcoxon Signed-Rank Test.

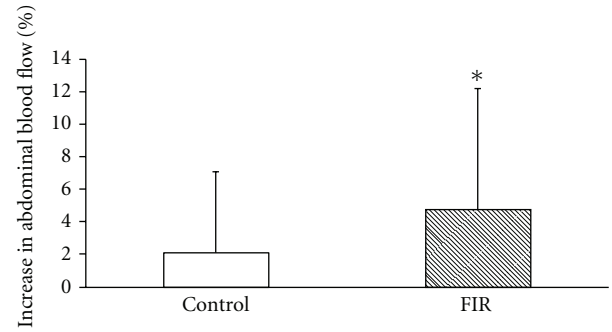


Figure 4: The average increase in abdominal blood flow after wearing the control belt and FIR belt. The symbol * indicates a significant difference between groups using the Wilcoxon Signed-Rank Test.

the whole day during menstruation, but could remove it in order to bathe. Application of an FIR belt or a control belt in sequence was also determined by random sampling (double-blind). The six precisely worded descriptions of pain degrees in the VRS (Chinese version) are scored as follows: none (0), very mild (1), mild (2), moderate (3), severe (4), and very severe (5). The 11-point NRS (Chinese version) is scored using eleven levels of pain, ranging from no pain (0) to the worst pain (10). For assessments of pain levels, there may be individual differences in subjects' feelings and cognition. Thus, this study adopted both the VRS and NRS as complementary indices so that participants' pain levels could be more precisely assessed.

2.5. Statistical Analysis. All data between groups before and after applying belts were analyzed by the Wilcoxon Signed-Rank Test using the SPSS 12.0 software package for statistical analysis. A value of $P < 0.05$ was considered statistically significant (*) and $P < 0.01$ was highly significant (**).

3. Results and Discussion

3.1. Abdominal Temperature. Figure 2 shows the results of temperature differences between pretreatment and posttreatment in the FIR belt group. There was a temperature increase after treatment which was probably due to the thermal insulation effect of the belt. The statistical analysis indicates that the average increases in temperature in the control group and FIR group were 0.36°C and 0.93°C, respectively (Figure 3). The temperature increase in the FIR group was thus enhanced by 0.57°C compared with that found in the control group, which demonstrates that the FIR treatment was associated with a greater rise in abdominal temperature than the control condition. It is reasonable to conclude that the thermal insulation properties of the FIR belt resulted in an increase in the subjects' temperatures. This finding is consistent with previously reported results in similar studies [24–26].

3.2. Abdominal Blood Flow. With the rise in body temperature, there was a corresponding rise in abdominal blood flow after using the belts for 30 minutes. The average blood perfusion units measured by the MoorLDI2-IR Laser Doppler Imager were 116.5 and 119.0 before and after intervention in the control group, and 115.9 and 121.4 in the FIR group, respectively. There were increases in blood flow of 2.12% and 4.78% in the control group and FIR group, respectively (Figure 4). Thus, the increase in abdominal blood flow in the FIR group was enhanced by 2.66% compared with that in the control group.

3.3. VRS and NRS Scores. Figures 5 and 6 show the subjects' distribution of VRS and NRS scores on the first day of menstruation. The results of both VRS and NRS showed a general trend for lower scores in the FIR group. Compared to the blank group, the percentage of subjects with a VRS score above 3 was reduced from 27% to 10% and the percentage

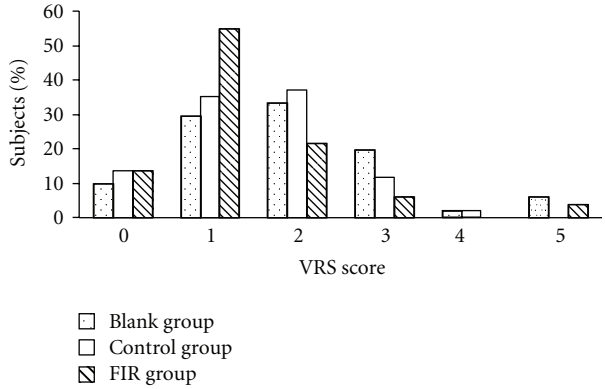


Figure 5: The distribution of subjects' VRS scores on the first day of menstruation.

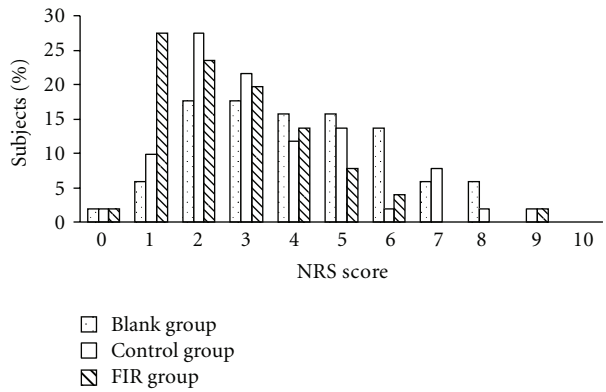


Figure 6: The distribution of subjects' NRS scores on the first day of menstruation.

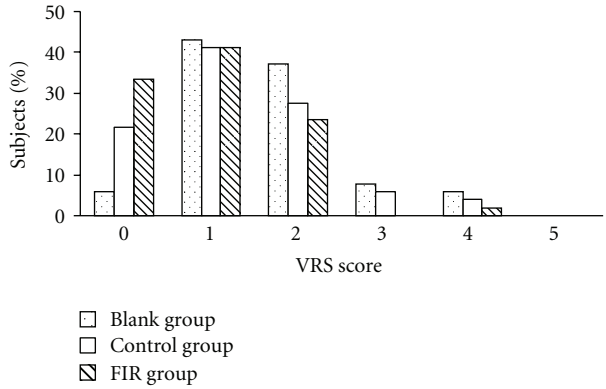


Figure 7: The distribution of subjects' VRS scores on the second day of menstruation.

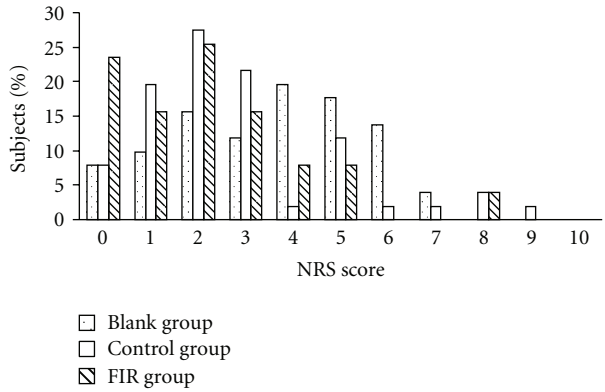


Figure 8: The distribution of subjects' NRS scores on the second day of menstruation.

of those with an NRS score above 5 was reduced from 41% to 14% in the FIR group. The distribution of scores in the control group was between those of the blank and FIR groups. Therefore, the placebo belt showed some efficacy for pain relief, but a better reduction in pain was reported by patients wearing the FIR belt.

The results of the second day (Figures 7 and 8) were similar to those found on the first day. After the belt intervention, pain intensity was reduced with respect to baseline. A greater reduction of pain was observed in the FIR belt group as compared with that in the control belt group. Compared with the blank group, the percentage of subjects with a VRS score above 3 was reduced from 14% to 2% (Figure 7) and the percentage of those with an NRS score above 5 was reduced from 35% to 12% (Figure 8) in the FIR group.

The results of the third day (Figures 9 and 10) were comparable to the results of the first and second days. The FIR group appeared to be the most efficient in terms of pain reduction. Compared to the blank group, the percentage of subjects with a VRS score above 3 was reduced from 10% to 2% (Figure 9) and the percentage of subjects with an NRS score above 5 was reduced from 14% to 6% (Figure 10) in the FIR group.

Figures 11 and 12 are the box-and-whisker plots of VRS and NRS, respectively. The median levels of VRS and NRS in the FIR group were significantly lower than those of the blank group. The VRS and NRS scores in the FIR group showed highly significant differences compared with those of the control and blank groups. The scores in the control group also showed significant differences compared with those in the blank group on the second day (NRS) and the third day (VRS and NRS). Figure 13(a) shows the pain duration data and Figure 13(b) indicates their average and standard deviation. The lower and upper quartiles in the FIR group were significantly lower than those in the blank and control groups. Compared to the blank group, it can be seen that duration of pain in the FIR group was significantly decreased in the menstrual period from 2.4 to 1.8 days. However, there was no significant difference between the blank and control groups. Based on these findings, the authors speculate that the application of sufficient warmth to the belly region can improve blood circulation and shorten the menstrual pain duration.

Previous research has shown that the dysregulation of endometrial blood flow can cause menstrual disorders [35]. Strong and abnormal uterine contractions in women with primary dysmenorrhea can decrease uterine blood flow,

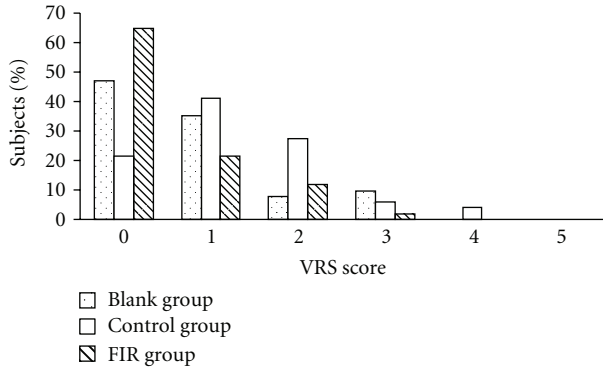


Figure 9: The distribution of subjects' VRS scores on the third day of menstruation.

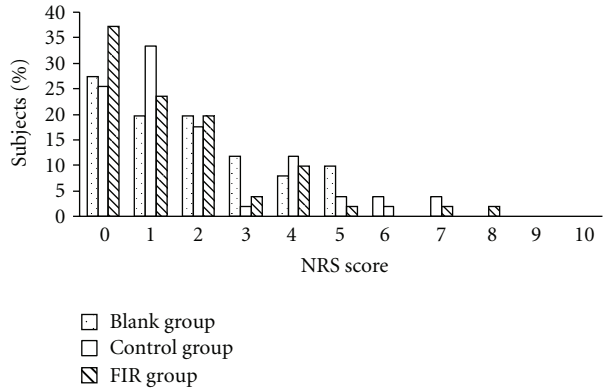


Figure 10: The distribution of subjects' NRS scores on the third day of menstruation.

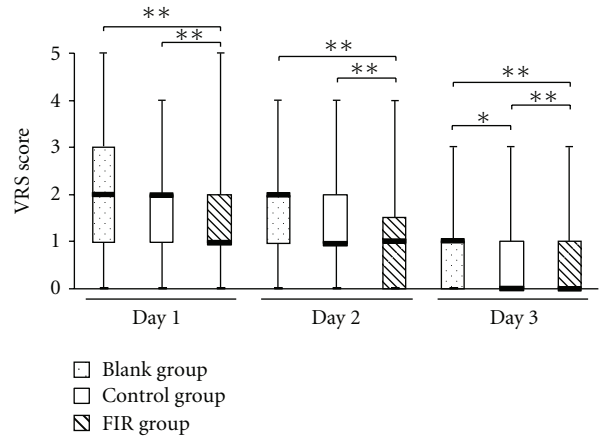


Figure 11: The VRS scores on the first three days of menstruation. The symbols * and ** indicate a significant and highly significant difference between groups using the Wilcoxon Signed-Rank Test, respectively.

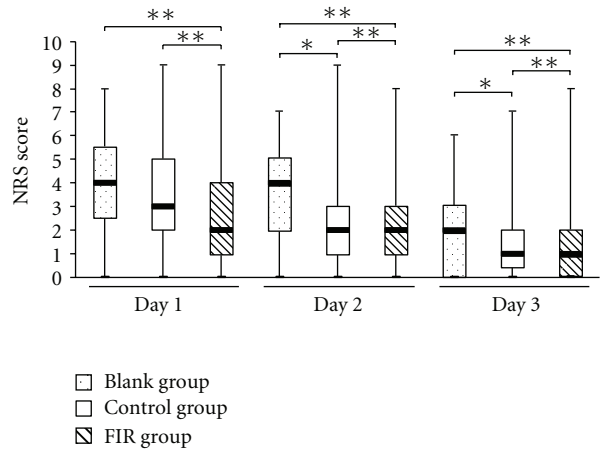


Figure 12: The NRS scores on the first three days of menstruation. The symbols * and ** indicate a significant and highly significant difference between groups using the Wilcoxon Signed-Rank Test, respectively.

resulting in pain in the uterus and the development of ischemia [4]. The application of FIR increases abdominal temperature and blood circulation and reduces muscle tension, leading to the relief of menstrual pain. Related studies also indicate that local heat treatment can alleviate dysmenorrhea [14].

In addition to the aforementioned thermal effects, FIR also exerts nonthermal effects in pain relief of dysmenorrhea. Previous studies showed that FIR could increase generation of nitric oxide (NO) and calmodulin in cells [19, 20, 36]. Associated physiological roles of NO include immune regulation [37, 38], neurotransmission [39], and vascular smooth muscle relaxation [40, 41]. Previous reports have demonstrated that NO in endothelial cells plays an important role in regulating smooth muscle [42]. Endothelial NO in smooth muscle cells can activate guanylyl cyclase to produce cyclic guanosine monophosphate (cGMP). The cGMP sequentially activates protein kinase G, reduces smooth muscle intracellular calcium concentration, inhibits myosin light chain phosphorylation, and ultimately promotes smooth muscle relaxation [42–44]. NO has also been shown to help relax the uterus, thereby reducing the degree of dysmenorrhea [45].

Ischemia is mainly a consequence of decreased microcirculation or a reduction in local muscle blood flow. This scenario has important consequences for cellular metabolic status, as metabolic and acid-base status is significantly worsened with increased acidosis. During dysmenorrhea, the corresponding regional organic ischemia is associated with increased oxidative stress due to increased levels of reactive oxygen species (ROS), such as superoxide and hydrogen peroxide, which are responsible for destructive processes in organic tissues. We demonstrated that the FIR ceramic material exerted an antioxidant effect by increasing hydrogen peroxide-scavenging activity [21, 22].

We also found that FIR induced anti-inflammatory effects by inhibiting prostaglandin E2 (PGE2) [46], and FIR irradiation caused significant inhibition of COX-2 elevation during inflammation [47]. Prostaglandin synthesis is mediated primarily by cyclooxygenase (COX-1 and COX-2), which catalyzes the metabolism of arachidonate to prostaglandin H2 and then produces PGE2 [48], stimulating

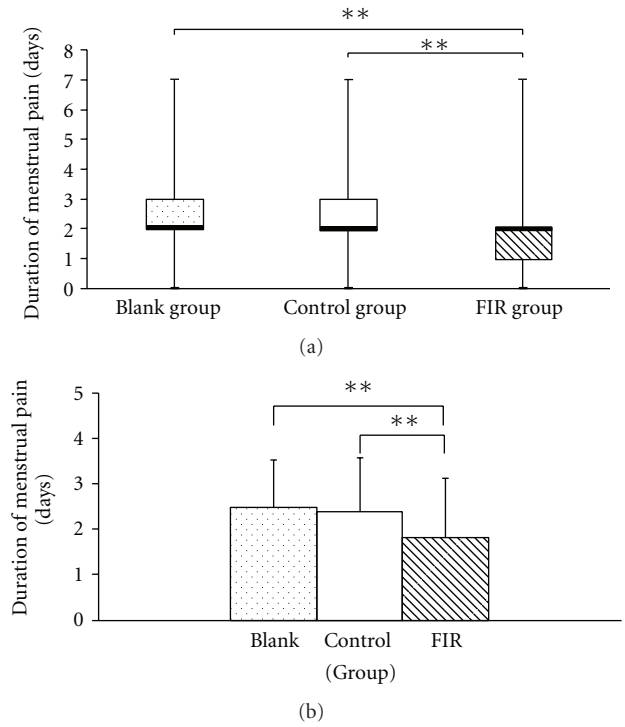


Figure 13: The duration of menstrual pain presented by (a) a box-and-whisker plot and (b) a bar chart indicating the average and standard deviation. The symbol ** indicates a highly significant difference between groups using the Wilcoxon Signed-Rank Test.

uterine contractility, which causes the pain. COX-2 and prostaglandin are strongly related to the severity of primary dysmenorrhea. Previous studies also reported that the main cause of primary dysmenorrhea is an abnormal increase in uterine prostaglandin level, leading to pain caused by uterine smooth muscle contraction [49]. Therefore, reducing PGE2 by applying FIR may lessen the discomfort caused by dysmenorrhea.

4. Conclusions

These findings of this study showed that the somatothermal FIR belt was effective as a novel alternative therapy for relief of primary dysmenorrhea. The FIR belt provided better therapeutic effects in terms of pain relief as well as greater elevation of skin temperature and promotion of blood circulation compared with those observed in patients wearing the sham belt. Compared with traditional complementary and alternative medicine therapies for pain reduction in patients with primary dysmenorrhea, our method has a number of advantages, which include convenience and ease of use, and no external energy source is required. We believe that this novel, noninvasive, and convenient FIR therapy may be of practical use in a clinical setting and suggest that further studies be conducted to confirm our results.

Conflict of Interests

The authors declare that they have no significant conflict of financial, professional, or personal interests.

Acknowledgments

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Research Article

In Vivo and *In Vitro* Antinociceptive Effect of *Fagopyrum cymosum* (Trev.) Meisn Extracts: A Possible Action by Recovering Intestinal Barrier Dysfunction

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Fagopyrum cymosum (Trev.) Meisn (Fag) is a herb rhizome which has been widely used to treat diseases. To investigate the effects and mechanisms of the Fag on irritable bowel syndrome (IBS), *in vivo* neonatal pups maternal separation (NMS) combined with intracolonic infusion of acetic acid (AA) was employed to establish IBS rat models. Fag reduced their visceral hyperalgesia and the whole gut permeability, ameliorated colonic mucosa inflammation and injury, and upregulated the expression of decreased tight junction proteins (TJs) of claudin-1, occludin, and ZO-1 (except ZO-2) in colonic epithelium. Caco-2 monolayer cells were incubated with TNF-α and IFN-γ *in vitro* to establish an epithelial barrier dysfunction model whose transepithelial electrical resistance (TER) depended more on dose of Fag than that of the controls, and whose TJs levels were lower than those of the controls. Fag upregulated the NP-40 insoluble and soluble components of the four TJs markedly in a dose-dependent manner. These data suggest that Fag alleviated the hyperalgesia of IBS rats by reducing intestinal inflammation and enhancing mucosal epithelial function after regulating the structure and function of TJs.

1. Introduction

Irritable bowel syndrome (IBS) is a chronic functional bowel disorder featured in abdominal pain and disturbed bowel habits. One of the pathomechanism is gastrointestinal motility dysfunction accompanied by visceral hyperalgesia [1]. Recent studies have found that chronic low-grade mucosal inflammation potentially led to IBS [2], as well as coexistent immune abnormalities. The T lymphocytes in descending colon mucosa significantly increased in the case of postinfectious IBS (PI-IBS) and non-PI-IBS [3]. Furthermore, the cytokine concentrations in the colonic mucosa of IBS patients increased due to activated immune system [4], and expression of mast cells adjacent to intestinal plexus in non-PI-IBS patients was highly expressed, which [2] resulted in hyperalgesia.

It has been reported that the intracolonic infusion of supernatants from the colonic biopsies of IBS patients led to somatic and visceral hyperalgesia [5] as well as impaired colonic permeability and sensitivity of mice [6]. Repeated stress increased $CD4^+/CD8T^+$ cells [7] and IFN-γ, which directly affected the tight junction proteins (TJs), and altered colonic mucosal barrier functions eventually [8]. However, adding supernatants of the culture from human colonic biopsies to Caco-2 cells reduced transepithelial resistance (TER), decreased ZO-1 mRNA in Caco-2 evidently, and increased paracellular permeability (PP) which correlated with abdominal pain [9]. Dunlop [10] and Spiller [11] have demonstrated that the intestinal permeability in PI-IBS and non-PI-IBS subgroups of diarrhea-predominant IBS elevated. Kong et al. [12] also showed that downregulated

claudin-1 and -4 expressions were associated with changed defecation habits of D-IBS patients [13]. Finally, intestinal mucosal inflammation may contribute to altering TJs structures and PPs in IBS patients.

Fagopyrum cymosum (Trev.) Meisn (Fag), which is a herb rhizome of the Polygonaceae family and buckwheat species, has been widely used to treat bacterial dysentery, menorrhalgia and abdominal pain in Chinese medicine. Our previous clinical practices have revealed that Fag effectively mitigated abdominal pain, diarrhea, and bloating of IBS, and we have a Chinese authorized patent on Fag treatment of IBS. We assumed that Fag alleviates hyperalgesia of IBS by preventing intestinal mucosal immune disorders or decreasing colonic permeability via affecting TJs. Thereby motivated, we extracted the active ingredients of Fag and established animal and cell models to verify the hypothesis and to clarify the pharmacological targets and mechanisms.

2. Materials and Methods

2.1. Animal Experiments

2.1.1. Preparation of Fag Extracts. Fag was provided by Nantong Jinghua Pharmaceutical Co., Ltd., Jiangsu Province of China, and was extracted by conventional refluxing in 3 fold of 50% ethanol for 3 h, 2 fold of 50% ethanol for 2 h, and 1.5 fold of 50% ethanol for 1 h, respectively. Then the product was decompressively concentrated and then spray-dried. The resulting powders were dissolved in 1% NaOH, the pH of which was then adjusted to 7.4 by HCl. The product was then diluted to a constant volume by saline and finally filtered prior to sterilization. The samples were identified by the Jiangsu Provincial Institute for pharmaceutical inspection of China.

2.1.2. Animals and Neonatal IBS Modeling. Neonatal Sprague-Dawley rats weighing 5–6 g in average were provided by the Animal Center of Nanjing University of Chinese Medicine. Experiments were conducted in accordance with the standards of Animal Ethics Committee of Nanjing University of Chinese Medicine and the regulations of animal welfare of NIH in the USA. IBS rat models were established referring to neonatal maternal separation (NMS) pups [14] plus intracolonic infusion of 0.5% acetic acid [15]. Postnatal days 4 and 21 pups were subjected to maternal deprivation for 3 h (from 9:00 to 12:00 am), during which they were transferred from the home cage to a new plastic cage individually equipped with constant-temperature bedding ($37 \pm 0.5^{\circ}$C, heat source: a hot plate). During NMS, pups were intracolonically infused with 0.2–0.5 mL of 0.5% acetic acid daily at the same time by an angioplasty catheter (3 mm in diameter and 20 mm in length, Cordis Inc., USA) that was inserted from anus to descending colon (2 cm from anus). The control pups were left undisturbed and intracolonically infused with the same amount of saline. The pups were weaned on 22nd day. The males were selected and housed in the same cage undisturbed until they grew to 160 g (6–8 weeks old). The adult model rats in drug groups were treated with Fag (6 g/kg, 24 g/kg, resp.) and VLS#3 (VLS Pharmaceuticals. Ft, Lauderdale, FL, USA. 0.08 g/kg, 3.0 g of each tablet containing 450 billion freeze-dried bacteria) orally once daily for two weeks. The normal rats and model rats were only treated with saline.

2.1.3. Evaluation of Viscera Hyperalgesia. Intense colorectal distension (CRD) is a nociceptive stimulus which enhances the contractile activities of abdomen and lowers those of limb muscles. Abdominal withdrawal reflex (AWR) evaluates the visceral sensitivity of IBS model during CRD. Rats were anaesthetized by ethyl ether, and a catheter-balloon assembly (made from latex glove; 2.5 cm in diameter and 4.0 cm in length, attached to 6 F catheter) was inserted into their descending colon (1 cm away from anus; exterior catheter was connected to a sphygmomanometer via a three-way pipe) with paraffin oil lubricant. Then the rats were put into transparent Lucite Cubicles (18 cm × 5 cm × 7 cm) and prevented from turning around. Thirty minutes after the rats' regained consciousness, air was injected into the balloon to produce the pressures of 20, 30, 40, 50, 60, 70, and 80 mmHg, respectively. Each expansion was lasted for 20 s with the intermittent of 2 min. The experiments were performed in triplicate, and the average values were recorded. Al-Chaer's method [16] was utilized as the AWR evaluation standard: 0 for no behavior responses, 1 for action pause followed by short head movement after stimulation, 2 for visible abdominal muscles without abdomen lifting off the platform, 3 for abdominal lifting off the platform, and 4 for body arching and pelvic structures or scrotum lift off.

2.1.4. Total Gut PP. ^{51}Cr-Ethylene diamine tetraacetic acid (^{51}Cr-EDTA, Perkin Elmer Life Science, Paris, France) was used as the selective paracellular permeation marker according to the Barreau's method, aiming to determine the total gut permeability towards large molecules [14]. 0.7 μ Ci of ^{51}Cr-EDTA diluted in 500 μL of saline was slowly administered orally. Then the animals were placed in metabolic cages, the faeces and urine of which were collected for 24 h. 1 mL of the collected urine and 1 mL of the 100 times diluted standard were subjected to γ counting measurements for 1 min to calculate the ^{51}Cr-EDTA excretory rate:

$$\begin{aligned} &51\text{Cr} - \text{EDTA excretory rate of urine during 24 h (\%)} \\ &= \left(\frac{(\text{counts of urine sample} - \text{background}) \times \text{urine volume}}{(\text{counts of diluted standard sample} - \text{background}) \times \text{dilution multiples}} \right) \times 100\%. \end{aligned} \tag{1}$$

2.1.5. Evaluation of Colonic Damage/Inflammation. The myeloperoxidase (MPO) activities of colon tissues were measured as described previously [17]. Frozen pieces of distal colon (5 cm from anus) were homogenized in the phosphate buffer (in 50 mM/L, pH = 6) containing hexadecyl trimethyl ammonium bromide (0.5% w/v). Homogenates were subjected to 3 cycles of freezing and thawing (−196°C, 1 min and 37°C, 10 min) and then were further disrupted with a sonicator (Kunshan Hechuang Ultrasonic CO., LTd., China) and then centrifuged (6000 g at 4°C for 15 min). The supernatants were collected for MPO activity measurements. Protein concentrations were determined according to the modified Lowry's method (Bio Rad DC Protein Assay, France) and MPO activities were expressed as units/per gram of protein. In IFN-γ and TNF-α assay, distal colon tissues were homogenized in ice buffer solution (1mL/0.1 g) and then centrifuged for 10 min (600 g at 4°C). The supernatants were collected and detected using an ELISA kit (BIO-RAD Laboratories, Inc., USA) according to the instructions. The absorbance was measured at 492 nm.

2.1.6. Colon Histological Staining. Distal colon fresh tissues were fixed in 10% paraform for 12 h, then dehydrated in 50%, 60%, 70%, 80%, 90%, and 100% alcohol separately, and embedded in paraffin at 56–58°C. The samples were cut into sections (4 μM for each), deparaffinized in dimethyl benzene, gradiently dehydrated in alcohol and stained with hematoxylin, eosin (H&E), dehydrated in 70%, 90%, and 95% ethanol, and finally cleared in xylene [18]. A microscope (Nikon 80, Japan) was used to collect pictures. The positive expressing areas and the positive cells were counted by Image-J software.

2.1.7. Western Blot of Colonic Claudin-l, Occludin, ZO-1 and ZO-2. Distal colonic tissues were quickly taken from sacrificed rats and stored in liquid nitrogen at −170°C and processed into a homogenate. Then lysate was added into the sample (500 μL: 100 mL), to which was also added phosphatase inhibitor to protect phosphorylated proteins. The protein concentrations in the supernatants centrifuged (12500 r/min, 15 min at 4°C) and the sample were determined using a BCA protein assay kit (Pierce, Rockford, IL). The primary antibodies included rabbit polyclonal anti-ZO-1, ZO-2 (1 : 1000, Santa Cruz, Inc. America), rabbit anti-claudin-1, occludin (1 : 1000, abcam, Inc., USA) and rabbit anti-GAPDH antibody (1 : 1000, Bioworld, Inc., USA). The secondary antibody was sheep polyclonal anti-rabbit IgG (1 : 10000, IRDye800 Conjugated, Rockland, Inc., USA). LI-COR Odyssey Infrared Imaging System and LI-COR Odyssey Analysis software were utilized for scanning and analysis, respectively.

2.1.8. Immunofluorescence Staining of ZO-1 and Occludin. Rat distal colon samples were embedded in paraffin, cut into sections (thickness: 4 μM), deparaffinized in dimethyl benzene, and then dehydrated in 100%, 95%, and 70% alcohol for 5 min. The antigens of the sections were restored by being placed on a metal staining rack in a pressure kettle containing boiling water to which was added phosphate buffer (0.01 mol/L, pH 6.0). The samples were processed for 5 min by gradually increasing pressure, removed, and quickly washed with cool water, followed by an additional wash 3 times with PBS (0.01 mol/L PH7.4) for 2 min. Goat serum was added to the samples, which was incubated for 20 min at 37°C, and the residual fluid was discarded. Then the samples were incubated with primary antibody (1 : 1000, rabbit anti-ZO-1, Santa Cruz, Inc. America; rabbit anti-occludin, abcam, Inc., USA) overnight at 4°C, then washed 3 times with PBS for 2 min. Thereafter, the samples were incubated with secondary antibody (1 : 200, TRITC or FITC goat anti-rabbit Ig G (H + L) conjugate, Invitrogen Inc., USA) at 37°C and then washed 3 times with PBS for 2 min. 50% glycerin was added to seal up the sections, which were then fluorescently visualized using a laser scanning confocal microscope (LSCM, Nikon Inc., Japan).

2.2. Caco-2 Cell Experiments

2.2.1. Cell Cultures. Caco-2 cells (Cell Banks of Chinese Institute of Sciences) were grown in a culture medium consisting of Dulbecco's modified Eagle's medium (DMEM, Gibco, USA), 4.5 g/L glucose, 4.0 mmol/L glutaminate, 10^5 U/L penicillin, 100 mg/L streptomycin, 20% Fetal Bovine Serum (FBS, Wisen, Canada) and nonessential amino-acid (Invitrogen, USA) [19]. After being digested with 0.25% trypsin and 0.02% EDTA in Ca^{2+} free and Mg^{2+} free PBS, the cells were inoculated in plug-in type 96-well plates at the density of 1×10^5 cells/cm^2 (PCF membrane, pore size 0.4 μm, Millipore, USA).

2.2.2. Determination of TER. Barrier functions were evaluated by TER which reflects the colonic mucosal permeability indirectly. The apical sides of the cells were administrated with cytokines (TNF-α 100 ng/mL and IFN-γ 100 ng/mL) and incubated for 72 h. The TER of Caco-2 monolayers was measured using the Millicell-ERS (Electrical Resistance System) (Milipore, USA) after 0, 12, 24, 36, 48, and 72 h. Then the monolayers were incubated with 100 ng/mL TNF-α, 100 ng/mL IFN-γ, and Fag (0, 5, 10, 30 μg/mL) for 24 h. The resistance values (RV) were measured consecutively at least 3 times. TER was determined according to:

$$\mathrm{TER}(\Omega \cdot \mathrm{cm}^2) = (R_{\mathrm{total}} - R_{\mathrm{blank}}) \times A, \tag{2}$$

where R_{total} represents the measured RV; R_{blank} represents the RV without cells; A represents the membrane surface area [20].

2.2.3. Assessment of TJs Expression by Western Blot. After digestion, the cells were incubated in 24-well plates, adhered to bottom overnight, and starved in serum-free medium for 12 h. Some cells were incubated only with Fag (0, 5, 15, 30 μg/mL), while the others were incubated with TNF-α, IFN-γ and Fag (0, 5, 15, 30 μg/mL) for 24 h. After intervention, NP-40 soluble protein and NP-40 insoluble protein in the cells were extracted [21] for TJs immunoblotting essay. Protease and phosphate inhibitors (Kang as Item) were added, and the protein concentrations were determined. The samples were transferred to a membrane (wet transfer)

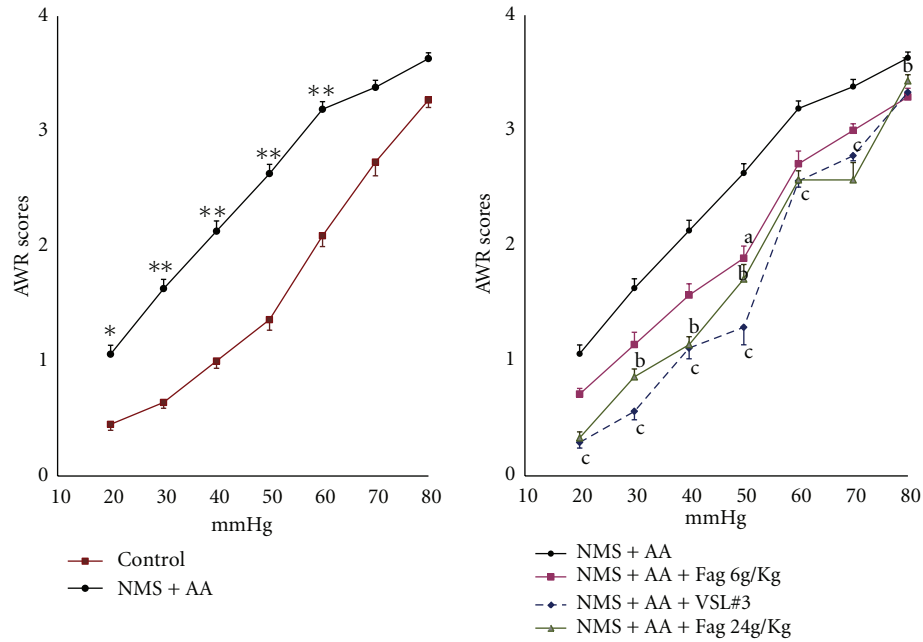

Figure 1: Sensitivities of the rats to CRD. (a) AWR scores of the rats in response to graded CRD. $^{*}P < 0.05$, $^{**}P < 0.01$, NMS + AA rats (model group) versus controls. (b) AWR scores of NMS + AA rats treated with Fag (6 g/kg, 24 g/kg) and VLS#3 $^{a}P < 0.05$, Fag (6 g/kg) treated rats versus NMS + AA rats; $^{b}P < 0.05$, Fag (24 g/kg) treated rats versus NMS + AA rats; $^{c}P < 0.05$, VLS#3 treated rats versus NMS + AA rats. The data are expressed as mean ± SEM (n = 8–10 in each group).

followed by SDS-Polyacrylamide gel electrophoresis, sealed with 1% bovine serum albumin at room temperature for 2 h, incubated with primary antibody (rabbit polyclonal anti-ZO-1, ZO-2, 1 : 2000, Santa Cruz, Inc., America; rabbit anti-claudin-1, occludin 1 : 2000, abcam, Inc., USA), and diluted with Tris-buffered saline (TBS) overnight at 4°C. The residual was discarded, washed 3 times with TBS containing 0.1% Tween-20 (TBST) for 5 min, administrated with secondary antibody (rabbit anti-GAPDH antibody, 1 : 1000, Bioworld, Inc., USA), diluted with TBS, and incubated at room temperature for 1 h. LI-COR Odyssey Infrared Imaging System was used for scanning.

2.2.4. Fluorescein Localization of Claudin-1. Caco-2 monolayers grown on transwell filters were washed with PBS, fixed with 4.0% formaldehyde for 10 min, permeabilized in NP-40 Lysis Buffer for 30 min, and washed 3 times with PBS. The monolayers were then labeled with rabbit anti-claudin-1 antibody (1 : 2000, abcam, Inc., USA) overnight at 4°C, followed by incubation for 30 min with secondary antibodies (1 : 200, Goat Alexa Fluor 488-conjugated anti-rabbit IgG). The images were visualized using an LSCM (Nikon, Inc., Japan).

2.3. Statistical Analysis. All values were expressed as mean ± SEM or mean ± SD. The statistics of the two groups were compared by t-test or Mann-Whitney U-test using GraphPad-InStat, version 5.01, and $P < 0.05$ was considered as statistically significant.

3. Results

3.1. Assessment of Visceral Sensitivity to CRD. The adult NMS + AA rats responded to graded CRD (20, 30, 40, 50, 60 mmHg) were significantly different from the controls ($P <$ 0.05 or <0.01) (Figure 1(a)). The responses of NMS + AA and 6 g/kg Fag rats to CRD did not differ significantly except AWR decreased when CRD = 50 mmHg. The AWR score of 24 g/kg Fag rats was significantly lower (when CRD at 30, 40, 50, 80 mmHg, $P < 0.05$) than that of NMS + AA rats. Besides, the AWR score of VSL#3 rats was effectively lower ($P < 0.05$) than that of the controls (Figure 1(b)).

3.2. Effect of Fag on Gut PP and Colonic Damage/Inflammation. In NMS + AA rats, ^{51}Cr-of EDTA excreted in urine over 24 h (GPP) significantly increased compared to that in control rats ($P < 0.001$), while gut PP in 6 g/kg, 24 g/kg Fag and VLS#3 rats decreased significantly ($P < 0.05$, $P < 0.01$) (Figure 2(a)). The colonic MPO activity of control rats was 157.6 ± 95 U/g of protein, which was significantly lower ($P < 0.001$) than that of NMS rats. The activities of 24 g/kg Fag and VLS#3 rats decreased ($P < 0.01$) (Figure 2(b)). The levels of TNF-α and IFN-γ in the colon of NMS + AA rats were significantly higher ($P < 0.01$) than those of the controls. TNF-α and IFN-γ in Fag treated rats exhibited a dose-dependent recovery to normal significantly compared with those in NMS rats did. VLS#3 decreased the contents of both TNF-α and IFN-γ (Figures 2(c) and 2(d)). These data demonstrated that Fag reduced gut PP by mitigating intestinal damage and inflammation.

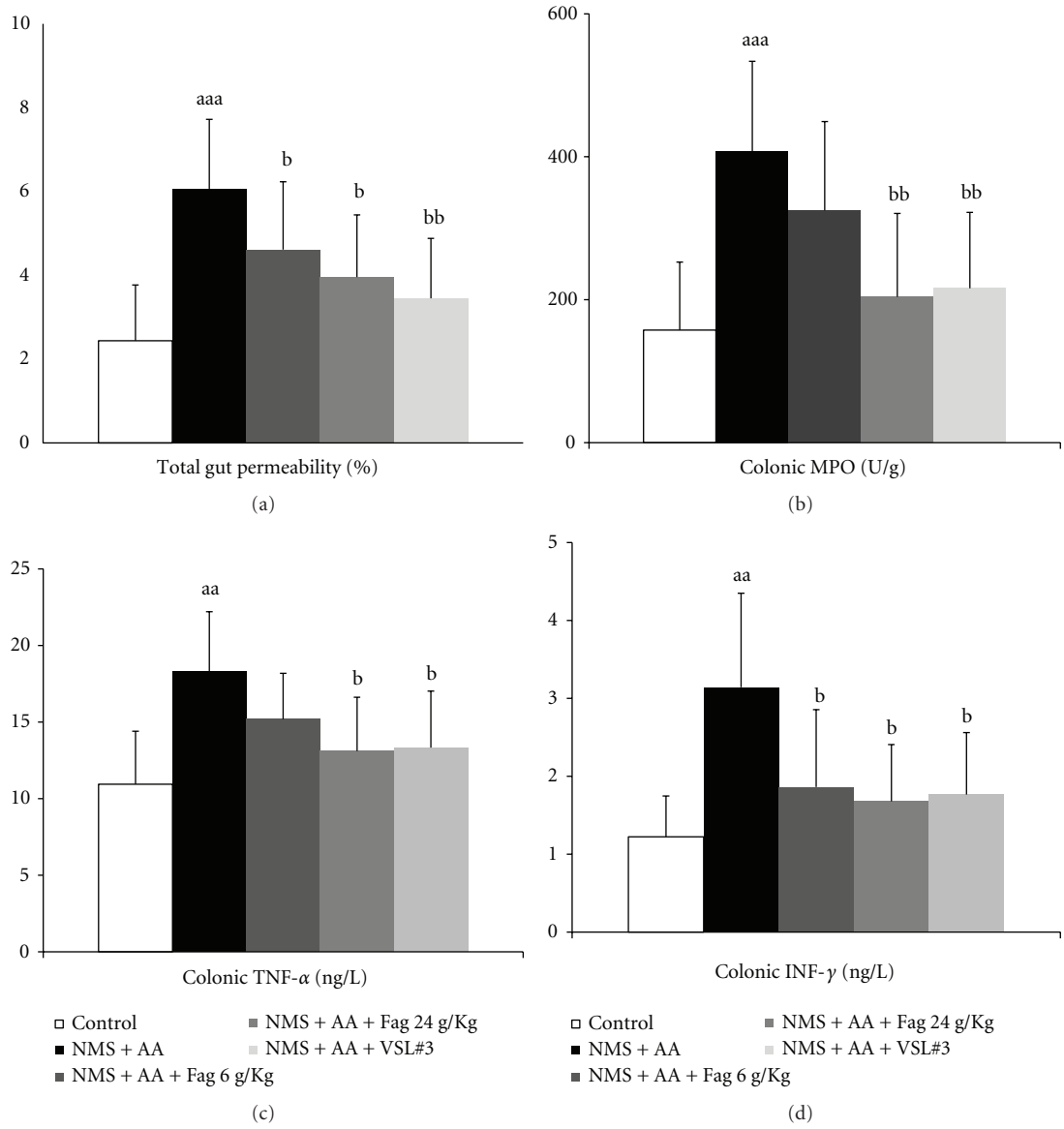

Figure 2: Effects of Fag on the total gut permeability (a), colonic myeloperoxidase (MPO) activity (b), colonic cytokines TNF-α (c) and IFN-γ (d) in NMS + AA rats. $^{aa}P < 0.01$, $^{aaa}P < 0.001$, compared with control rats; $^{b}P < 0.05$, $^{bb}P < 0.01$, compared with NMS + AA rats. The values are expressed as mean ± SD ($n = 8$ in each group).

3.3. Effect of Fag on Colonic Lamina Propria Inflammatory Cell Counts. HE staining shows a small number of lamina propria inflammatory cells infiltrated in the distal colon of the controls, but there were numerous inflammatory cells in NMS+AA rats (Figure 3(a)), which differed significantly ($P < 0.01$) from those in the controls. Compared with NMS + AA rats, the cell counts in Fag 24 g/kg ($P < 0.05$) and VLS#3 rats ($P < 0.01$) decreased significantly, whereas those in Fag 6 g/kg rats only decreased slightly ($P > 0.05$) (Figure 3(b)).

3.4. TJs Expression of Colon Tissues. The colonic TJs of claudin-1, occludin, ZO-1, ZO-2 were analyzed by western blotting (Figure 4(a)). Compared with the controls (100%), the total protein of the four TJs in NMS + AA rats significant decreased ($P < 0.05$). Besides, the TJs of occludin ($P < 0.05$), and ZO-1 ($P < 0.05$) in Fag-treated rats as well as the TJs of claudin-1, occludin and ZO-1 in VSL#3 rats ($P < 0.01$) were significantly higher than those in NMS + AA rats (Figure 4(b)).

Moreover, we examined the expression and distribution of TJs by immunofluorescence. The four types of TJs were localized at both the surface and crypts connecting colonic cells, which is consistent with TJs distribution. However, the TJs of occludin, ZO-1 in NMS + AA rats drastically reduced. The membrane fluorescence intensities and discontinuities of NMS + AA rats declined or even, lost compared to those of the controls. In addition, 24 g/kg Fag and VLS#3 considerably

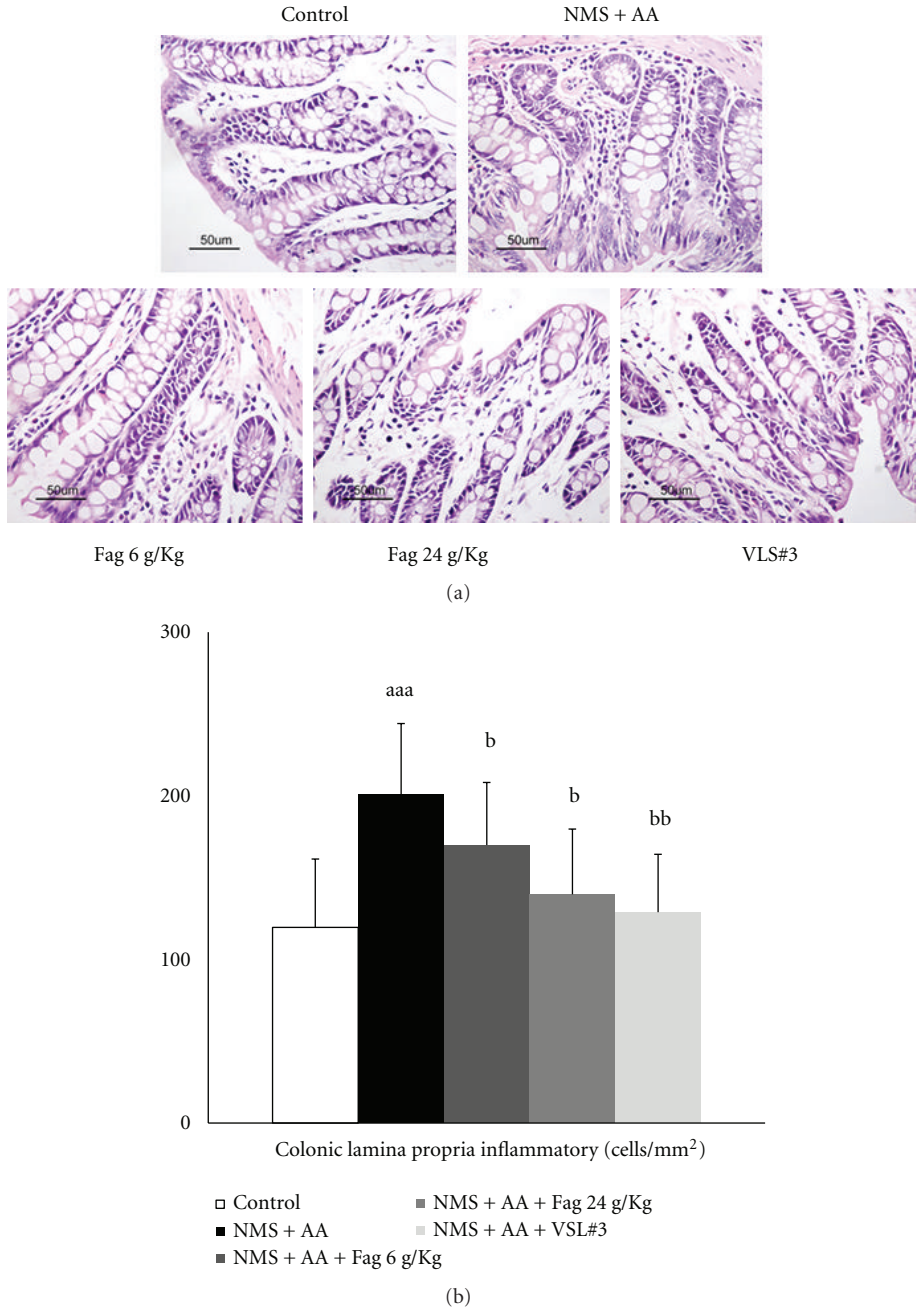

Figure 3: Effects of Fag treatment on colonic lamina propria inflammatory cells of rats (×200). (a) HE staining of colonic lamina propria inflammatory cells; (b) quantification of inflammatory cells. The data are expressed as cell counts per square millimeter of colon lamina propria. The values are expressed as mean ± SD ($n = 8$), $^{aa}P < 0.01$ compared with control rats. $^{b}P < 0.05$, $^{bb}P < 0.01$, compared with NMS + AA rats.

prevented the loss of occludin and ZO-1, the densities of which were remarkably higher than those in NMS + AA rats (Figure 5).

3.5. Effect of Fag on TER of Caco-2 Cell Monolayers. TER was lower time dependently in the cell monolayers when incubated with (TNF-α 100 ng/mL and IFN-γ 100 ng/mL at 24, 36, 48, and 72 h than controls without any intervention ($P < 0.001$) (Figure 6(a)). Without cytokines, the values were dose-dependently higher after administration of cells with Fag treatment ($P > 0.05$) for 24 h than controls (Figure 6(b)). TER was dose dependently higher ($P < 0.05$) when monolayers incubated with both cytokines (TNF-α 100 ng/mL and IFN-γ 100 ng/mL) and Fag for 24 h than controls incubated with only cytokines (Figure 6(b)).

3.6. Effect of Fag on TJs in Detergent-Insoluble and Soluble Fractions of Caco-2 Cells. The protein levels of ZO-1, ZO-2,

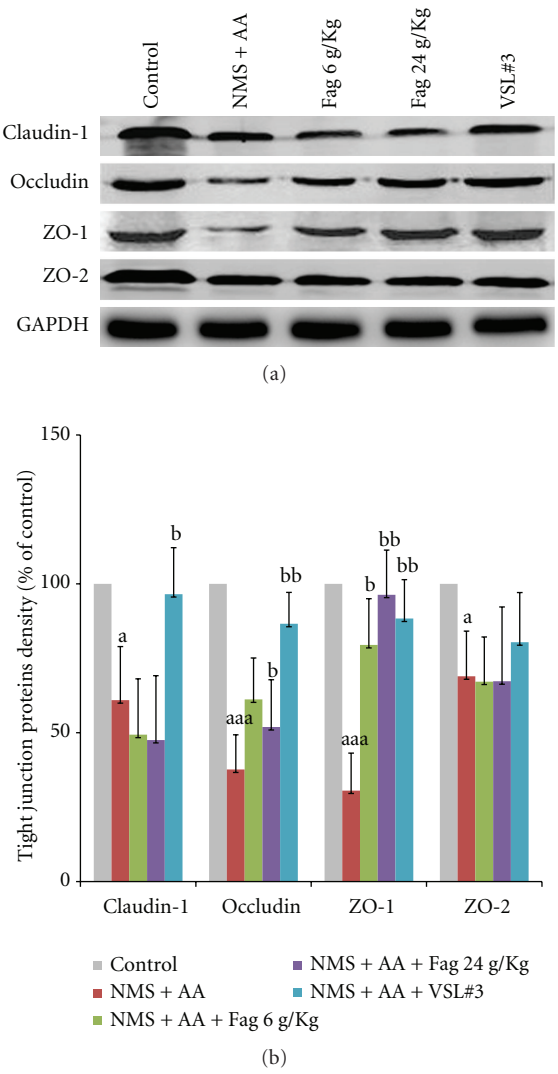

Figure 4: Western blot of Fag treated rat colon TJs. (a) Protein of claudin-1, occludin, ZO-1, ZO-2. (b) Relative density analysis of concentration-dependent TJs of drug-treated groups and the controls. The values are expressed as mean ± SD ($n = 3$). $^{a}P < 0.05$, $^{aaa}P < 0.001$, compared with control rats; $^{b}P < 0.05$, $^{bb}P < 0.01$ compared with NMS + AA rats.

occludin, claudin-1 were observed after incubating the cells with Fag (0, 15, or 30 μg/mL) in the absence of or in the presence of cytokines (100 ng/mL TNF-α and 100 ng/mL IFN-γ) for 24 h (Figure 7(a)). The densitometric analysis reveals that Fag and cytokines-free treatment increased the TJs of claudin-1, occludin, ZO-1, and ZO-2 in the NP40 detergent-insoluble fraction (Figure 7(b1)) and those of occludin and ZO-2 in the NP40-soluble fraction (Figure 7(b2)) of cells in a dose-dependent manner ($P < 0.05$). Particularly, the level of ZO-1 in the NP40-insoluble fraction was 3–9 times higher than that in the controls. When treated with cytokines, the TJs of claudin-1, occludin, ZO-1, ZO-2 in the NP40-insoluble fraction ($P < 0.05$) and those of claudin-1, ZO-1 in the NP40-soluble fraction ($P < 0.05$) were lowered. The four TJs except ZO-1 in the NP40-insoluble fraction were positively proportional to ($P < 0.05$) the doses of Fag and cytokines (Figure 7(b3)), and the TJs in the NP40-soluble fraction also increased ($P < 0.05$) when treated with cytokines and Fag (15 or 30 μg/mL) (Figure 7(b4)).

3.7. Effect of Fag on Immunofluorescence of Claudin-1 in Caco-2 Cells. The claudin-1 in the cells incubated with Fag (15, 30 μg/mL) without cytokines (100 ng/mL TNF-α and 100 ng/mL IFN-γ) for 24 h exhibited more intense immunofluorescence than the control monolayers did (Figure 8(a)). However, the fluorescence intensity decreased after being treated with cytokines. Therefore, Fag raised the fluorescence intensity of the inflammatory monolayers (Figure 8(b)).

4. Discussion

Previous studies have shown that NMS predisposed adult rats to colonic barrier dysfunction in response to mild stress [22] and altered the long-term colonic sensitivity to rectal distension [23]. Barreau et al. [14] found that NMS continuously altered the colonic epithelial barrier as a stress factor owing to the exaggerated expression of cytokines. Winston et al. [15] infused ten-day-old rat pups with saline containing 0.5% acetic acid intracolonically, which resulted in higher sensitivities and IFN-γ levels in the proximal colon in adult rats compared to those of the controls. In our study, a new IBS model of intestinal barrier dysfunction was established via repeatedly stimulating AA based on NMS, which excessively activated the intracolonic immunity. The results show that adult rats had visceral hypersensitivity and high permeability of the colonic mucosa, which are associated with the increased colonic MPO activity, lamina propria inflammatory cells, and cytokine expression, as well as the declined expressions of colonic epithelial claudin-1, occludin, ZO-1, and ZO-2.

We assume that the combination of NMS with AA activated the hypothalamic-pituitary-adrenal (HPA) axis, which may account for the increased translocation of pathogenic bacteria in gut rather than the reduced probiotic bacteria protection [7, 24]. As a result, intestinal immunity was interfered, mucosa was destructed, and PP increased ultimately [25].

Complicatedly structured TJs, which comprise over 50 proteins, form plasma membrane-crossing fibrils and interact with proteins in the adjoining cells and the adherens junctions that are linked to the perijunctional actomyosin ring relating to the assembly of TJs and paracellular permeability [26, 27]. TJs are controlled by various signaling pathways, including protein kinase C (PKC), mitogen-activated protein kinases (MAPK), myosin light chain kinase (MLCK), and the Rho family of small GTPases. Phosphorylated TJs are active and exhibit augmented epithelial barrier function. The phosphorylation of threonine residues in occludin plays a crucial role in the assembly and maintenance of TJs in Caco-2 and MDCK cell monolayers [28]. The assembly and integrity of adherens junctions and the level of TER depend on the phosphorylation of tyrosine residues in Caco-2 cells [29].

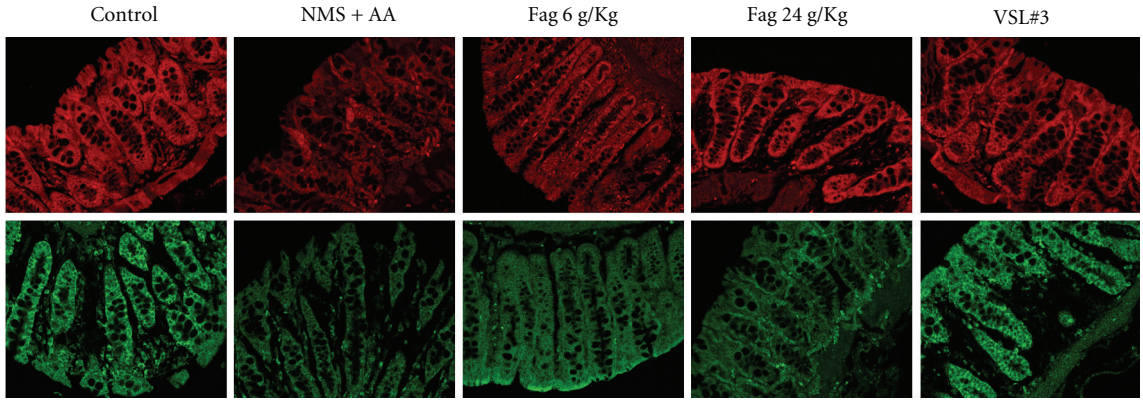

Figure 5: Immunofluorescence localization of ZO-1 (above) and occludin (below) in rat colon mucous membrane. The images were collected by LSCM (×40).

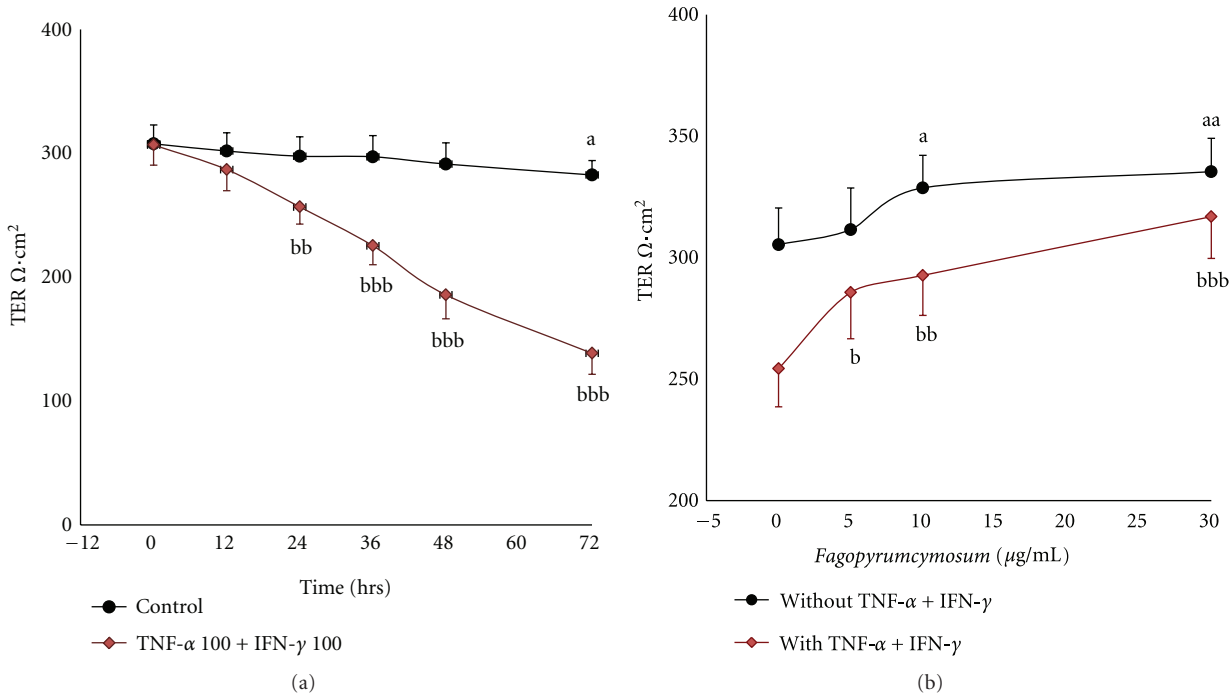

Figure 6: TER of Caco-2 cell monolayers ($\Omega \cdot cm^2$). (a) Effects of treatment time of cytokines: measured at 0, 12, 24, 36, 48, and 72 h after incubating the cells with or without cytokines (100 ng/mL TNF-α and 100 ng/mL IFN-γ). All data are expressed as mean ± SD, $n = 6$. $^{a}P < 0.05$, controls without cytokines: 72 h versus 0 h; $^{bbb}P < 0.001$, at the same time: cells with cytokines versus controls without cytokines. (b) Effects of drug concentration gradient: measured after administrating the cells with Fag (0, 5, 10, or 30 μg/mL) plus cytokines or removing cytokines during the last 24 h. The values are expressed as mean ± SD, $n = 6$. $^{a}P < 0.05$, $^{aa}P < 0.01$, "a" represents 10 and 30 μg/mL Fag versus 0 μg/mL Fag without cytokines, $^{b}P < 0.05$, $^{bb}P < 0.01$, $^{bbb}P < 0.001$, "b" represents 5, 10, and 30 μg/mL Fag versus 0 μg/mL Fag with cytokines.

Nonphosphorylated occludin concentrates on the basolateral membranes while phosphorylated occludin is mainly distributed on the membranous surface as NP-40-insoluble form, almost whole cell occludin is NP-40 soluble though [30]. Fujibe et al. [31] also confirmed that phosphorylated claudin-1 was one of the main detergent-insoluble constituent in rat lung endothelial cell line RLE. TNF-α upregulated MLCK through initiating NF-κB-mediated response, leading to ZO-1 downregulation and increased colonic epithelial permeability [32, 33]. IFN-γ increased the responses of epithelial monolayers to TNF-α, thus they synergetically disrupted TJs morphology and barrier function via MLCK up-regulation and myosin light chain (MLC) phosphorylation [34, 35]. Sappington [36] and Han [37] found that the ZO-1, ZO-3, occludin protein and ZO-1 mRNA levels decreased after exposing enterocytes to the proinflammatory mixture of TNF-α, IFN-γ and IL-1β.

VSL#3 treatment significantly lowers the visceral hypersensitivity and epithelial permeability of IBS rats, and inhibits the decreased expression of TJs of occludin and ZO-1

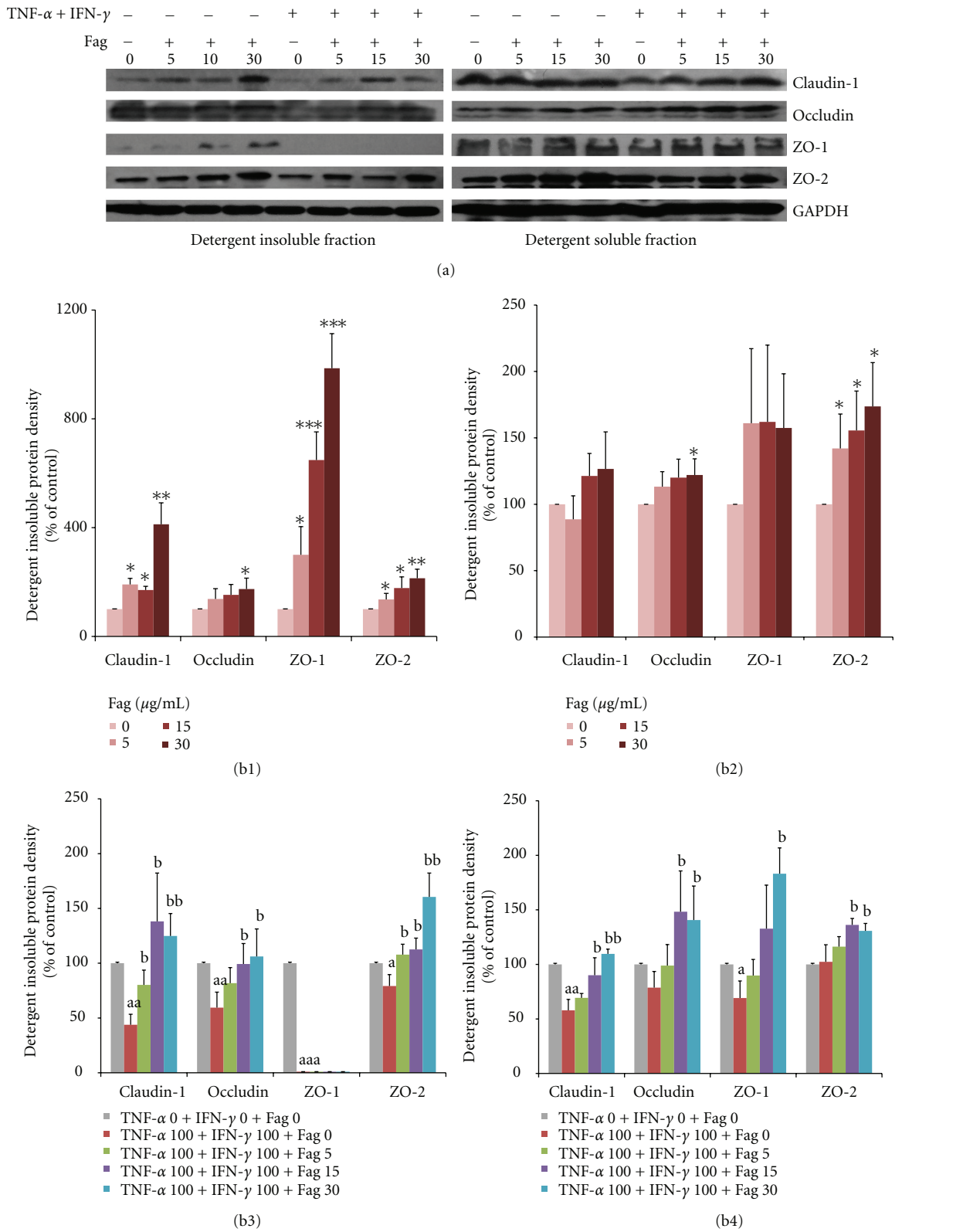

Figure 7: Immunoblot of TJs (claudin-1, occludin, ZO-1, ZO-2) in Caco-2 cells. (a): TJs of ZO-1, ZO-2, occludin, claudin-1 in the NP40-insoluble and soluble fractions of cells incubated with 0, 15, or 30 μg/mL Fag without cytokines or with cytokines (100 ng/mL TNF-α and 100 ng/mL IFN-γ) for 24 h. (b): Relative grey values (% of the controls) of TJs. (b1)-(b3) Detergent insoluble; (b2)-(b4) detergent soluble; (b1) and (b2) were incubated with or without Fag; (b3) and (b4) were incubated with or without Fag and cytokines (100 ng/mL TNF-α and 100 ng/mL IFN-γ). The density values are normalized to those of the corresponding controls. The values are expressed as mean ± SD ($n = 3$). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, the symbols indicate the differences from the controls (Fag = 0); $^{a}P < 0.05$, $^{aa}P < 0.01$, $^{aaa}P < 0.001$, $^{b}P < 0.05$, $^{bb}P < 0.01$, "a" represents TJs in the cells incubated with only cytokines versus controls (TNF-α 0 + IFN-γ 0 + Fag 0); "b" represents TJs in the cells incubated with Fag and cytokines versus those incubated with cytokines alone.

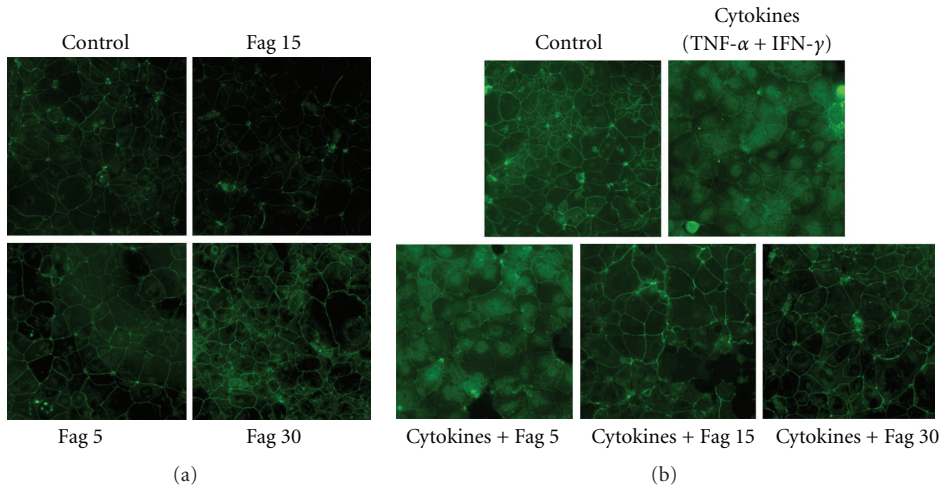

Figure 8: Immunofluorescence of claudin-1 in Caco-2 cell monolayers. (a) The cells were incubated with Fag (0, 5, 15, 30 μg/mL) and stained by claudin-1 for 24 h. (b) The cells were incubated with Fag (0, 5, 15, 30 μg/mL) and cytokines (100 ng/mL TNF-α and 100 ng/mL IFN-γ) or Fag alone for 24 h. The images were collected by LSCM ($\times$200).

[38]. Moreover, probiotics facilitate the redistribution of TJs from the cytoplasm to the membrane [39] and TJs gene expressions [40], compete for adhesion space with intestinal pathogens [41], and antagonize cytokines-induced epithelial barrier dysfunction [42]. In this study, we found that Fag and positive control drug VLS#3 not only relieved the hyperalgesia of IBS rats, but also decreased the levels of TNF-α and IFN-γ, and promoted ZO-1, occluding or claudin-1 expression, which reduced the overall gut PP in a dose-dependent manner. In other words, Fag and VLS#3 integrated the intestinal barrier in IBS rats.

To further investigate the Fag mechanism, we found that *in vitro* TER decreased depending on time during the 24 h of incubation of the monolayer cells with TNF-α and IFN-γ, suggesting the intestinal epithelial permeability was proportional to the duration in which cells were incubated with cytokines. Moreover, cytokines downregulated four target insoluble components of TJs, indicating inhibited phosphorylation and membrane localization. We assumed that cytokines may influence the claudin-1 and ZO-1 of monolayers at the transcriptional level due to the decreased NP-40 soluble components that represent the whole cell protein level. Nevertheless, the total levels of TJs in IBS models all decreased owing to cytokines. The immunoblotting alteration of TJs *in vitro* was not completely consistent with that *in vivo*, which may be attributed to the longer-preserved and more sophisticated colonic inflammatory microenvironment *in vivo* than those *in vitro*. The underlying mechanisms need to be further explored.

Previous research revealed that buckwheat hulls and flour are rich in total flavonoids. Flavanols (including catechins and procyanidins) mainly contain (−)-epicatechin, (−)-epicatechin gallate, and dimeric procyanidin B-2. Flavonoids mainly contained rutin, quercetin and hyperoside [43]. The high-performance liquid chromatography (Supplemental Figure 1 avaliable on at doi:10.1155/2012/983801) reveals that Fag contains four standard procyanidins dimmers procyanidin B-2 (PB2), epicatechin, rutin and quercetin, in which procyanidin B-2 is most abundant. Both PB2 and epicatechin are classified into tannins. Procyanidins potently possess strong antioxidant activity [44] and immunomodulate IL-1β, IL-2, IL-4, IL-5, IFN-γ, and so forth [45, 46]. Green tea polyphenol (−)-epigallocatechin gallate (EGCG) prevents IFN-γ-induced increase of permeability in T84 and THP-1 cells [47]. However, Obara found that (−)-epicatechin gallate (ECG) induced the phosphorylation of protein phosphatase inhibitor by activating PKCδ, thereby inhibiting MLC phosphatase and enhancing MLC phosphorylation [48]. Thus, TJs were internalized due to actomyosin contractility [49]. Quercetin and rutin (quercetin glycosides) are pharmacologically versatile. Rutin normalizes the increased vascular permeability and fragility, prevents vascular edema [50], and lowers the risks of inflammation, lipoperoxidation, and hyperalgesia in biliary obstruction-induced acute pancreatitis [51]. Isoflavone genistein prevents TNF-α-induced TER reduction in colon cell line HT-29/B6, but it does not impact TER *per se* [52]. Suzuki and Hara [53] demonstrated that quercetin augmented the phosphorylation of ZO-2, claudin-1, and occludin in Caco-2 cells by inhibiting PKCζ activity, which led to actomyosin redistribution, thereby refreshing the intestinal epithelial function [54].

The *in vitro* experiments show that Fag increased TER of the monolayer cells depending on concentration, and upregulated detergent-insoluble components in claudin-1, occludin, ZO-1 and ZO-2 with or without cytokines' intervention. We speculated that flavonoids such as quercetin directly intensified the phosphorylation of TJs, via integration, benefited the membrane assembly of TJs and improved intestinal barrier function. Fag might promote the expression

of TJs at transcriptional level because it could sustainably restore the epithelial barrier dysfunction caused by combination treatment with TNF-α and IFN-γ and dose-dependently increase the detergent soluble components components of TJs. Probably, tannins in Fag indirectly protected the intestinal barrier from impairing by immunomodulation and prevented TJs from blocking by cytokines. The reason for the untouched total protein levels of claudin-1 and ZO-1 by Fag in the absence of cytokines is still unclear and merits further studies. So we speculated Fag may exert the direct and indirect role in reducing the colonic epithelial barrier permeability *in vivo*. Fag perhaps further avoided the exposure of intraepithelial nerve plexus and restored sensitizing process due to inflammation, which thus alleviated the visceral hypersensitivity of IBS rats. In a word, Fag may treat IBS via multi-target and multi-channel analgesia.

In summary, our study has demonstrated that Fag soothed the visceral hypersensitivity of model rats, possibly by inhibiting the colonic epithelium inflammation and injury, as well as by facilitating membrane localization and expressions of colonic epithelial TJs which strengthened the colonic barrier. This study herein provides a theoretical basis for Fag-treated IBS due to its multi-target and multichannel pharmacological values.

Conflict of Interests

The authors have no conflict of interests to disclose.

Authors' Contribution

L. Liu and X. Cai contributed equally to this work.

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Research Article

Inhibitory Activities of *Cudrania tricuspidata* Leaves on Pancreatic Lipase *In Vitro* and Lipolysis *In Vivo*

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To identify effective herb to treat obesity, we screened 115 herbal extracts for inhibition of porcine pancreatic lipase (triacylg-ycerol acylhydrolase, EC 3.1.1.3) activity *in vitro*. Of the extracts tested, *Cudrania tricuspidata* leaves exhibited the most pronounced inhibitory effect on lipase activity with an IC_{50} value of 9.91 μg/mL. Antilipid absorption effects of *C. tricuspidata* leaves were examined in rats after oral administration of lipid emulsions containing 50 or 250 mg *C. tricuspidata*/kg body weight. Plasma triacylglycerol levels 2 h after the oral administration of emulsions containing *C. tricuspidata* were significantly reduced compared to the untreated group ($P < 0.05$). These results suggest that *C. tricuspidata* leaves may be useful for the treatment of obesity.

1. Introduction

Obesity is a significant risk factor for increased morbidity and mortality from cardiovascular disease and diabetes; however, it is also associated with many other medical conditions including cancer, liver and kidney diseases, sleep apnea, and depression [1]. The recent National Health and Nutrition Examination Survey showed that 68.0% of those studied were considered overweight (basal metabolic rate (BMI) $\geq$ 25) and 33.8% were obese (BMI $\geq$ 30) [2]. The inhibition of dietary fat absorption is a logical target for managing obesity, and pancreatic lipase is a key enzyme involved in triglyceride absorption in the small intestine. It is secreted from the pancreas and hydrolyzes triglycerides into glycerol and free fatty acids. Thus, inhibitors of digestive lipases are suggested to function as antiobesity agents [3]. Orlistat, which can be found in global markets, inhibits the action of gastrointestinal lipase and thus reduces absorption of dietary fat. However, it has serious side effects, such as steatorrhea, stomach pain, irregular menstrual periods, and headaches [4]. Recently, studies have searched for new lipase inhibitors in natural resources with minimal adverse effects. In a series of investigations to evaluate potential lipase inhibitors derived from plants, researchers showed that certain plant extracts significantly inhibited porcine pancreatic lipase *in vitro* [5, 6]. In this study, as a preliminary evaluation of natural antiobesity products, we tested 115 herbal extracts for inhibition of pancreatic lipase activity *in vitro* and verified the suppression of lipid absorption by *C. tricuspidata* leaves *in vivo*. The fruits of *C. tricuspidata* suppress development of atopic dermatitis in animal model and the roots of it exhibit immunomodulatory and antioxidant activities *in vitro* [7, 8]. These results show that *C. tricuspidata* leaves extracts have on lipase and dietary fat absorptionactivities and may be useful in the treatment of obesity and metabolic disease.

2. Material and Methods

2.1. Plant Materials and Chemicals. Herbs were collected from Republic of Korea from September 2005 to July 2009 and identified by Professor Kim, Division of Life Science, Gachon University, Republic of Korea. Samples

were deposited at the Herbarium of Diabetic Complication Research Team, Korea Institute of Oriental Medicine. Porcine pancreatic lipase (type II), orlistat, and *p*-nitrophenyl butyrate were purchased from Sigma-Aldrich (St. Louis, MO, USA). All reagents were of biochemical grade.

2.2. Animals. Male Wistar rats (6 weeks of age) were purchased from Koatech (Kyungkido, Korea) and housed for 1 week in a 12-h/12-h light/dark cycle in a temperature- and humidity-controlled room. The animals were given free access to food and water. After adaptation to these conditions for 1 week, healthy animals were used in the present study. The Animal Studies Committee of Korea Institute of Orient Medicine approved the experimental protocol.

2.3. Preparation of Herbal Extracts. Dried and ground herbs (200 g) were extracted with 1 L of 80% EtOH 3 times by maceration. The extracts were concentrated and dried *in vacuo* at 40°C. Concentrated extracts were stored at −20°C for further studies. Extracts were dissolved in dimethyl sulfoxide at concentrations that in the total volume (3%) did not affect enzyme activity.

2.4. Measurement of Porcine Pancreatic Lipase Inhibitory Activity. The ability of the herbs to inhibit pancreatic lipase was measured using the method previously reported by Kim et al. [9, 10]. Briefly, an enzyme buffer was prepared by the addition of 6 μL porcine pancreatic lipase solution (Sigma-Aldrich) in buffer containing 10 mM MOPS (morpholinepropanesulphonic acid) and 1 mM EDTA, pH 6.8, to 169 μL Tris buffer (100 mM Tris-HC1 and 5 mM $CaCl_2$, pH 7.0). Then, 20 μL of either the herbal extracts at the test concentration (0, 0.313, 0.625, 1.25, 2.5, 5, 7.5, 10, 50, and 100 μg/mL) or orlistat (Roche, Basel, Switzerland) were mixed with 175 μL enzyme buffer and incubated for 15 min at 37°C with 5 μL substrate solution (10 mM *p*-NPB (*p*-nitrophenylbutyrate) in dimethyl formamide); the enzymatic reactions were allowed to proceed for 15 min at 37°C. Lipase activity was determined by measuring the hydrolysis of *p*-NPB to *p*-nitrophenol at 405 nm using an ELISA reader (BIO-TEK, Synergy HT, Winooski, VT, USA). Inhibition of lipase activity was expressed as the percentage decrease in OD when porcine pancreatic lipase was incubated with the test materials. Lipase inhibition (%) was calculated according the following formula:

$$\text{Inhibition (\%)} = 100 - \left(\frac{B-b}{A-a} \times 100\right), \quad (1)$$

where A is the activity without inhibitor, a is the negative control without inhibitor, B is the activity with inhibitor, and b is the negative control with inhibitor. The results were expressed as an average ($n = 3$).

2.5. Estimation of Plasma Triacylglycerol after Oral Administration of Lipid Emulsion in Rats. Plasma triacylglycerol levels were estimated using the method previously reported by Kim et al. [11]. Rats (7 weeks of age, body weight 190 ~ 230 g) that had fasted overnight were orally administered 3 mL lipid emulsion consisting of corn oil (6 mL), cholic acid (80 mg), cholesteryloleate (2 g), and saline (6 mL) with or without *C. tricuspidata* leaves (at doses of 50 or 250 mg *C. tricuspidata* leaves/kg body weight). Blood was taken from the tail vein at 0, 1, 2, 3, and 4 h after oral administration of the lipid emulsion and centrifuged at 5500 ×g for 5 min to obtain the plasma. Triacylglycerol levels were determined using the Cleantech TS-s kit (ASANPHARM, Seoul, Korea).

2.6. Statistical Analysis. All experiments were repeated three times, and representative data are shown. Data are expressed as the mean ± S.D. Differences between groups were analyzed using a one-way ANOVA followed by the Tukey multiple comparison test (PRISM software, Graph Pad, CA, USA). Values of $P < 0.05$ were considered statistically significant.

3. Results and Discussion

3.1. Pancreatic Lipase Activity of Herbal Extracts. Currently, obesity is considered a global epidemic, and many medications have been studied and developed to treat this condition. However, there is presently only one drug—orlistat—globally approved for long-term treatment of overweight patients after sibutramine was withdrawn in January 2010 from the European market [12, 13]. Although this compound strongly inhibits the activity of pancreatic lipase, which is an important enzyme associated with fat digestion, orlistat may cause serious adverse effects on the gastrointestinal, nervous, endocrine, and renal systems and interferes with the absorption and effectiveness of many drugs and vitamins [4, 14]. Therefore, researching a safe and effective natural inhibitor of pancreatic lipase has been a major target for the development of new drugs to treat obesity [15]. Among them, extracts isolated from natural sources such as *Sorbus commixta*, *Morus bombycis*, *Panax ginseng*, and *Ginkgo biloba* have been reported as potential agents in pancreatic lipase inhibition action [16–19]. Our previous studies have also identified some natural products as new pancreatic lipase inhibitors [11, 18, 19]. In this study, 115 herbal extracts were prepared from selected parts of plants and tested at various concentrations as inhibitors of pancreatic lipase. The lipase inhibitory effects of the extracts are indicated by percentage (%) and IC_{50} values (Table 1). Eighteen extracts had IC_{50} values less than 50 μg/mL, and of these extracts, three samples (i.e., the whole *Solidago serotina* plant, the branches and leaves of *Acer mono*, and the leaves of *C. tricuspidata*) had IC_{50} values less than 10 μg/mL. Notably, *C. tricuspidata* leaves exhibited an IC_{50} value of 9.91 μg/mL (Figure 1).

3.2. Inhibitory Effect of C. tricuspidata on Lipolysis In Vivo. Next, we focused on *C. tricuspidata* on lipolysis *in vivo*. *C. tricuspidata* has been used as an important folk medicine for the treatment of cancer in Korea and has also been used as a traditional medicine for the treatment of hypertension, neuritis, and inflammation in Asia [20–22]. To evaluate the antilipolytic effects of *C. tricuspidata* leaves *in vivo*, we analyzed plasma triacylglycerol levels after oral administration

Table 1: Lipase inhibitory activities of extracts from herbs.

Scientific name	Family	Part used	Conc. (μg/mL)	Inhibition (%)[a]	IC_{50} (μg/mL)
Solidago serotina	Compositae	Whole plant	2.5	41.76 ± 2.48	5.16
			5	49.70 ± 1.44	
			7.5	55.70 ± 1.81	
Acer mono	Aceraceae	Branch, leaf	5	46.17 ± 3.03	7.7
			7.5	48.87 ± 3.09	
			10	53.16 ± 0.93	
Cudrania tricuspidata	Moraceae	Leaf	5	26.55 ± 0.52	9.91
			7.5	38.97 ± 2.92	
			10	50.72 ± 1.05	
Kalopanax pictus	Araliaceae	Bark	10	49.77 ± 1.00	10.51
			50	70.52 ± 1.70	
			100	76.34 ± 0.36	
Cudrania tricuspidata	Moraceae	Branch, stem	5	32.34 ± 2.04	13.8
			10	48.29 ± 1.19	
			50	65.83 ± 0.29	
Oenothera odorata	Onagraceae	Whole plant	10	45.06 ± 1.81	23.34
			50	59.58 ± 0.70	
			100	61.07 ± 0.63	
Platycarya strobilacea	Juglandaceae	Branch, stem	10	45.08 ± 4.01	25.51
			50	56.72 ± 1.74	
			100	61.74 ± 1.26	
Actinidia arguta	Actinidiaceae	Fruit	10	41.62 ± 7.54	26.7
			50	59.30 ± 0.80	
			100	67.23 ± 3.20	
Tilia amurensis	Tiliaceae	Branch, leaf	10	41.72 ± 2.86	28.5
			50	59.26 ± 0.55	
			100	67.17 ± 1.03	
Actinidia arguta	Actinidiaceae	Stem	10	36.79 ± 0.82	28.51
			50	63.38 ± 2.42	
			100	66.84 ± 2.70	
Euscaphis japonica	Staphyleaceae	Branch	20	43.12 ± 4.05	28.62
			30	50.91 ± 1.29	
			40	56.29 ± 2.10	
Actinidia arguta	Actinidiaceae	Root	10	34.08 ± 1.94	31.34
			50	63.93 ± 1.94	
			100	71.03 ± 0.89	
Carpinus cordata	Betulaceae	Branch, stem	10	44.19 ± 3.68	31.39
			50	54.25 ± 1.11	
			100	58.91 ± 1.62	
Rhus sylvestris	Anacardiaceae	Branch, leaf	10	41.57 ± 2.64	32.14
			50	57.23 ± 4.33	
			100	57.43 ± 2.28	

Table 1: Continued.

Scientific name	Family	Part used	Conc. (μg/mL)	Inhibition (%)[a]	IC_{50} (μg/mL)
			10	41.52 ± 1.71	
Celtis sinensis	Ulmaceae	Branch, stem	50	54.56 ± 0.52	35.89
			100	54.09 ± 3.37	
			10	34.40 ± 2.70	
Prunus serrulata	Rosaceae	Branch, leaf	50	53.53 ± 0.62	42.55
			100	56.43 ± 3.18	
			10	28.48 ± 4.40	
Potentilla fragarioides	Rosaceae	Whole plant	50	54.81 ± 2.36	42.58
			100	61.88 ± 1.34	
			10	32.90 ± 4.37	
Tilia mandshurica	Tiliaceae	Flower, leaf	50	51.59 ± 2.07	48.21
			100	52.74 ± 2.30	
			10	19.86 ± 2.15	
Actinidia arguta	Actinidiaceae	Stem, leaf, fruit	50	50.25 ± 2.65	54.09
			100	56.92 ± 2.15	
			10	28.85 ± 6.19	
Hypericum ascyron	Hypericaceae	Whole plant	50	49.57 ± 5.42	56.12
			100	57.57 ± 3.13	
			10	37.15 ± 0.50	
Rhus chinensis	Anacardiaceae	Branch, leaf	50	49.65 ± 0.66	56.9
			100	52.06 ± 1.66	
			10	23.97 ± 2.01	
Picrasma quassioides	Simaroubaceae	Branch, stem	50	48.78 ± 0.80	60.47
			100	54.89 ± 1.38	
			10	26.90 ± 1.18	
Prunus persica	Rosaceae	Branch, leaf	50	48.04 ± 0.94	62.12
			100	56.27 ± 1.46	
			10	12.22 ± 5.84	
Actinidia arguta	Actinidiaceae	Root	50	45.58 ± 3.38	69.17
			100	56.48 ± 1.93	
			10	24.96 ± 2.54	
Spiraea pubescens	Rosaceae	Branch, leaf, flower	50	47.25 ± 3.35	74.62
			100	52.19 ± 1.37	
			10	17.77 ± 3.99	
Tilia mandshurica	Tiliaceae	Branch, stem	50	44.39 ± 2.14	79.67
			100	54.07 ± 2.85	
			10	17.93 ± 2.59	
Acer ginnala	Aceraceae	Branch, leaf	50	43.30 ± 3.02	82.29
			100	53.89 ± 2.92	
			10	20.95 ± 3.37	
Elsholtzia splendens	Labiatae	Root	50	44.64 ± 1.74	83.98
			100	52.58 ± 1.67	

Table 1: Continued.

Scientific name	Family	Part used	Conc. (μg/mL)	Inhibition (%)[a]	IC_{50} (μg/mL)
			10	28.75 ± 5.25	
Staphylea bumalda	Staphyleaceae	Branch, leaf	50	42.55 ± 2.40	84.28
			100	53.45 ± 2.55	
			80	49.17 ± 1.04	
Pinus densiflora	Pinaceae	Stem	90	49.77 ± 3.57	87.58
			100	52.63 ± 2.09	
			10	29.96 ± 8.94	
Machilus thunbergii	Lauraceae	Leaf, branch	50	45.82 ± 0.31	90.9
			100	50.93 ± 0.00	
			10	27.34 ± 8.43	
Deutzia glabrata	Saxifragaceae	Branch, leaf, flower	50	42.85 ± 2.09	91.09
			100	51.51 ± 1.46	
			10	22.19 ± 1.39	
Indigofera kirilowii	Leguminosae	Branch, leaf, flower	50	39.83 ± 0.73	94.98
			100	51.24 ± 1.32	
Opuntia ficus-indica	Opuntiacae	Stem	100	28.17 ± 1.66	>100
Hibiscus syriacus	Malvaceae	Root	100	13.95 ± 0.72	>100
Actinidia arguta	Actinidiaceae	Bark	100	26.02 ± 8.63	>100
Euonymus oxyphyllus	Celastraceae	Branch	100	47.50 ± 0.76	>100
Eucommia ulmoides	Eucommiaceae	Branch, leaf	100	37.76 ± 0.89	>100
Asarum sieboldii	Aristolochiac	Root	100	15.50 ± 5.18	>100
Bupleurum longeradiatum	Umbelliferae	Whole plant	100	34.69 ± 2.52	>100
Plantago asiatica	Plantaginacea	Root	100	−14.66 ± 4.59	>100
Alisma plantago-aquatica	Alismataceae	Root	100	22.03 ± 4.65	>100
Duchesnea chrysantha	Rosaceae	Whole plant	100	36.69 ± 1.07	>100
Cuscuta japonica	Convolvulaceae	Whole plant	100	2.43 ± 1.75	>100
Clematis apiifolia	Ranunculaceae	Stem, leaf, flower	100	−19.96 ± 1.10	>100
Prunus serrulata	Rosaceae	Branch	100	43.47 ± 0.18	>100
Colocasia antiquorum	Araceae	Aerial part	100	−12.08 ± 3.87	>100
Lespedeza cuneata	Leguminosae	Aerial part	100	−8.62 ± 2.65	>100
Lespedeza cuneata	Leguminosae	Root	100	−4.14 ± 1.86	>100
Mallotus japonicas	Euphorbiaceae	Aerial part	100	11.45 ± 3.84	>100
Alisma canaliculatum	Alismataceae	Aerial part	100	16.36 ± 2.85	>100
Alisma canaliculatum	Alismataceae	Root	100	26.99 ± 0.41	>100
Magnolia denudata	Magnoliaceae	Flowers	100	−5.01 ± 2.23	>100
Scopolia japonica	Solanaceae	Stem, leaf	100	−10.52 ± 0.76	>100
Scopolia japonica	Solanaceae	Root	100	−18.32 ± 1.18	>100
Chloranthus japonicus	Chloranthaceae	Whole plant	100	31.04 ± 2.37	>100
Barbarea orthoceras	Cruciferae	Whole plant	100	−27.85 ± 2.32	>100
Caulophyllum robustum	Berberidaceae	Stem, leaf	100	−4.46 ± 3.06	>100
Caulophyllum robustum	Berberidaceae	Root	100	−23.10 ± 6.27	>100
Carduus crispus	Compositae	Stem, leaf	100	30.13 ± 3.47	>100
Carduus crispus	Compositae	Flower	100	44.24 ± 2.47	>100
Styrax japonica	Styracaceae	Flower	100	31.62 ± 4.47	>100
Cornus controversa	Cornaceae	Branch, leaf	100	39.65 ± 5.62	>100
Cornus controversa	Cornaceae	Flower	100	40.45 ± 0.66	>100
Magnolia sieboldii	Magnoliaceae	Branch, leaf	100	4.84 ± 5.72	>100

Table 1: Continued.

Scientific name	Family	Part used	Conc. (μg/mL)	Inhibition (%)[a]	IC_{50} (μg/mL)
Magnolia sieboldii	Magnoliaceae	Flower	100	−7.03 ± 8.14	>100
Prunus persica	Rosaceae	Fruit	100	27.35 ± 1.98	>100
Rhamnus yoshinoi	Rhamnaceae	Branch, leaf	100	43.98 ± 7.76	>100
Erigeron annuus	Compositae	Whole plant	100	26.14 ± 0.86	>100
Styrax japonica	Styracaceae	Branch, leaf	100	27.88 ± 0.97	>100
Quercus aliena	Fagaceae	Branch, leaf	100	45.95 ± 1.73	>100
Callicarpa japonica	Verbenaceae	Branch, leaf	100	11.36 ± 2.56	>100
Ligustrum obtusifolium	Oleaceae	Branch, leaf	100	4.18 ± 1.41	>100
Lindera obtusiloba	Lauraceae	Branch, leaf	100	41.98 ± 1.40	>100
Lespedeza bicolor	Leguminosae	Branch, leaf	100	47.02 ± 2.78	>100
Carpinus laxiflora	Betulaceae	Branch, leaf	100	39.49 ± 5.62	>100
Machilus thunbergii	Lauraceae	Bark	100	36.58 ± 3.17	>100
Hedera rhombea	Araliaceae	Whole plant	100	29.92 ± 0.78	>100
Arenaria serpyllifolia	Caryophyllaceae	Whole plant	100	13.09 ± 1.54	>100
Paulownia coreana	Paulowniaceae	Flower	100	35.25 ± 1.77	>100
Thlaspi arvense	Brassicaceae	Whole plant	100	0.32 ± 0.92	>100
Vicia villosa	Leguminosae	Whole plant	100	28.71 ± 1.94	>100
Descurainia pinnata	Brassicaceae	Whole plant	100	7.88 ± 1.21	>100
Ribes fasciculatum	Saxifragaceae	Branch, leaf, fruit	100	33.67 ± 2.10	>100
Corydalis speciosa	Fumariaceae	Whole plant	100	9.30 ± 3.47	>100
Clematis fusca	Ranunculaceae	Whole plant	100	−1.24 ± 5.89	>100
Deutzia parviflora	Saxifragaceae	Branch, leaf, stem, flower	100	34.77 ± 3.21	>100
Rosa multiflora	Rosaceae	Branch, leaf, stem, flower	100	42.42 ± 0.26	>100
Parthenocissus tricuspidata	Vitaceae	Leaf, stem	100	48.73 ± 1.62	>100
Chelidonium majus	Papaveraceae	Whole plant	100	10.93 ± 1.55	>100
Platycarya stobilacea	Juglandaceae	Leaf	100	47.97 ± 1.14	>100
Platycarya stobilacea	Juglandaceae	Flower	100	46.63 ± 0.54	>100
Carpinus cordata	Betulaceae	Leaf	100	45.84 ± 1.30	>100
Celtis sinensis	Ulmaceae	Leaf	100	40.23 ± 0.47	>100
Orixa japonica	Rutaceae	Leaf	100	−0.19 ± 2.17	>100
Orixa japonica	Rutaceae	Branch, stem	100	15.79 ± 3.07	>100
Orixa japonica	Rutaceae	Fruit	100	25.89 ± 5.92	>100
Picrasma quassioides	Simaroubaceae	Leaf	100	40.51 ± 0.74	>100
Picrasma quassioides	Simaroubaceae	Fruit	100	25.21 ± 2.08	>100
Tilia mandshurica	Tiliaceae	Leaf	100	42.08 ± 1.27	>100
Aralia cordata	Araliaceae	Whole plant	100	32.27 ± 4.39	>100
Viburnum sargentii	Caprifoliaceae	Branch, leaf	100	27.00 ± 1.59	>100
Polygonatum odoratum	Liliaceae	Root	100	36.72 ± 0.40	>100
Astragalus membranaceus	Leguminosae	Root	100	−4.26 ± 0.91	>100
Pleuropterus multiflorus	Polygonaceae	Root	100	−17.48 ± 1.88	>100
Torilis japonica	Umbelliferae	Fruit	100	−20.02 ± 4.86	>100
Phaseolus angularis	Leguminosae	Fruit	100	−58.89 ± 0.70	>100
Phaseolus radiates	Leguminosae	Fruit	100	−98.96 ± 9.06	>100
Artemisia scoparia	Compositae	Aerial part	100	−21.76 ± 3.22	>100
Solanum tuberosum	Solanaceae	Tuber	100	−38.90 ± 4.60	>100
Brassica juncea	Cruciferae	Leaf	100	−34.85 ± 7.98	>100
Arctium lappa	Compositae	Root	100	−38.38 ± 7.90	>100
Cucumis sativus	Cucurbitaceae	Fruit	100	−138.86 ± 0.64	>100

Table 1: Continued.

Scientific name	Family	Part used	Conc. (μg/mL)	Inhibition (%)[a]	IC_{50} (μg/mL)
Diospyros kaki	Ebenaceae	Fruit	100	−136.26 ± 6.37	>100
Artemisia princeps	Compositae	Aerial part	100	12.82 ± 2.47	>100
			0.0005	5.53 ± 3.21	
Orlistat (positive control)			0.005	21.40 ± 10.76	0.036 (0.073 μM)
			0.05	63.19 ± 7.04	

[a] Results are the mean ± SD ($n = 3$).

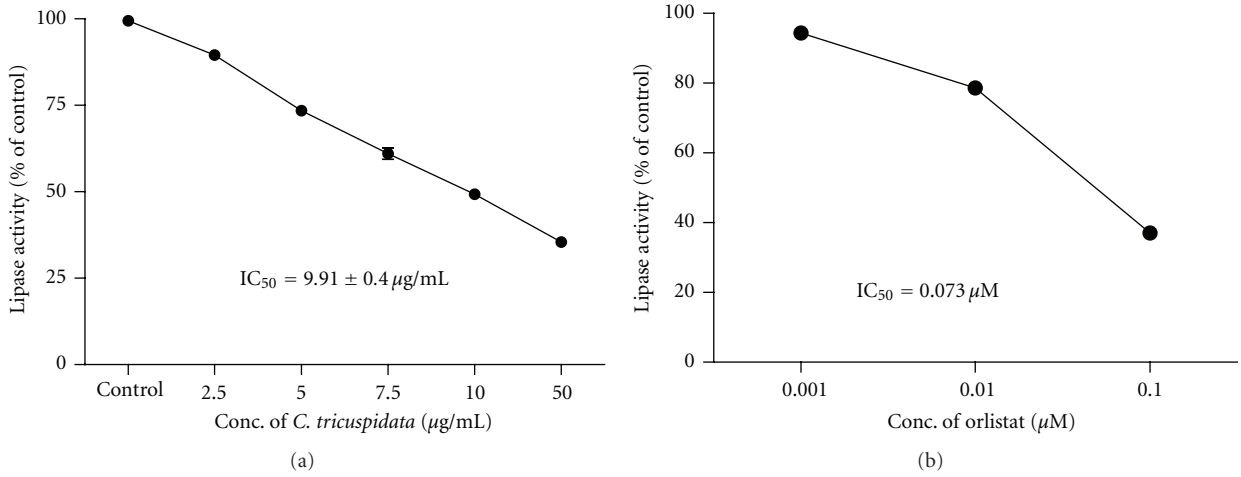

Figure 1: Inhibitory effect of *Cudrania tricuspidata* leaf extract on porcine pancreatic lipase. (a) Porcine pancreatic lipase activity at different concentrations of *C. tricuspidata* leaves. (b) Orlistat was used as a positive control. Data are the mean ± S.D. ($n = 3$).

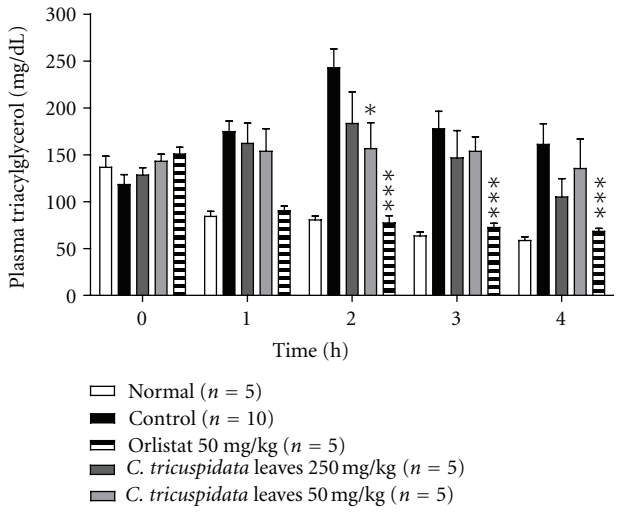

Figure 2: Inhibitory effect of *Cudrania tricuspidata* leaves on rat plasma triacylglycerol levels. Plasma triacylglycerol levels, at the time marked by an asterisk, significantly differ between the control and *C. tricuspidata* (250 mg/kg) groups ($P < 0.05$). Orlistat (a lipase inhibitor) was used as a positive control ($P < 0.001$ versus control).

of lipid emulsions with or without the *C. tricuspidata* leaves to rats. Figure 2 shows plasma triacylglycerol levels after oral administration of lipid emulsion with or without *C. tricuspidata* as a function of time. After oral administration, low concentrations of *C. tricuspidata* (50 mg/kg body weight) reduced plasma triacylglycerol levels and high concentrations of *C. tricuspidata* (250 mg/kg body weight) delayed lipid absorption significantly; however, these effects were weaker than that of the positive control, orlistat.

C. tricuspidata is a rich source of xanthones and flavonoids, including cudraflavone C [23]. A recent study reported that cudraflavone C from *Artocarpus nitidus* inhibited pancreatic lipase activity ($IC_{50} = 17.0 \pm 0.7\,\mu$M) [24]. Thus, cudraflavone C may be a potential as one of active compounds for preventing and treating obesity.

4. Conclusion

In this paper, we screened 115 herbal extracts for inhibition of porcine pancreatic lipase to identify effective herb to treat obesity. *C. tricuspidata* leaves show the most pronounced effect on pancreatic lipase activity and are able to suppress dietary fat absorption *in vivo*. Up until now, *C. tricuspidata* leaves extracts have not been reported on lipase and dietary fat absorptionactivities. Thus, it is worthwhile to further investigate these extracts for their potential pharmacological effect in antiobesity and attempt should be made to characterize phytoactive compounds to be used as safer therapeutic agents in future.

Authors' Contribution

Y. S. Kim and Y. Lee contributed equally to this work.

Conflict of Interests

The authors declare no conflict of interests.

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Research Article

Mozart K.545 Mimics Mozart K.448 in Reducing Epileptiform Discharges in Epileptic Children

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Mozart K.448 has been shown to improve cognitive function, leading to what is known as the Mozart Effect. Our previous work reveals positive effects of Mozart K.448 in reducing epileptiform discharges in epileptic children. In this study, we evaluated the effect of Mozart K.545 and compared the effects with those of Mozart K.448 on epileptiform discharges in children with epilepsy. Thirty-nine epileptic children with epileptiform discharges were included in the study. They received electroencephalogram examinations before, during, and after listening to Mozart K.448 and K.545, one week apart, respectively. The frequencies of epileptiform discharges were compared. There was a significant decrease in the frequency of epileptiform discharges during and right after listening to Mozart K.448 and K.545 (reduced by 35.7 ± 32.7% during Mozart K.448 and 30.3 ± 44.4% after Mozart K.448; and 34.0 ± 39.5% during Mozart K.545 and 31.8 ± 39.2% after Mozart K.545). Spectrogrammatic analysis of the two pieces of music demonstrated that both share similar spectrogrammatic characteristics. Listening to Mozart K.448 and K.545 decreased the epileptiform discharges in epileptic children. This suggests that Mozart K.448 is not the only piece of music to have beneficial effects on children with epilepsy. Other music with lower harmonics may also decrease epileptiform discharges in epileptic children.

1. Introduction

Music has been used to improve physical and mental illnesses. Rauscher et al. first report the "Mozart Effect" in 1993. They note that Stanford-Binet spatial task scores improve immediately after listening to Mozart's Sonata for Two Pianos in D major, K.448 (Mozart K.448) for ten minutes, when compared to the same time of silence or relaxation instruction [1]. Rauscher suggests that cognitive processing is improved by listening to Mozart's music. Subsequent studies demonstrate the beneficial effects of listening to music for many neurologic diseases, including Parkinson's disease, senile dementia, and sleep disorder [2–4]. Regarding epilepsy, Hughes et al. and our previous study show that the epileptiform discharges decrease when listening to Mozart K.448 in patients with epilepsy [5, 6]. In addition, our study shows that harmonics are associated with decreasing epileptiform discharges. However, whether Mozart K.448 is the only piece of music that can effectively reduce epileptiform discharges remains unclear. In the present study, we used another piece of Mozart's music, Mozart Piano Sonata No. 16 in C major (Mozart K.545), with similar harmonics to Mozart K.448, to study the role of the harmonics of the musical stimulus in reducing epileptiform discharges. We analyzed the relationships between the decrease in epileptiform discharges with the foci of epileptiform discharges, mentality, state of wakefulness, epileptic etiology, seizure type, and gender.

Table 1: Profile comparison between patients effective and noneffective in decrease epileptiform discharges during Mozart K.448 and K.545 exposure.

	K.448 Effective	K.448 Noneffective	*P* value	K.545 Effective	K.545 Noneffective	*P* value
Sex						
Male (%)	13 (68.4)	6 (31.6)	0.915	15 (79)	4 (21)	0.113
Female (%)	14 (70)	6 (30)		11 (55)	9 (45)	
Mentality						
IQ $\geqq$ 70 (%)	23 (71.9)	9 (28.1)		22 (68.8)	10 (31.2)	
IQ < 70 (%)	3 (60)	2 (40)	0.722	3 (60)	2 (40)	0.813
Undetermined (%)	1 (50)	1 (50)		1 (50)	1 (50)	
Seizure type						
Generalized (%)	16 (84.2)	3 (15.8)	0.048	16 (84.2)	3 (15.8)	0.024
Focal (%)	11 (55)	9 (45)		10 (50)	10 (50)	
Classification						
Idiopathic (%)	21 (72.4)	8 (27.6)	0. 092	20 (69)	9 (31)	0.826
Probably symptomatic (%)	0 (0)	2 (100)		1 (50)	1 (50)	
Symptomatic (%)	6 (75)	2 (25)		5 (62.5)	3 (37.5)	
Conscious state						
Awake (%)	17 (70.8)	7 (29.2)	0.784	16 (66.7)	8 (33.3)	1.000
Sleep (%)	10 (66.7)	5 (33.3)		10 (66.7)	5 (33.3)	

2. Patients and Methods

2.1. Subjects. Thirty-nine Taiwanese children (19 boys and 20 girls) diagnosed with epilepsy were enrolled. The mean age of these children was 7 years 3 months ±3 years 5 months (ranging from 2 years 9 months to 17 years 3 month). The diagnosis of epilepsy was made according to the criteria established by the International League Against Epilepsy (ILAE). Informed consent was given by a family member or legal guardian in each case. This study was approved by the Institutional Review Board of Kaohsiung Medical University Hospital.

2.2. Electroencephalogram Examinations. The patients in this study received electroencephalogram (EEG) examinations with three sections of parallel periods; before, during, and after listening to Mozart K.448 (8 min 22 sec) and K.545 (9 min 7 sec) in random order, one week apart, respectively. They received 60–70 dB of musical stimuli via loudspeakers [7] that was measured with a decibel meter (DSL332, Taipei, Taiwan). Each EEG was recorded digitally (Harmonie DVN V5.1, Montreal, Canada). Electrodes were placed according to the International 10–20 System. Two neurologists counted the number of discharges in each of the three sections of the experiment. Changes in epileptiform discharge were expressed as (baseline discharge − discharge during/after music/baseline discharge) ×100. Each patient maintained the same state of wakefulness throughout the recording period. We defined an "effective" result as exposure to the music resulting in a reduction of epileptiform discharges by more than 20% (about half the value of one standard deviation of decreased epileptiform discharges in this study).

2.3. Spectrogrammatic Analysis of Mozart K.448 and K.545. Spectrogrammatic analyses of Mozart K.448 and K.545 were performed with the MATLAB program (Mathworks, Inc., MI, USA). Short-time Fourier transformations of the time signals were computed to generate the time series of spectra (spectrogram). A hamming window was used to truncate 100 s of time data, which was sampled at a rate of 44.1 kHz for each spectrogram. The frequency resolution for the analyzed 20 kHz frequency range was 1 Hz.

2.4. Statistical Analysis. Data are shown as means ± SD. Differences in the distribution of effective and noneffective results were calculated using the chi-square test. The two-sample *t*-test and ANOVA were used to compare the percentages of epileptiform discharge reduction while listening to the music by mentality, seizure type, epileptic etiology, state of wakefulness, and gender. Paired *t*-tests were used to compare epileptiform discharge frequencies before, during, and after listening to the music. Pearson correlation coefficients were used to test the correlations of the effects between the two pieces of music. A *P* value less than 0.05 was considered statistically significant.

3. Results

Thirty-nine patients with epilepsy were recruited for this study (19 males and 20 females). Thirty-two patients demonstrated normal intelligence, five patients had a reduced IQ, and two patients had undetermined IQ levels. The majority of patients ($n = 29$) were idiopathic in etiology, two patients were probably symptomatic, and eight patients were symptomatic (Table 1). None of the patients were suffering

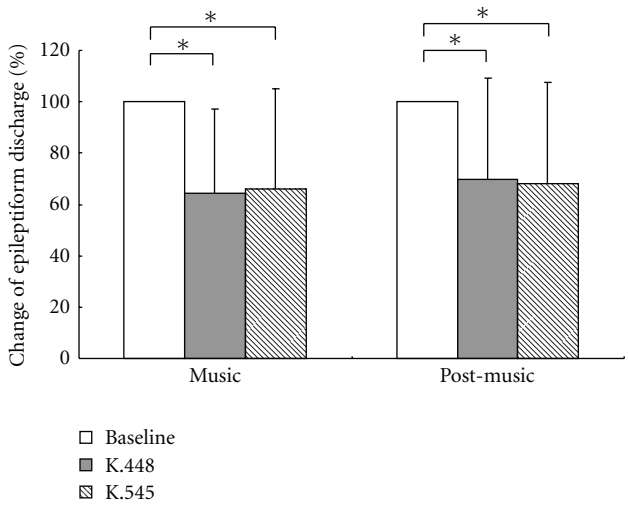

Figure 1: Epileptiform discharges during and after listening to Mozart K.448 and K.545. Comparisons made with baseline EEG (before music). Percentages of the decrease observed in epileptiform discharges in all patients during ($n = 39$) and after listening to Mozart K.448 ($n = 33$) and after listening to K.545 ($n = 34$). $^*P < 0.001$.

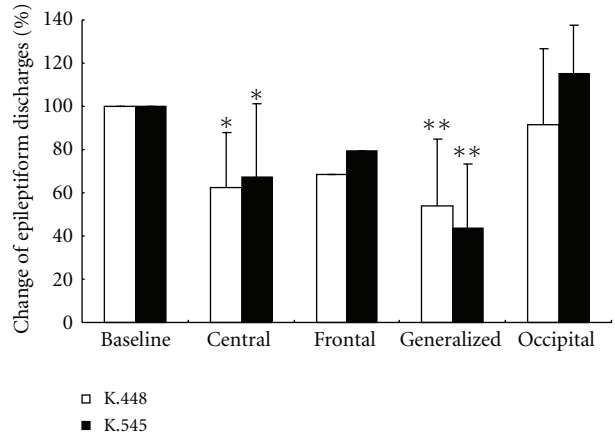

Figure 2: Epileptiform discharge reduction by area of epileptic focus. Comparisons made with baseline EEG (before music) baseline, central ($n = 11$), frontal ($n = 1$), generalized ($n = 19$) and occipital ($n = 8$). $^*P < 0.01$, $^{**}P < 0.001$.

from musicogenic epilepsy or active seizures at the time of study.

3.1. Change of Epileptiform Discharge during and after Music Exposure. Since the average epileptiform discharge frequency in each patient before music listening was highly variable, ranged from 1.1/min to 88.7/min (average 20.9 ± 22.5/min), and 0.4/min to 85.2/min (average 16.9 ± 22.7/min) before Mozart K.448 and Mozart K.545, respectively. The changes of epileptiform discharges were expressed as percentage of reduction. There was a significant decrease in the frequency of epileptiform discharges during listening to Mozart K.448 and K.545 (35.7 ± 32.7% reduction during Mozart K.448 and 34.0 ± 39.5% reduction during Mozart K.545). Thirty-three and thirty-four patients maintained the same stage of sleep or wakefulness during EEG examinations after the conclusion of Mozart K.448 and K.545, respectively. After excluding data from the six and five patients who changed their wakefulness state, the reductions of epileptiform discharges were significant right after listening to Mozart K.448 and K.545 (30.3 ± 44.4% a reduction after Mozart K.448 and 31.8 ± 39.2% after Mozart K.545) (Figure 1). Most patients (84.6% and 82.1% during Mozart K.448 and K.545, resp.) demonstrated decreased interictal discharge frequencies when listening to either piece of music. The patients with generalized epilepsy had more effective results than those with focal seizures when listening to Mozart K.448 and K.545 ($P = 0.048$ and 0.024, resp.) (Table 1). However, six and seven patients had an increase in interictal discharge frequencies in the Mozart K.448 and K.545 music group, respectively. Most of them (66.7% and 71.4%) had occipital discharges. There was no significant difference in the change of epileptiform discharges between patients who were awake and asleep during the EEG recordings. In addition, there were no significant differences in epileptiform discharges by gender, epilepsy etiology, and IQ (Table 1).

When the epileptiform discharge foci in patients were considered, eleven showed central origin, one had frontal origin, nineteen demonstrated generalized discharges, and eight had occipital origin. The average reductions of epileptiform discharges in different foci when listening to Mozart K.448 and K.545 were 37.6 ± 25.2% and 32.7 ± 34.1% from central origin; 31.4%, and 20.4% from the frontal origin; 46.2 ± 31.1% and 56.1 ± 29.3% from generalized discharges; and 8.8 ± 35.7% and −15.2 ± 22.3% from occipital origin, respectively. Compared with the baseline data before music stimulation, the reductions were significant in patients with central and generalized discharges for both pieces of music (Figure 2).

3.2. Spectrograms and Correlations of the Reduction in Epileptiform Discharges between Mozart K.448 and Mozart K.545. Although Mozart K.448 has a more complex music structure and time variation than K.545, both still share similar spectrogrammatic characteristics (Figures 3(a) and 3(b)). The similarities are particularly noteworthy at lower frequencies, where both concentrate more energy in the fundamental frequencies and the lower harmonics, and both peak at around 1 kHz. Significant correlations in decreased epileptiform discharges were observed between Mozart K.448 and K.545 during (correlation coefficient = 0.337, $P = 0.036$) and right after (correlation coefficient = 0.531, $P = 0.002$) listening to the music (Figures 4(a) and 4(b)).

4. Discussion

There are a number of recent studies reporting the positive effects of music in patients with neurological diseases. Patients with migraines receive 12 weeks of music-aided

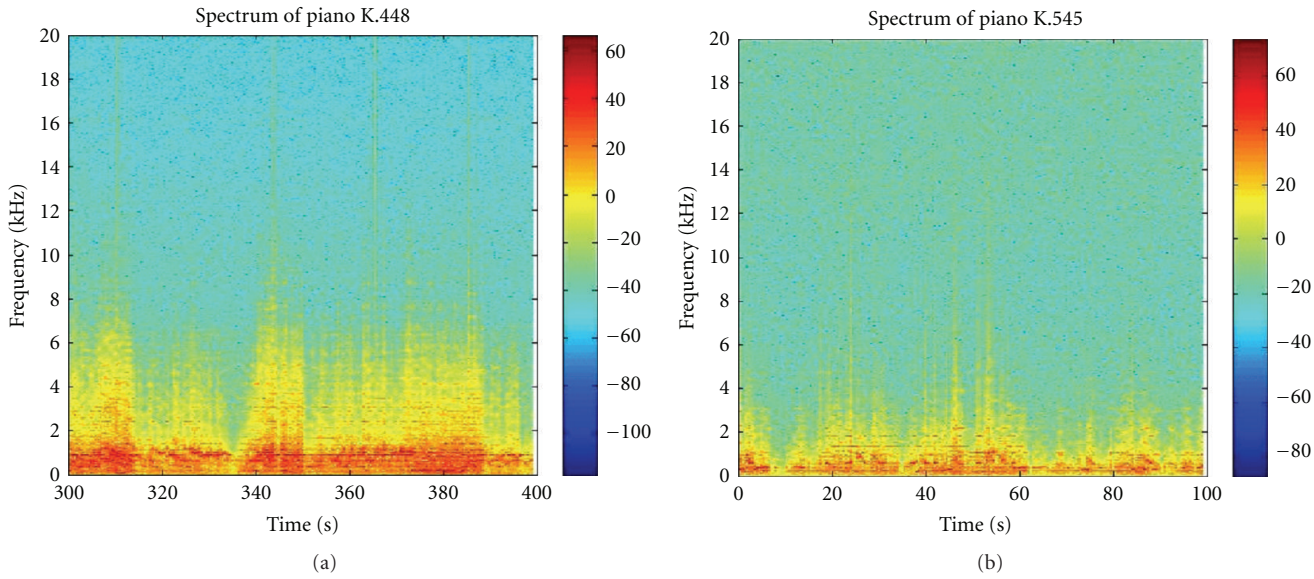

Figure 3: Spectrogram analysis for Mozart K.448 and K.545. The spectrograms showed similar low frequency harmonics during one section of Mozart K.448 (a) and K.545 (b). Data were averaged in 5-second periods in the middle (300 s) of the first movement of K.448, and initial (0 s) of the first movement of K.545.

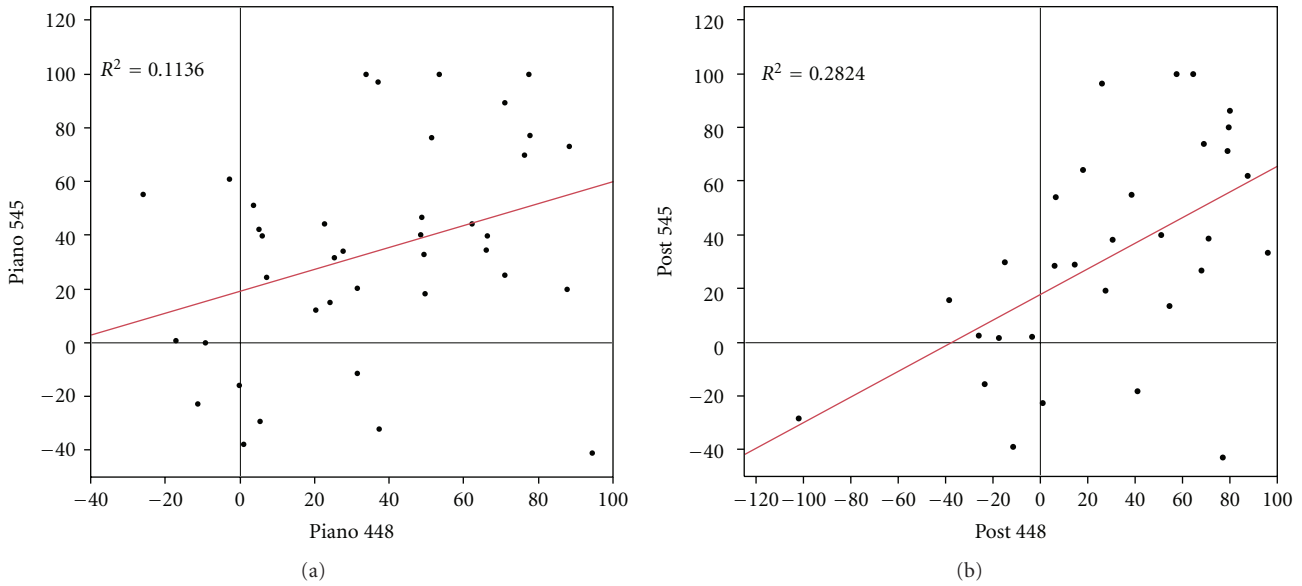

Figure 4: Correlation of reduction of epileptiform discharges between Mozart K.448 and K.545. There was a significant correlation in epileptiform discharge reduction between Mozart K.448 and K.545 during (a) and right after (b) listening to the music.

relaxation training and demonstrate a significant reduction in headache frequencies, suggesting a possible mechanism of relaxation [8]. Patients with middle cerebral artery stroke show a significant improvement in recovery of verbal memory and focused attention after listening to their favorite music for two months [9]. It is postulated that the enhanced cognitive recovery is possibly the result of positive emotions induced by the music. Another study shows that short-term, self-selected music improves aim and line tracking in patients with Parkinson's disease [10]. Our previous studies show that listening to Mozart K.448 reduces epileptiform discharges as well as seizure frequencies [6, 11]. It is clear, therefore, that music has positive effects on several neurological diseases.

Musical characteristics including rhythm, melody, texture, form, tone color, and tonality may play roles in the beneficial effects of music. Hughes reports that the long-term periodicity of the melody line in Mozart's music has a significantly higher value when compared with music by Wagner and three other composers. Hughes suggests this may be the reason why Mozart's music reduces epileptiform

discharges [12]. Zhao and Chen report that the valence of music melodies, rather than the mood it induces, may be the mediator in the pain-relief effect of different pieces of music [13]. Our previous study shows that listening to string K.448 with higher harmonics does not decrease epileptiform discharges when compared with the patients who listen to piano K.448 [6]. In our current study, we hypothesized that the similar lower harmonics in Mozart K.448 and K.545 may be the crucial factor responsible for the reduction in epileptiform discharges. In this study, we confirmed that both Mozart K.448 and K.545 showed significant correlations in the reduction of epileptiform discharges.

Although the human auditory cortex is found in the temporal area, listening to music is a complex process for the brain, since it triggers a sequence of cognitive and emotional responses [14]. Neural activity associated with listening to music extends beyond the auditory cortex. It involves a wide-spread bilateral network of frontal, temporal, parietal, and subcortical areas associated with attention, semantic and music-syntactic processing, memory, motor functions, even extending to the limbic and paralimbic regions related to emotional processing [15–23]. Since our results demonstrated that the greatest decrease in epileptiform discharges occurred in patients with generalized and central discharges, the results are clearly not limited to an effect in the auditory cortex.

In this study, most patients who had increase in epileptiform discharges during music stimulation demonstrated the epileptic foci of occipital origin. The reason patients with occipital foci did not have significant decreases or even increases in epileptiform discharges after music listening remains unclear. On the basis of the evidence from mirror neuron studies, when musical stimuli enter the temporal cortex, areas of the ventral premotor cortex and areas of the inferior parietal lobule are activated and considered to form a frontoparietal mirror neuron system [24]. The results from this study suggested that the occipital cortex did not appear to be involved in this auditory network.

There were no significant differences in gender, epileptic etiology, and state of wakefulness in this study, which we also report in our previous study [6, 25] and is noted in other studies. In addition, Dureau reports no significant gender differences in heart rate and behavioral state after listening to music for 3 minutes [26]. Significant differences were not found when analyzing IQ levels in our study. Research indicates that music therapy is a useful therapeutic approach, regardless of mentality, and is commonly used with mentally retarded children or adults. Our previous study shows that IQ levels were not associated with the reduction of epileptiform discharges in epileptic children when listening to Mozart K.448 [6]. In children with Rett syndrome, active music therapy improves fine motor tasks and social behavior [27], and in a study by Heal and O'Hara, handicapped women with Down's syndrome show improvement in their relationship to the external world and in anorectic behavior after music therapy [28].

Recently, several theories have been introduced regarding the effects of sound on the brain. It is reported that poor health is associated with lower parasympathetic tone in several medical conditions, including epilepsy [29]. Mukherjee et al. report that lower parasympathetic tone, lower parasympathetic reactivity, and more severe dysautonomia are found in patients with intractable epilepsy than in those with well-controlled epilepsy [30]. One study shows that a two-hour music intervention in cancer patients increases their relaxation scores and parasympathetic activities [31]. Another study shows that forty-five minutes of music therapy once per week in patients with cerebrovascular disease enhances parasympathetic activities and decreases congestive heart failure events by reducing plasma cytokine and catecholamine levels [32]. It is possible that musical enhancement of parasympathetic tone may account for the beneficial effects on epilepsy.

Neurotransmitter pathways may also be involved in the effect of Mozart K.448 and K.545 on epilepsy. Musical exposure is known to increase the expression of dopamine levels in the brain [33]. In recent years, dopamine is reported to play a crucial role in the pathophysiology of epilepsy. The reduced binding capacity of dopamine receptors in the basal ganglion is hypothesized to contribute to seizures in autosomal dominant frontal lobe epilepsy and juvenile myoclonic epilepsy [34]. In a recent animal study, the authors report that pilocarpine induces seizures by altering the affinity of dopaminergic receptors in striatal and hippocampal areas, facilitating the propagation and maintenance of seizures [35]. It is possible that listening to music modifies the dopaminergic pathways contributing to the beneficial effects in epilepsy therapy.

5. Conclusions

In conclusion, listening to Mozart K.448 and K.545 decreased the epileptiform discharges in children with epilepsy. The effect was most pronounced when the discharges originated from the central cortex or were generalized. Our findings suggest that Mozart K.448 is not the only piece of music to have beneficial effects on children with epilepsy, and that listening to Mozart K.545 with similar lower harmonics can decrease epileptiform discharges in epileptic children as well.

Conflict of Interests

None of the authors has any conflict of interests to disclose.

Acknowledgments

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Research Article

Repurposing of Yunnan Baiyao as an Alternative Therapy for Minor Recurrent Aphthous Stomatitis

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The study was designed to evaluate the efficacy and safety of an herbal extract of Yunnan Baiyao formulated in toothpaste as an alternative therapy for minor RAS. A randomized, double-blind, placebo-controlled clinical trial (from March 2010 to March 2011) was conducted on a cohort of 227 minor RAS patients. The toothpaste containing Yunnan Baiyao was used twice daily as part of the patient's routine oral hygiene for 5 days. An assessment of ulcerative size and pain was recorded on day 0 (baseline), day 3, and day 5. Any noted adverse reactions were recorded. All data were analyzed using the SAS software 8.0. As a result, the toothpaste containing Yunnan Baiyao began to present noticeable effectiveness on ulcer healing (ulcer size) by day 3 (27.5% versus 15.8%, $P < 0.05$), which further improved by day 5 when compared to the placebo (66.4% versus 50.0%, $P = 0.01$). A significant difference in alleviating pain was noted on day 5 for those who used the toothpaste containing Yunnan Baiyao (66.4% versus 51.8%, $P < 0.05$). No side effects were noted as a result of the Yunnan Baiyao. Therefore, Yunnan Baiyao may provide an alternative therapy for minor ulcers by promoting healing.

1. Introduction

Recurrent aphthous stomatitis (RAS) is a common oral disorder with prevalence of 25% [1], characterized by recurrent ulcers on unkeratinized oral mucosa. Three clinical types were classified as minor, major, and herpetiform [2]. Minor aphthous represents 80–85% of RAS, presenting with 3–10 mm painful ulcers in diameter up to 5 lesions concurrently and usually lasting for 10–14 days each [3]. Currently, there is no definitive curative treatment for RAS, given the unclear etiology, but the consensus recommendation is to lessen the pain and duration of ulcers by suppressing the local immune response and preventing secondary infection [1]. Therefore, topical agents including corticosteroids, antimicrobials, and analgesics make up the first choice for RAS patients due to the minimum serious adverse effects [4]. Topical corticosteroids such as triamcinolone acetonide, prednisolone, and betamethasone [5] are the major remedies that are available and have helped in reducing local inflammation and hastening the healing process. Chlorhexidine and tetracyclines, being antimicrobials, provide another local anti-inflammatory approach for RAS [1]. However, with the prolonged and frequent exposure to certain topical corticosteroids or antimicrobials, relevant drug resistance, oral flora imbalance, and secondary fungal infection may lead to the prescription of more potent therapeutic agents and even systemic administration [6]. Consequently, more potential risks will arise for health such as adrenal suppression, myelosuppression, and even inducing neoplasm.

In this study, we suggest that an herbal medicine approach may offer an alternative therapy for minor RAS. Yunnan Baiyao is a well-known traditional Chinese medicine, formulated in a powder or capsule form. It was initially and widely used in wounds for its antihemorrhagic hemostatic function [7, 8] and further in gastrointestinal bleeding. Yunnan Baiyao powder has been generally applied on RAS among Chinese population [9]. However, because of the absence of reliable clinical data, together with relevant non-English publications, there is limited widespread recognition on its clinical utility. In this study, we conducted

a randomized, double-blind, placebo-controlled clinical trial that included 227 patients with minor RAS to assess its efficacy and safety when formulated in toothpaste.

2. Materials and Methods

2.1. Ethical Approval. Ethical approval for this study was obtained by Peking University Institutional Review Board. Each patient agreed and signed the informed consent prior to the study. The study design and protocol were in accordance with both the Helsinki Declaration and related Chinese regulatory laws in reference to conducting clinical trials.

2.2. Participants. A randomized, double-blind, placebo-controlled study was performed in three stomatology clinical centers: School and Hospital of Stomatology, Peking University (Peking Center); Stomatological Hospital, Capital Medical University (Capital Center); Stomatological Hospital, Nankai University (Nankai Center). A total of 240 patients were enrolled in the study. The breakdown from the sites is as follows: 84, 78, and 78 patients were from Peking, Capital, and Nankai Centers, respectively. Patients enrolled into the study had a verified history of at least two episodes of RAS in the past 12 months without any definitive cause. Below are the inclusion and exclusion criteria.

Inclusion criteria: (1) both male and female aged 18 to 65 years old; (2) patients diagnosed as minor recurrent aphthous stomatitis with the duration of each ulcer in excess of 5 days; (3) fresh ulcers available with less than 72 hours eruption.

Exclusion criteria: (1) hypersensitive to various medical agents; (2) concurrent acute infectious disease; (3) pregnancy or lactation; (4) concurrent other immunology disorders; (5) accepting systemic administration of corticosteroids or immunosuppressive agents within 3 months; (6) aphthous-like ulcers related to certain systemic disorders such as ulcerative colitis, Crohn's disease, Behçet's syndrome, and serious anemia; (7) aphthous-like ulcers related to drug such as nonsteroidal anti-inflammatory drugs (NSAIDs) and antihistamines; (8) accepting anaesthetic therapy within 24 hours, systemic antibiotics within 2 weeks, or other management for oral ulcers within 72 hours prior to the study; (9) neoplasm patients; (10) volunteers of other clinical trials on medical agents or toothpaste within one month.

2.3. Materials. Experimental toothpaste (120 grams weight prepackaged; offered by Yunnan Baiyao Group Co., Ltd., China) contained 0.65% (about 0.78 gram) of the active extract from Yunnan Baiyao, along with sodium phosphate dibasic dehydrate, sorbitol, hydrated silica, sodium lauryl sulfate, flavoring essence, pectin, and sodium benzoate. Placebo toothpaste (120 grams weight prepackaged; offered by Yunnan Baiyao Group Co., Ltd., China) contained the above ingredients, except for the active Yunnan Baiyao extract, though it exhibited similar color, odor, and flavor to the experimental toothpaste.

2.4. Randomization. Both experiment and placebo toothpastes (1 : 1 allocation ratio) were randomized using a computer-based random number generator and distributed to the centers. The randomized list was sealed, and its contents were recorded in the protocol which was conserved by the third party (an assigned statistical company). Both patients and investigators were blinded until the end of study.

2.5. Study Intervention. The patients were instructed to brush the teeth twice daily (in the morning after getting up and before going to bed) for 5 days, using 1 gram of toothpaste provided (equivocal to approximately 6-7 lines on a standard toothbrush) for 3 minutes, while other analogues were forbidden. The ulcer size and pain level were measured and recorded at the respective stomatology clinics on days 0 (baseline), 3, and 5 by two assigned independent clinical investigators. Any oral mucosal complications and side effects during the study as a result of the toothpaste, as well as body temperature, pulse, respiration, and blood pressure, were recorded at the time of the visit, accompanied by a detailed oral examination. If the ulcer disappeared within the 5-day study period, the experimental and/or placebo toothpaste was collected.

Prior to the study, all assigned clinicians from the different clinical centers were trained by the principal investigator as to the standard operating procedures, which included measuring the ulcers, conducting the visual analog scale (VAS), and recording/documenting the data. To calibrate the values measured by different investigators, a kappa statistical analysis was performed on ten patients ($\kappa = 0.8$). A standard brushing duration was enforced, and calibrated timers were given to the participants of the study. The study was standardized in reference to toothpaste consumption, where 2 grams were recommended per day for each patient, and patients who used less than 80% or more than 120% of the 2 grams per day of toothpaste were removed from the study. The daily consumption of the toothpaste was calculated by weighing the remainder of the toothpaste at the end of the study.

2.6. Clinical Evaluation. The fresh ulcers, as described as being developed within 72 hours of onset and must be clearly visible and accessible to the investigators, were documented. The assessment of the surface area of the ulcer was measured in millimeters by a dental probe (Shanghai Dental Instrument Factory, China). Ulcer size was assessed as the product of maximum diameter and its vertical diameter. Pain intensity was measured using a VAS, where the amount of pain recorded ranged from 0 (no pain) to 10 (unbearable pain). Pain was assessed by irritating the ulcer with the periodontal probe. The values were collected by the assigned investigators.

The efficacy index (EI) was measured as a function of either ulcer size index (EI size) or pain intensity index ($\mathrm{EI}_{\mathrm{pain}}$), calculated as follows:

$$\mathrm{EI(size)} = \frac{E0 - (E3/E5)}{E0} \times 100\%,$$
$$\mathrm{EI(pain)} = \frac{E0 - (E3/E5)}{E0} \times 100\%. \quad (1)$$

Table 1: The criteria of response to therapy.

Criteria	Definition
EI = 100%	Either ulcer healed or pain disappeared
EI = 70–100%	More than 70% of either ulcer surface area closed or VAS value decreased, including 70% but not 100%
EI = 30–70%	More than 30% of either ulcer surface area closed or VAS value decreased, including 30% but not 70%
EI = 0–30%	Less than 30% of either ulcer surface area closed or VAS value decreased

$E0$, $E3$, and $E5$ represent the respective data values (either based on ulcer size or pain) that were collected on days 0, 3, and 5. The response to therapy (experimental versus placebo) was broken down to four criteria (Table 1) (ClinicalTrials.gov registration number: NCT01652625).

2.7. Statistical Analysis. Parametric and nonparametric statistical tests were used for the analysis of comparing the ulcer size or pain scale between experimental and placebo groups. The *t*-test was used in the analyses of ulcer size, *Wilcoxon rank-sum* test for categorical data of VAS (pain), and *chi-square* test for the comparison of gender and efficacy index (EI). All data were analyzed using the SAS software 8.0 (SAS Institute Inc., USA). $P < 0.05$ was considered statistically significant.

3. Results

A total of 240 patients were enrolled into the study (from March 2010 to March 2011), and after the start of the study, thirteen patients withdrew due to personal reasons, leaving a total of 227 patients. The patients were randomly placed into one of two groups, experimental (113 patients) or placebo (114 patients) group. At the initial visit (day 0-baseline), no differences were noted based on age, gender, duration of previous ulcers, existing ulcer size, and pain intensity (Table 2). The 227 patients enrolled had compliant for the 5-day study period.

3.1. Effect of Yunnan Baiyao on Size of Ulcer. Observations on the effect of Yunnan Baiyao on ulcer size were assessed for both experimental and placebo groups during day 3 and day 5 using the efficacy index (EI) as described in Section 2. Table 2 provides a summary of these results. On day 3, starting with those that were in the EI_{size} = 100% category, 11 and 6 from the experimental group and placebo group, respectively, were healed fully. In the EI_{size} = 70–100% category, 20 and 12 from the experimental group and placebo group, respectively, had moderate improvement. If we paired EI = 100% and EI = 70–100% categories as considered significant improvement of ulcer and EI = 0–30% and EI = 30–70% as nonsignificant improvement, an obvious between-group difference was identified in the rate of significant improvement relative to the size of the ulcer, as displayed by 27.4% in the experimental group compared to 15.8% in the placebo group ($P < 0.05$). On day 5, 39 patients from the experimental group (34.5%, including the 11 from day 3) fell into the EI_{size} = 100% category, and 31.9% (36/113) of patients fell into the EI_{size} = 70–100%, whereas in the placebo group, 21.5% (24/114) and 28.9% (33/114) fell into the EI_{size}= 100% and EI_{size} = 70–100% categories, respectively. Consequently, a relatively higher rate of significant improvement was exhibited in experimental group than in placebo group (66.4% in experimental group versus 50.0% in placebo group, P = 0.01). Meanwhile, the between-group difference in reference to average size of ulcers was displayed on day 3 (3.4 mm^2 versus 5.3 mm^2, P = 0.01) and further on day 5 (2.1 mm^2 versus 3.7 mm^2, $P < 0.01$) (see Table 3 and Figure 1). The results suggested a potential effectiveness of the active extract from Yunnan Baiyao on reducing the size of ulcer.

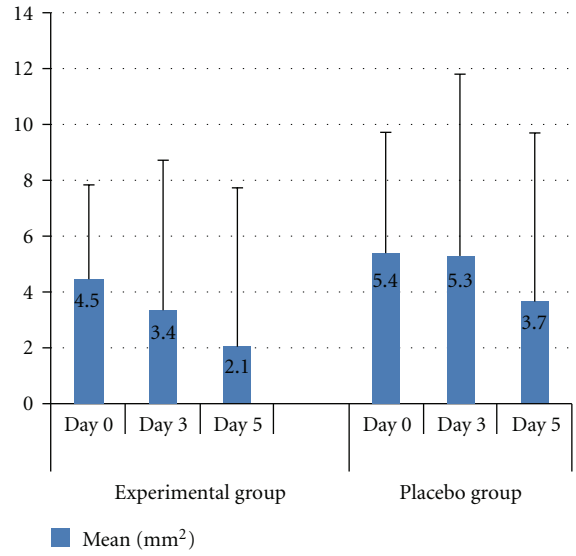

Figure 1: A comparison of ulcer size between experimental and placebo groups for day 0, day 3, and day 5.

3.2. Effect of Yunnan Baiyao on the Levels of Pain. The level of pain was recorded using the VAS for both groups. The measurement was performed on 113 experimental patients and 114 placebo patients. Based on the EI criteria for pain, by day 3, the VAS values have decreased by 24.8% (28/113) from the experimental group for both EI_{pain} = 100% and EI_{pain} = 70–100% categories, as compared to 14.9% (17/114)

Table 2: The baseline demography data of the patients.

	Experimental group ($n = 113$)	Placebo group ($n = 114$)	P
Age (year) (mean ± SD)*	31.9 ± 10.9	31.4 ± 11.8	0.75
Gender**			0.17
Male	22 (19.5%)	31 (27.2%)	
Female	91 (80.5%)	83 (72.8%)	
Duration of previous ulcer (day)*	9.1 ± 3.0	9.4 ± 3.8	0.98
Ulcer size (mm^2) (mean ± SD)*	4.5 ± 3.3	5.4 ± 4.3	0.21
Ulcer pain (VAS)***			0.21
0	4	1	
1	8	6	
2	9	11	
3	23	25	
4	16	9	
5	27	23	
6	9	16	
7	9	14	
8	5	7	
9	2	1	
10	1	1	

VAS: visual analog scale.
*: *t*-test was used in analysis of quantitative data, $P > 0.05$.
**: *chi-square* test was used in analysis of enumeration data, $P > 0.05$.
***: *Wilcoxon rank-sum* test was used in analysis of categorical data, $P > 0.05$.

Table 3: The number of patients in experimental and placebo groups on day 3 and day 5 based on ulcer size.

	Day 3		P	Day 5		P
	Experimental group (%)	Placebo group (%)		Experimental group (%)	Placebo group (%)	
Number	113	114		113	114	
Size (mean ± SD)	3.4 ± 5.3	5.3 ± 6.5	0.01*	2.1 ± 5.6	3.7 ± 6.0	<0.01**
Significant improvement	31 (27.4)	18 (15.8)	0.03***	75 (66.4)	57 (50.0)	0.01****
$EI_{size} = 100\%$	11 (9.7)	6 (5.3)		39 (34.5)	24 (21.1)	
$EI_{size} = 70–100\%$	20 (17.7)	12 (10.5)		36 (31.9)	33 (28.9)	
Nonsignificant improvement	82 (72.6)	96 (84.2)		38 (33.6)	57 (50.0)	
$EI_{size} = 30–70\%$	35 (31.0)	38 (33.3)		16 (14.2)	21 (18.4)	
$EI_{size} = 0–30\%$	47 (41.6)	58 (50.9)		22 (19.5)	36 (31.6)	

* and **: P values represent the comparisons of ulcer size between groups on days 3 and 5, respectively.
*** and ****: P values represent the comparisons of significant improvement and nonsignificant improvement on days 3 and 5, respectively.
( ): it represents percentage per group.

in the placebo group. By day 5, the VAS values had further decreased for majority of the patients for both $EI_{pain} = 70–100\%$ and $EI_{pain} = 100\%$ categories, 66.4% (75/113) and 51.8% (59/114) for the experimental and placebo groups, respectively. An obvious difference was shown by day 5 but not by day 3 (see Table 4).

There were no pathological changes related to body temperature, blood pressure, pulse, and respiration as a result of the study. We noted two patients from placebo group complained of lingual numbness (not related to the study) though, after further followup, they recovered without any medical intervention.

4. Discussion

In this study, we examined the repurposing of a Chinese herbal medicine to alleviate RAS in terms of its efficacy and safety. Yunnan Baiyao is a combination of compounds from a variety of Chinese medical herbs. It has been known to be effective for its unique antihemorrhagic hemostatic function/capacities in the Chinese population [7, 8]. For nearly a century, the recipe remains protected by Chinese government due to intellectual property concerns.

A total of 13 milligrams of the Yunnan Baiyao extract is present in 2 grams of the toothpaste that was designed for

Table 4: The number of patients in the placebo and experimental groups on day 3 and day 5 based on the level of pain.

	Day 3		P	Day 5		P
	Experimental group (%)	Placebo group (%)		Experimental group (%)	Placebo group (%)	
Number	113	114		113	114	
Significant improvement	28 (24.8)	17 (14.9)	>0.05*	75 (66.4)	59 (51.8)	<0.05**
EI_{pain} = 100%	16 (14.2)	6 (5.3)		48 (42.5)	45 (39.5)	
EI_{pain} = 70–100%	12 (10.6)	11 (9.6)		27 (23.9)	14 (12.3)	
Nonsignificant improvement	85 (75.2)	97 (85.1)		38 (33.6)	55 (48.2)	
EI_{pain} = 30–70%	34 (30.1)	43 (37.7)		24 (21.2)	32 (28.1)	
EI_{pain} = 0–30%	51 (45.1)	54 (47.4)		14 (12.4)	23 (20.2)	

* and **: P values represent the comparisons of significant improvement between groups on days 3 and 5, respectively.

adult minor RAS patients in the present paper. By weighing the rest of the toothpaste at the end of the study, the permitted toothpaste consumption was confined to 1.6–2.4 grams daily (within 80–120% of 2 grams). Consequently, there was 0.8 gram difference at most between the minimum and maximum allowed amount, containing 5.2 milligrams of Yunnan Baiyao, in different patients. Along with the similar amount of toothpaste consumption, the standardized brushing approach and brushing duration instructed by the investigators prior to the study, together with the ulcer site accessible to the foam from brushing, were warranted to the same amount of foam from brushing presenting per unit of area of the ulcers. Future studies will be needed to evaluate the dose/responses and titrations of Yunnan Baiyao based on toothpaste amount. The results presented in the study were reliable due to the between-group similarity of ulcer size and ulcer pain level on initial investigation and the duration of ulcers within 72 hours onset.

As a result of the study, by day 3, the effect of Yunnan Baiyao began to display on reducing ulcer size compared to the placebo, as a noticeable decrease on the average size of the ulcer, and by day 5, the size was insignificant. Additionally, by day 3, and day 5, the surface area of ulcer closed by no less than 70% in larger proportion of the patients with Yunnan Baiyao toothpaste than that with the placebo. Apart from the active extract of Yunnan Baiyao, there was no difference between experimental and placebo toothpastes in both ingredients and amount of the components. Therefore, it was suggested that Yunnan Baiyao active extract might play an important role in reference to ulcer healing. Yunnan Baiyao formulated in toothpaste could be recommended as an adjunctive treatment of minor RAS or may even be effective for patients with severe RAS in conjunction with other remedies. The effect of Yunnan Baiyao on ulcer healing based on our study will give impetus to utilizing another approach of a carrier of the extract that would be focused (increased concentration) at the sight of the ulcer which includes an ointment approach or a delivery as in the form of a gel or mouthwash. However, just the toothpaste formulation of Yunnan Baiyao, might hinder more patients (27.4% and 66.4% of the patients on days 3 and 5, resp.) in the present study getting a significant improvement on ulcer size. The foam from brushing teeth limited the duration of Yunnan Baiyao extract contacting with the surface of lesions, and subsequently the penetration towards subepithelial layer infiltrated with amounts of inflammatory cells. With the closing of more ulcers, pain from the ulcers disappeared. The effectiveness of Yunnan Baiyao toothpaste on pain, exhibited in the present study on day 5, might be the result of healing of ulcer, but not directly related to the analgesic function.

RAS is an etiologically complex disorder and considered to be the common manifestation of a group of disorders with different causes, rather than a single entity [10]. Immune mechanism including local immune may involve in the development of ulcers, which was supported by the histological infiltration of neutrophils, lymphocytes, and plasma cells in the epithelium affected [11]. Various cytokines may contribute to the pathogenesis of ulcers [12], as elevated IL-2, IFN-γ, and TNF-α, while lower concentrations of IL-10 were reported in lesions of RAS patients [13, 14]. Although the exact mechanism is unknown, the effect of Yunnan Baiyao on ulcer size in our study might be attributable to its role in the anti-inflammatory or immunosuppressive pathways, hence accelerating the healing process. The property of Yunnan Baiyao in promoting intestinal mucosal cell spreading, confirmed by more extensive lamellipodia at the leading edges of the wounds [15], may benefit the healing of oral ulcers starting from the periphery. Moreover, the immunosuppressive activity of Yunnan Baiyao was demonstrated by a highly selective cytotoxicity towards B and T lymphocytes and an inhibitory ability on the expressions of TNF-α and IFN-γ in colonic mucosa and serum of experimental colitis mice, discovered lately by Li and his colleagues [15]. The upregulated level of TNF-α in oral lesions was considered to play an important role in RAS eruption, which is produced by gamma-delta T cells [16], macrophages, and mast cells [17] and may induce inflammation by its chemotactic action on neutrophils [18]. Thus, as a final therapeutic strategy, a group of anti-TNF-α agents were employed by oral clinicians for severe and refractory RAS patients, including pentoxifylline, thalidomide, adalimumab, and infliximab [1] and eventually obtained therapeutic success in shortening the duration of ulcer and lengthening the free ulcer course. Therefore, Yunnan Baiyao may be a potential alternative anti-TNF-α agent for RAS.

Ulcers in RAS patients were generally exposed to plenty of microorganisms which can induce and promote the

inflammatory reaction. Protecting the lesions against microbes may benefit the healing of these ulcers. Although the antimicrobial activity of Yunnan Baiyao on inflammatory bowel disease was denied by Li et al. for its ineffectiveness on *E. coli* growth [15], the effect of Yunnan Baiyao on RAS-related microbes was not detected in this study.

In our previous results (not shown), 21.6 mg/kg/day of Yunnan Baiyao active extract was administered intragastrically in Sprague-Dawley rats (SD rats) (170–210 grams weight) for a duration of 30 days. The extract was diluted into 2 milliliters of water and provided to the rats by rat stomach-cleaning apparatus. Based on the human equivalent dose (HED) conversion formula recommended by FDA [19], 13 milligrams of Yunnan Baiyao extract permitted daily in present study was far more less than the HED of 222 milligrams for an adult patient weighing 70 kilograms. The safety evaluation of Yunnan Baiyao on adult RAS patients, whose oral mucosa interacts with a maximum dose of 13 milligrams of Yunnan Baiyao for a total of 6 minutes daily for 5 days, was assessed by monitoring body temperature, blood pressure, pulse, and respiration, as well as any side effects. The absence of pathological changes of the above values observed in the present paper suggested the safety of Yunnan Baiyao for topical use as a toothpaste carrier. The lingual numbness noted by few patients in the placebo group may be as a result of the common constituents of toothpaste, but not related to the Yunnan Baiyao extract.

5. Conclusion

In conclusion, the repurposing of Yunnan Baiyao may have a beneficial effect on healing of minor RAS by short-term topical application without significant side effects, which was demonstrated by our present randomized, double-blind, placebo-controlled clinical trial.

Conflict of Interests

The authors declare that they have no conflict of interests.

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Research Article

Different Effects of Resveratrol on Dose-Related Doxorubicin-Induced Heart and Liver Toxicity

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The aim of the study was to evaluate the effect of resveratrol in doxorubicin-induced cardiac and hepatic toxicity. Doxorubicin was administered once a week throughout the period of 7 weeks with 1.0 or 2.0 mg/kg body weight or concomitantly with resveratrol (20 mg/kg of feed). Heart and liver toxicity was histologically and biochemically evaluated. Resveratrol protected from the heart lipid peroxidation caused by 1 mg doxorubicin and it sharply diminished superoxide dismutase activity. An insignificant effect of resveratrol on the lipid peroxidation level and the superoxide dismutase activity was observed in the hearts of rats administered a higher dose of doxorubicin. However, resveratrol attenuate necrosis and other cardiac histopathological changes were induced by a high dose of doxorubicin. Interestingly, it slightly intensified adverse cardiac histological changes in rats receiving a lower dose of doxorubicin. Resveratrol did not have any protective effect on the hepatic oxidative stress, while exerting a mild beneficial effect on the morphological changes caused by doxorubicin. All in all, this study has shown different effects of resveratrol on dose-related doxorubicin-induced heart and liver toxicity. Resveratrol may modulate the hepatic and cardiac effect of doxorubicin, depending on the drug dose.

1. Introduction

Drug-drug and drug-food interactions are specially important in the anticancer therapy because of the very narrow therapeutic index of these chemotherapeutics. Moreover, many patients suffering from cancer in the terminal state decide to take unconventional agent, very frequently beyond of a doctor knowledge.

Doxorubicin (DOX) is a very efficient antitumor drug, but its administration is limited by a dose-dependent, irreversible, and progressive cardiomyopathy, which may become evident years after completion of the therapy [1–5]. The pathomechanism of DOX-related late cardiotoxicity is multifactorial [6, 7], but the prevalent hypothesis ascribes the dominant role to oxidative stress linked to redox-cycling of the drug [8, 9]. The DOX redox-cycling is started from one-electron reduction with the formation of DOX radical (DOX*) [10]. Many NADPH and NADH-dependent enzymes catalyze that reaction, for example, NADPH cytochrome P450 reductase [9], NOS [11–13], NADPH oxidase [14, 15], and catalase [16]. Subsequently, DOX* are reoxygenated to the nonradical parent compound while at the same time the superoxide anion radical is formed (O_2^{-*}). This cycle of reactions may repeat many times leading to overproduction of O_2^{-*}, which is the source of hydrogen peroxide and much more toxic hydroxyl radical

[6, 17]. These reactive oxygen species (ROS) are responsible for oxidative stress. The above-mentioned enzymes involved in DOX* production are abundant in hepatocytes [18], suggesting that liver may be specially involved in DOX* generation. Although no such intense DOX synthase takes place in the heart, relatively low antioxidant defense of cardiomyocytes makes the heart a target for DOX toxicity [19]. The studies of the last decade have been focussed towards the understanding of the latent period of DOX-mediated cardiomyopathy caused by oxidative stress. Redox cycling transformation occurs in cytoplasm, endoplasmatic reticulum, and specially in mitochondria [20]. Oxidative changes in mitochondrial DNA are responsible for disturbances in mitochondrial protein synthesis. For that reason, the product of the electron transfer chain is not only four electron reduced oxygen (H_2O) but also ROS—one, two, and three electron products—$O_2{}^{-*}$, H_2O_2, and HO*, respectively [21]. On the other hand, the oxidation of mtDNA by ROS appears again. In the beginning, the symptoms during that latent period are not clinically overt [22, 23]. Insufficiency of mitochondria function becomes more visible with the pass of time. For that reason, participation of glycolysis pathway in ATP synthesis arises. Eventually, after months or years, the death of cardiomyocytes [24, 25] can consequently lead to heart failure [26, 27].

Resveratrol (3,5,4′-trihydroxystilbene) is a polyphenol, presents in some plants growing in Mediterranean countries (grapes), Minor Asia (hellebore), and Japan (knotweed), is easy achievable as a diet supplement. Resveratrol has been studied in many clinical trials [28, 29] and has shown anticancer [30–32], cardioprotective [33–35], and antioxidant activity [36–39]. Previous studies demonstrated that resveratrol causes resistance to oxidant injury in rats' neonatal cells (H9c2) [40], and it prevents from cardiomyocytes' cytotoxicity of DOX through mitochondrial stabilization [41].

Thus, resveratrol having antioxidative and anticancer activity can be additionally taken as a supplement by the doxorubicin-treated patient. The aim of this study was to verify the hypothesis according to which resveratrol protects against oxidative injury and pathomorphological changes caused by DOX.

2. Materials and Methods

2.1. Animals and Treatment. The experimental protocol was approved by the Local Bioethical Council of the Medical University in Lublin. The study was conducted on sexually mature male albino rats of Wistar CRL:(WI)WUBR strain, obtained from a commercial breeder (Warszawa-Rembertow, Poland). Animals with the initial body weight of 160–195 g were maintained in stable conditions at 22°C with a 12 h light/dark cycle and given standardized granulated fodder LSM (AGROPOL, Poland). The rats were exposed to doxorubicin (DOX; Ebewe, Austria) and resveratrol (RV, Sigma-Aldrich, USA). The animals were randomly divided into control ($n = 8$) and 4 study groups ($n = 6$): 1DOX and 2DOX where doxorubicin was injected intraperitoneally alone in a dose of 1.0 or 2.0 mg/kg body weight, respectively. In groups 1DOX+RV and 2DOX+RV, doxorubicin was administered according to the same scheme concomitantly with resveratrol given with the fodder LSM prepared by AGROPOL in the concentration 20 mg/kg of fodder (20 ppm). In the control group, a 0.9% NaCl was intraperitoneally injected. Doxorubicin was dissolved with saline 1 : 2 v/v and injected once a week for seven weeks in all study groups. Dietary resveratrol supplementation was started one week before the administration of doxorubicin and followed throughout the study. Since in most of previous studies [42, 43] resveratrol in the selected dose (20 mg/kg) did not show any significant effect on hepatic and cardiac morphology and function, secondary to the suggestion of the local Bioethical Committee the group exposed exclusively to resveratrol was not designated. 96 hours after administration of the last dose of doxorubicin, the animals were anesthetized by the intraperitoneal administration of 1 mg/kg pentobarbital, and the blood was collected from the left cardiac ventricle. Immediately after decapitation, the heart and liver samples were collected during autopsy. The organs were washed with saline and then the heart was sectioned along the interventricular and coronal grove. The wall of the left ventricle and the liver samples were placed in liquid nitrogen and stored at −75°C until the time of biochemical analysis. The right ventricular wall and the liver samples were fixed in buffered 10% formalin and routinely histologically processed to paraffin blocks.

2.2. Serum and Plasma Parameters. The concentrations of both rat heart fatty acid binding protein (H-FABP, Life Diagnostic, USA) and rat brain natriuretic peptide (BNP, Phoenix Pharmaceuticals, USA) were determined, respectively, in rat serum and plasma, using ELISA commercial kits. In both cases, two types of antibodies were used in the evaluation: antibodies covering microtiter plate and the secondary antibodies bound to horseradish peroxidase. The products of the catalytic reactions were spectrofotometrically detected at 450 nm.

Serum activity of aspartate aminotransferase (AST), alanine aminotransferase (ALT), kreatine kinase CK, lactated dehydrogenase (LDH) and alkaline phosphatase (ALP) was determined using standard kits of CORMAY (Poland).

2.3. Cardiac and Hepatic Oxidative Markers. All measurements were conducted on homogenates obtained from ~20 mg of frozen cardiac or hepatic samples using the extraction buffer provided by the manufacturer of each commercial kit. The evaluation of lipid peroxidation in cardiac and hepatic homogenates was based on malondialdehyde and 4-hydroxyalkenals concentration (MDA+4HAE) using the commercial kit (Bioxytech LPO-586; OxisResearch, USA). The concept of the method is based on the reaction between MDA and 4HAE with N-methyl-2-phenylindol. After mixing N-methyl-2-phenylindole and methanol with the supernatant acquired from the homogenization, methanesulfonic acid was added and all reagents were placed at the temperature of 45°C for 60 minutes. Next, the solution was centrifuged and the supernatant containing the product was

Table 1: Serum and plasma markers of heart and liver damage (M ± SD).

	FABP (μg/L)	BNP (μg/L)	AST (IU/L)	ALT (IU/L)	CK (IU/L)	LDH (IU/L)	ALP (IU/L)
Control	5.0 ± 1.59	0.40 ± 0.237	104.3 ± 9.07	57.63 ± 7.11	1536.75 ± 343.61	1275.9 ± 596.29	186.25 ± 31.62
1DOX	7.0 ± 1.97	0.54 ± 0.437	96.0 ± 9.03	68.20 ± 13.65	1099.00 ± 421.51	647.8 ± 179.96	89.80 ± 28.86[a]
1DOX + RV	5.6 ± 0.76	0.52 ± 0.672	130.8 ± 42.49	100.00 ± 23.05[a]	981.80 ± 377.63[a]	979.2 ± 85.56	91.60 ± 19.76[a]
2DOX	11.1 ± 3.26[a]	1.32 ± 0.759[a]	91.4 ± 45.88	48.80 ± 27.69	953.40 ± 142.70[a]	519.2 ± 151.58[a]	84.20 ± 20.09[a]
2DOX + RV	4.6 ± 2.11[b]	0.16 ± 0.257[b]	69.6 ± 18.72[a]	30.00 ± 19.44[a]	545.63 ± 368.32[a]	567.3 ± 211.00[a]	74.63 ± 33.58[a]

[a]$P \leq 0.05$ versus control. [b]$P \leq 0.05$ versus DOX.

transferred to the plastic plate used in the spectrophotometric reader PowerWave XS (BioTek, USA) at 586 nm. Subsequently, the procedure was conducted according to the manufacturer's description and the concentration of MDA+4HNE in the tested samples was calculated from the formula of the calibration curve $y = 0.0896x - 0.008$. The obtained date was calculated taking into account the recommendation described in the procedure. The obtained results were expressed in nmol/g cardiac or hepatic sample.

Glutathione determination was conducted using a commercial kit Bioxytech GSH/GSSG-412 (OxisResearch, USA). The frozen cardiac or hepatic samples (~20 mg) were homogenized in the extraction buffer provided by the manufacturer. Total glutathione (GSH_T : GSH (reduced glutathione) + GSSG (oxidized glutathione)) was determined in the enzymatic reaction, where Ellman's reagent (5,5′-dithiobis-2-nitrobenzoic acid) reacts with GSH forming a color product with the maximum of absorbance at 412 nm. The concentrations of GSH, GSSG, and GSH/GSSG ratio were assessed after measuring the speed of the reaction and establishing the calibrations curves. The concentrations of GSH and GSSG were determined based on the calibration curve described by the formulae: $y = 0.1447x + 0.0004$ and $y = 0.1475x$, respectively. The obtained data was used to calculate the GSH/GSSG ratio.

The activity of superoxide dismutase activity (SOD) was determined colorimetrically using Bioxytech SOD-525 kit (OxisResearch, USA). The method is based on SOD-mediated increase in the rate of autoxidation of 5,6,6 a,11b-tetrahydro-3,9,10-trihydroxybenzo[c]fluorine in alkaline solution to yield chromophore with the maximum of absorbance at 525 nm.

The activity was measured by kinetic spectrophotometric plate reader using a PowerWave XS (BioTek, USA). The mean value in the proper control groups (for cardiac or liver samples) was assumed to be 100%.

2.4. Preparation of Slides for Histological Evaluation. 4 μm histological slides obtained from paraffin blocks were routinely processed and stained with hematoxylin and eosin (H&E). To visualize cardiomyocyte necrosis, Selye's method was also used. Liver slides were also stained with van Gieson, paS (periodic acid-Schiff), and d-paS (diastase + paS).

2.5. Statistical Analysis. The obtained data was expressed as mean ± SD and analyzed by STATISTICA 5.0 software. Continuous data were compared among the experimental groups using the Kolmogorov-Smirnov test. The statistical significance of differences between control and study groups was evaluated by Student's t-test or Mann-Whitney U test. Group-to-group comparisons were made by one-way ANOVA. A value of $P < 0.05$ was considered as statistically significant.

3. Results

Lack of animal mortality was found during the study. Food and water consumption, as well as body weight gain, were insignificant between the xenobiotic-exposed and control groups. A significant increase of FABP and BNP levels was observed in rats exposed to the higher dose of doxorubicin (Table 1). Moreover, resveratrol significantly reduced the concentration of both parameters in animals receiving a higher dose of doxorubicin to the levels below of control.

Aspartate aminotransferase (AST) activity was significantly decreased in the group concomitantly exposed to a higher dose of doxorubicin and resveratrol. On the other hand, alanine aminotransferase (ALT) activity was increased in animals exposed to both resveratrol and a low dose of doxorubicin but decreased among the rats treated with resveratrol and a high dose of the drug. A statistically significant decrease of lactate dehydrogenase (LDH) activity was noted in the groups exposed to a high dose of doxorubicin with or without resveratrol. In the case of creatine kinase (CK) activity, an significant change was also revealed in the group treated with resveratrol and a higher dose of the drug. A significant decrease of the ALP activity was found in all the xenobiotic-exposed groups. However, no significant changes in the activity of all the above-mentioned enzymes were noticed between the groups the DOX+RV versus DOX group.

Unlike the insignificant changes of GSH_T in cardiac homogenates, all other parameters of oxidative stress were highly affected by the tested substances (Table 2). Significant changes of MDA+4HAE, GSH/GSSG ration, and SOD were found in groups exposed to a high dose of doxorubicin with or without resveratrol. An increase of MDA+4HAE was also noted among the animals exposed exclusively to a low dose of doxorubicin. A decrease of SOD activity was found in the group cotreated with resveratrol and a low dose of the drug as well. The value was also significantly lower when compared to the group exposed only to a low dose of doxorubicin. A similar effect among the groups 1DOX+RV and 1DOX were found in case of MDA+4HAE. Insignificant differences in hepatic GSH_T, and SOD level were found in all studied groups comparing to the control. However, the GSH/GSSG

Table 2: Markers of oxidative stress in the heart (M ± SD).

	MDA+4HAE (nmol/g)	GSH/GSSG	GSH_T (μmol/g)	SOD (% of control)
Control	18.38 ± 5.16	13.33 ± 5.130	4.30 ± 0.20	100.00 ± 17.525
1DOX	28.46 ± 2.41[a]	11.14 ± 5.142	4.16 ± 0.34	112.22 ± 11.123
1DOX + RV	20.54 ± 2.07[b]	8.83 ± 2.073	4.18 ± 0.19	26.54 ± 10.042[a·b]
2DOX	24.67 ± 2.25[a]	5.65 ± 1.606[a]	4.17 ± 0.26	46.50 ± 19.065[a]
2DOX + RV	33.83 ± 10.40[a]	6.09 ± 1.095[a]	4.14 ± 0.32	65.95 ± 9.606[a]

[a] $P < 0.05$ versus control, [b] $P < 0.05$ versus DOX.

Table 3: Markers of oxidative stress in the liver (M ± SD).

	MDA+4HAE (nmol/g)	GSH/GSSG	GSH_T (μmol/g)	SOD (% of control)
Control	21.90 ± 5.7	127.36 ± 72.34	13.13 ± 3.34	100.00 ± 34.434
1DOX	31.25 ± 4.91[a]	59.89 ± 83.48	16.05 ± 1.76	92.18 ± 18.579
1DOX+RV	34.54 ± 6.16[a]	162.03 ± 143.16	17.77 ± 3.57	94.47 ± 9.059
2DOX	35.32 ± 9.75[a]	48.59 ± 61.82[a]	10.65 ± 8.22	101.26 ± 27.269
2DOX+RV	33.62 ± 4.24[a]	54.07 ± 53.75[a]	13.21 ± 2.59	70.14 ± 10.647

[a] $P < 0.05$ versus control.

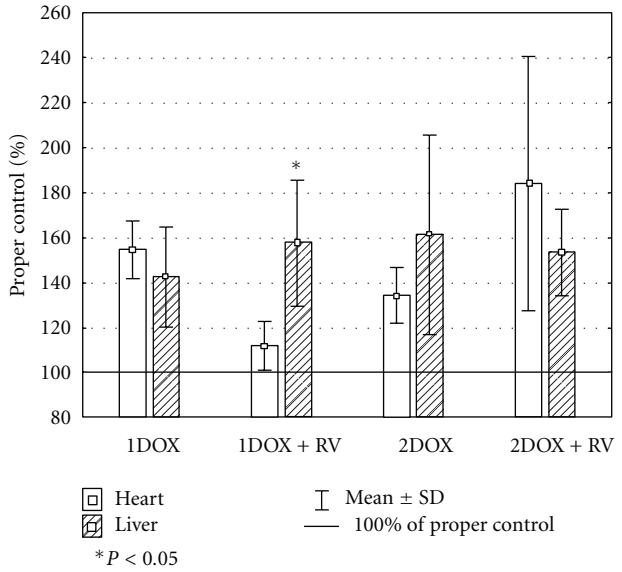

Figure 1: The relative differences of MDA+4HNE between heart and liver.

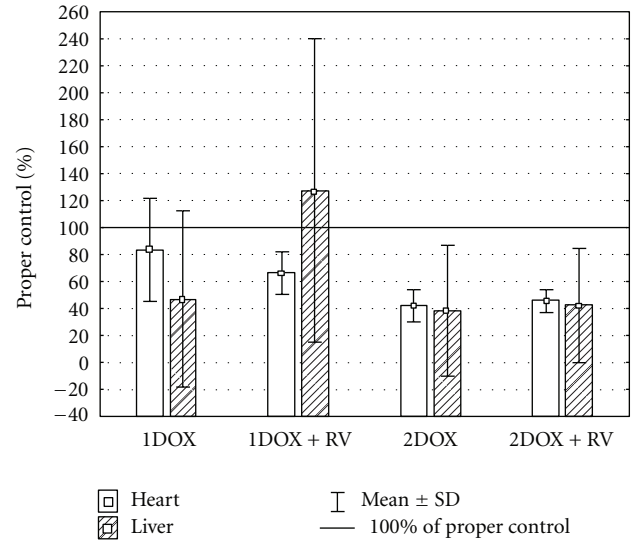

Figure 2: The relative differences of GSH/GSSG between heart and liver.

ratio was significantly lower in both groups receiving a higher dose of doxorubicin versus control (Table 3), while MDA+4HNE was significantly higher in all tested groups comparing to the control. No significant changes in liver-determined oxidative markers between the DOX+RV versus DOX group were found. Several complex changes in relative values of oxidative stress markers between heart and liver were observed only in the group of RV+1DOX (Figures 1, 2, 3, and 4). These rats had significantly higher relative changes in liver MDA+4HNE, GSH_T and SOD level than in the heart. A similar result referring to GSH_T was found in 1DOX and referring to the SOD in 2DOX. There were no significant changes between heart and liver in GSH/GSSG relative values in all studied groups.

Histological cardiac abnormalities in the untreated control group were limited mostly to single cases of irregular, wavy direction of cardiomyocytes (Table 4). Occasionally, a concomitant parenchymatous degeneration and interstitial edema were also revealed. The highest occurrence of pathological changes was found in groups exposed exclusively to doxorubicin (Figure 5). The effect was dose-dependent since massive necrosis was found only in 4 of 6 animals exposed to the high dose of the drug (Figure 6). In the remaining two animals and in 4 out of 6 ones from the group treated with a low dose of doxorubicin, the necrosis was limited to single, spread cardiomyocytes. In all cases an inflammatory infiltration was located around necrotic loci. Dose dependence was also confirmed in case of the eosinophilic degeneration and

Table 4: Histopathological cardiac changes in animals exposed to doxorubicin (DOX) with or without resveratrol (RV).

	n	Eosinophilic degeneration	Parenchymatous degeneration	Vacuolar degeneration	Irregular direction of cardiomyocytes	Pycnotic nuclei of cardiomyocytes	Interstitial edema	Necrosis	Inflammatory infiltration
Control	8	0	1	0	3	0	1	0/0[a]	0/0[b]
1DOX	6	2	3	2	3	1	3	0/4	0/4
1DOX + RV	6	4	4	1	5	0	2	0/2	0/2
2DOX	6	6	4	3	5	2	5	4/2	4/2
2DOX + RV	6	3	2	4	3	2	1	1/3	1/4

A single animal may be represented more than once in the listing of individual histological changes.
[a]Massive necrosis/changes limited to individual cardiomyocytes.
[b]Massive inflammatory infiltration/disseminate mononuclear cells between cardiomyocytes.

Table 5: Histopathological hepatic changes in animals exposed to doxorubicin (DOX) with or without resveratrol (RV).

	n	Eosinophilic degeneration	Parenchymatous degeneration	Vacuolar degeneration	Cellular edema	Pycnotic nuclei of hepatocytes	Necrosis	Inflammatory infiltration
Control	8	0	2	0	0	0	0/0[a]	0/1[b]
1DOX	6	5	5	0	4	0	0/0	0/0
1DOX + RV	6	4	5	2	0	2	0/0	0/2
2DOX	6	5	6	3	2	3	0/0	0/1
2DOX + RV	6	3	3	2	3	0	0/0	0/2

A single animal may be represented more than once in the listing of individual histological changes.
[a]Massive necrosis/changes limited to single hepatocytes.
[b]Massive inflammatory infiltration/disseminate mononuclear cells between hepatocytes.

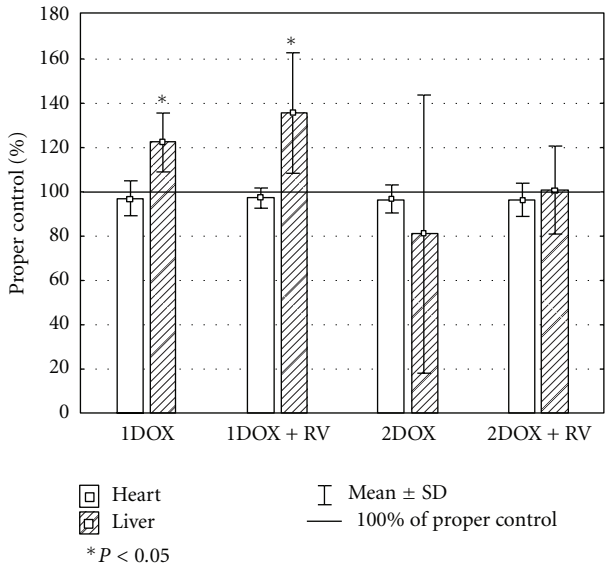

Figure 3: The relative differences of GSH_T between heart and liver.

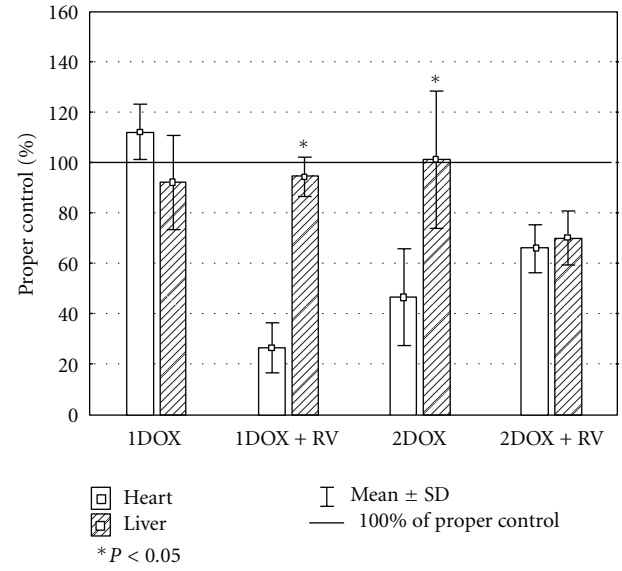

Figure 4: The relative differences of SOD between heart and liver.

interstitial edema. A lower incidence but similar histological changes were observed in groups concomitantly treated with doxorubicin and resveratrol (Figure 7). Insignificantly higher occurrence of the eosinophilic degeneration was found in the group cotreated with resveratrol and a low-dose doxorubicin, when compared with rats exposed only to the low dose of the drug, while a lower incidence of such abnormality was revealed in animals exposed to resveratrol and a high dose of doxorubicin. However, the incidence of necrosis and inflammatory infiltration was lower than that in the groups exposed only to doxorubicin.

Hepatic changes were occasionally observed in the control group (Table 5). The parenchymatous (Figure 8) and eosinophilic hepatic degeneration (Figure 9) was the most commonly observed among all the xenobiotic-exposed groups. Usually, they were limited to centri- and midlobular

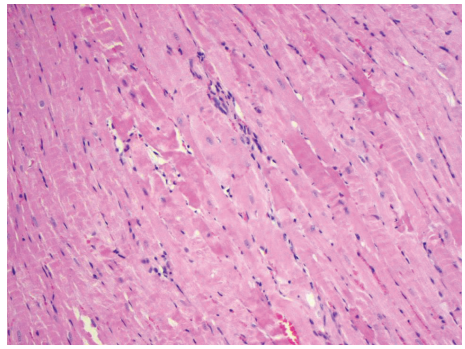

Figure 5: Interstitial edema and inflammatory infiltration between irregualar wavy-directed cardiomiocytes (H and E; objective mag. ×20; group 2DOX).

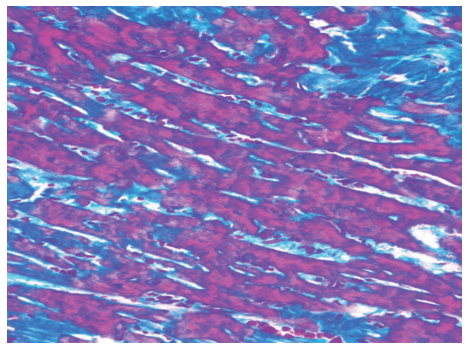

Figure 6: Necrosis of cardiomiocytes (Selly's staining; objective mag. ×40; group 2DOX).

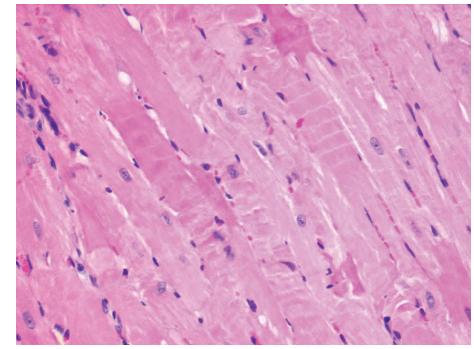

Figure 7: Intestitial edema eosynophylic cytoplasm and inflammatory infiltration between irregular wavy-directed cardiomiocytes (H and E; objective mag. ×40; group 2DOX+RV).

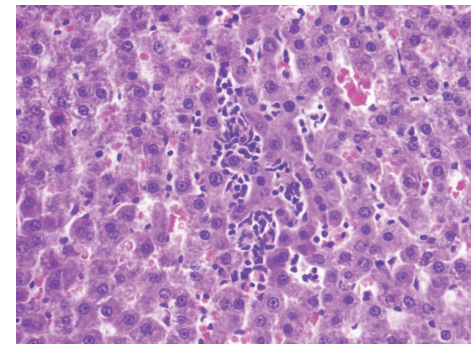

Figure 8: Eosinophilic degeneration and local inflammatory infiltration in a central zone of the hepatic lobule. Pycnotic nuclei in selected hepatocytes (H and E; objective mag. ×40; group 2DOX).

zones. Activation of cells lining to sinus zone was also observed in such cases. Many disseminated eosinophilic hepatocytes presented an intensive cytoplasmic paS reaction (Figure 10). Moreover, d-paS staining performed in the paS-positive cases and in organs with the eosinophilic degeneration showed an extracytoplasmic, intrahepatic capacity, which corresponds to the glycogen accumulation. However, some hepatocytes with the eosinophilic degeneration did not stain in the paS method before and after diastase digestion. The highest but similar occurrence of the parenchymatous and eosinophilic hepatic degeneration was found in two groups treated exclusively with doxorubicin. However, the vacuolar degeneration and pycnotic nuclei of hepatocytes were observed only in rats treated with a high dose of the drug and in groups exposed to both examined xenobiotics. The occurrence of hepatic changes was insignificantly lower among the animals co-treated with doxorubicin and resveratrol. Unlike that in the case of the hearts, lack of necrosis was found in the examined livers but occasionally some local accumulation of mononuclear inflammatory cells, mainly in mid- and centrilobular zone, was seen. In two animals exposed to the high dose of doxorubicin and resveratrol, an increased amount of the fibrous connective tissue around the central vein was found (Figure 11). There were no features of cholestasis or fibrosis in van Gieson's staining.

4. Discussion

As it was expected, DOX in the two tested doses induced oxidative stress in rat heart and liver. The protective effect of resveratrol against lipid peroxidation was only found in the heart of rats exposed to a lower dose of DOX. It is worth to stress that at the same time resveratrol slightly intensified adverse cardiac histological changes in rats receiving a lower dose of DOX, but it also attenuated necrosis and other histopathological changes in the heart induced by a higher dose of the drug. Moreover, resveratrol had no protective influence on the liver oxidative stress and while having a mild beneficial effect on the organ morphological changes caused by doxorubicin.

In this study, DOX was administered 7 times, every next week in the doses 1.0 or 2.0 mg/kg, which according to other studies represents no observed general toxic effect and general toxic effects without mortality, respectively [23]. However, a similar schedule using even 0.8 mg doxorubicin/kg results in adverse cardiac effects on the histological, ultrastructural and biochemical level [44]. According to the current hypotheses referring to prolonged DOX-dependent cardiotoxicity, oxidative damage caused directly by DOX leads to mitochondrial DNA (mtDNA) damage, which in turn is responsible for mitochondrial dysfunction [23].

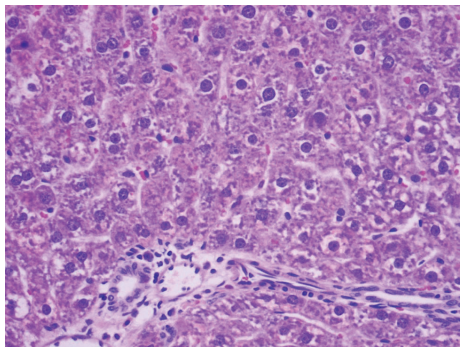

Figure 9: Parenchymatous and vacuolar degeneration in a peripheral zone of the hepatic lobule with a lesser foci of mononuclear inflammatory cells (H and E; objective mag. ×40; group 2DOX).

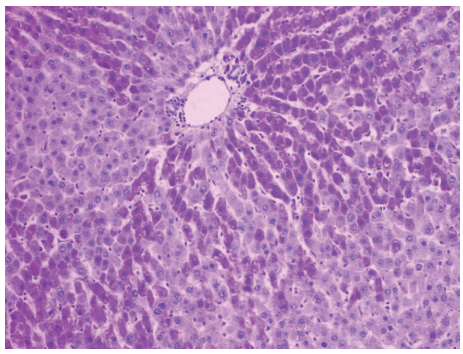

Figure 10: Irregular positive cytoplasm p.a.S reaction in the middle and central zone of the hepatic lobule (p.a.S.; objective mag. ×20; group 2DOX).

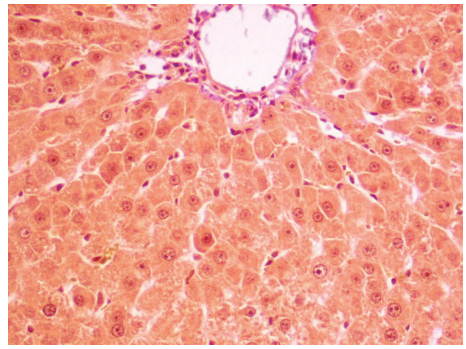

Figure 11: Single fibers of the connective tissue around the central vein. Pycnotyc nuclei in selected hepatocytes in the central zone of the hepatic lobule (van Gieson's; objective mag. ×40; group 1DOX-RV).

No changes in mitochondrial electron transfer result in oversynthesis reactive oxygen species [45]. For that reason we assessed the oxidative stress marker (MDA+4HNE) 96 hours after the last seventh dose of the drug, since the intramyocardial half-life of DOX appears to be only a few hours [46].

In this study resveratrol was taken by the rats in the fodder (20 ppm). Assuming that the average daily amount of fodder taken by rats weighing 100 g is 10 g, the dose of resveratrol is 2.0 mg/kg. It is a very low dose because NOAEL (no observed adverse effect level) for resveratrol estimated in a 28-day test, when rats were administered every day intragastrically, equals 300 mg/kg [43]. Clinically, a well-tolerated dose of resveratrol is up to 5 g/day (approximately 70 mg) [28]. In the studies with resveratrol the usually used doses are within the range of 3 mg/kg−120 mg/kg body weight or 5 g/kg of fodder [47–50].

To evaluate DOX cardiac and hepatic toxicity and to assess the effect of resveratrol on those changes, the markers of oxidative stress, histopathological features, and especially necrosis and blood biochemical parameters, were analyzed. Among the oxidative stress parameters the cardiac and hepatic lipid peroxidation products (MDA+4HNE), GSH/GSSG ratio, total glutathione GSH_T and activity of superoxide dismutase were determined. Resveratrol was found to have a significant protective role in the heart, since lipid peroxidation induced by a lower dose of doxorubicin was diminished in the group DOX+1RV to the level seen in the control. Similarly, residual activity of SOD—the key enzyme neutralizing O_2^{-*}, generated by doxorubicin was lower in cardiac homogenates in group 1DOX+RV than that in 1DOX group. The rational explanation of the SOD phenomena is difficult, especially in the light of a lack of such changes comparing 2DOX+RV with 2DOX. However, the aforementioned protective role of resveratrol referring to lipid peroxidation may be attributed to the ROS scavenging activity of resveratrol which was confirmed by Murias et al. [51] and Leonard et al. [52]. No sign of the protective effect of resveratrol against the cardiac lipid peroxidation of rats administered with a higher dose of doxorubicin (2DOX versus 2DOX+RV) may result from ROS overproduction, which cannot be composed by resveratrol. According to the study by Kitada et al. [53], resveratrol can improve the antioxidative potential via reduction of tyrosine-nitrated modification of SOD, but the applied dose was 1500-fold higher than that in this study. The absence of the effect of phytophenol on the oxidative stress in the liver induced by doxorubicin may have a similar background. There is much higher activity of enzymes taking part in one electron reduction of DOX in the liver [54]. Thus, abundance of ROS caused oxidative stress cannot be scavenged by resveratrol. Moreover, resveratrol undergoes intensive metabolism by sulfotransferases and glucuronosyltransferases highly expressed in the liver [55].

Different effects of resveratrol on cardiac histology were observed. However, it generally depended on the doxorubicin dose. Resveratrol strengthened the morphological adverse changes in the heart of rats given a lower dose of DOX and attenuated the pathological features when the dose of the chemotherapeutic drug was higher. The reasonable explanation of that is that resveratrol in some redox conditions may act as an antioxidative but in other conditions as a prooxidative factor. However, comparing the DOX versus DOX+RV groups, it may be found that changes in the morphological features of the heart are not accompanied by oxidative stress, thus, oxidative stress of the rats administered with DOX and resveratrol plays an important but not a

crucial role in morphological changes. It was clearly seen in the groups 2DOX+RV, when MDA+4HNE concentration was the highest among all the tested groups, but necrosis in the heart estimated histopathologically and biochemically (FABP) was significantly reduced. Moreover, the normalizing effect of resveratrol was also found referring to the contractility function. A higher dose of doxorubicin significantly elevated blood BNP level, but resveratrol given together with 2DOX significantly reduced BNP level. On the basis of the current knowledge, it is difficult even to speculate about the reason for that. On the other hand, there were no features of necrosis in the liver, but resveratrol showed a protective effect against other observed morphological changes in that organ.

Furthermore, there were no significant differences in serum activity of LDH, CK, AST, ALT, and ALP between, DOX and DOX+RV group. However, in the group 1DOX+RV, the level of CK, and in the group 2DOX + RV the levels of AST and ALT were lower than control in contrast with DOX groups, where there were no significant differences comparing to the control.

The range of changes in red-ox parameters may differ between the heart and the liver. In the group of 1DOX+RV, significantly higher relative changes in liver MDA+4HNE, GSH_T and SOD level comparing to the heart were found. However, there were no such complex changes between the heart and the liver in the other groups. The changes in MDA+4HNE level indicate that oxidative stress is stronger in the liver than in the heart and a higher level of GSH_T and SOD may be interpreted as an adaptation feedback on oxidative changes. In conditions of the conducted study, the effect of oxidative/antioxidative changes in the organs is dependent mainly on two mechanisms the first being the efficiency of xenobiotic-dependent ROS overproduction and the second, the potential of antioxidative organ defence. The liver, because of the xenobiotic metabolism, may produce an important amount of ROS but at the same time the activity of antioxidative defence is a few times higher than, for example, that in the heart [56–58]. Probably, the difference between the heart and the liver in the group of 1DOX+ RV results predominantly from the metabolism of doxorubicin and resveratrol by the liver. Both are metabolised by the monooxygenase system (cytochromes P450) which produces ROS [18, 59]. Interestingly, there were no morphological feature of necrosis in the liver contrary to the heart, where necrosis was observed in all the studied groups.

In the light of this study, the questions arise why a higher hepatic oxidative stress comparing to the heart did not cause necrosis and why despite a higher oxidative stress in the liver, the target of long-term toxicity is the heart and not the liver. Moreover, this comparison may indicate that oxidative stress is not a major necrosis predictor. The compartmentalization of the subcellular oxidative stress seems to be reasonable to explain these questions. Although, superoxide radical may freely diffuse through intracellular membranes, subcellular compartmentalization of glutathione may significantly modulate the harmful activity of ROS in subcellular compartments [60]. Therefore, probably more important is not the sum of oxidative stress in all cell organelles, as was measured in this study, but the level of mitochondrial oxidative stress. According to this assumption, a moderately higher level of ROS, for example, in cytoplasm, does not destroy the cell membrane—the key feature of necrosis—but mitochondria ROS overproduction may cause inhibition of the cell membrane pumps resulting from reduced ATP synthesis and triggers the necrosis pathway. That interpretation is also consistent with the obtained results and the current knowledge, according to which the target organ in long-term DOX toxicity is the heart but not the liver.

In conclusion, resveratrol had different effects on the oxidative stress and cardiac morphology, which was generally dependent on the dose of doxorubicin. The phytophenol reduced the incidence of cardiac necrosis in rats treated with a higher dose of doxorubicin. It also had an insignificant effect on the hepatic oxidative stress but the substance normalized the organ morphology. In sum, those results give a hope that future studies with higher doses of resveratrol can improve doxorubicin related-toxicity to a broader extent. Due to the number of differences between humans and laboratory rats, the obtained result cannot be directly applied in the human clinical practice. For this reason, more intensive studies, including other nonrodent species, are necessary.

Conflict of Interests

The authors declare that there is no conflict of interests.

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Research Article

Melanogenesis Inhibitor(s) from *Phyla nodiflora* Extract

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Overexpression of tyrosinase can cause excessive production of melanin and lead to hyperpigmentation disorders, including melasma and freckles. Recently, agents obtained from plants are being used as alternative medicines to downregulate tyrosinase synthesis and decrease melanin production. *Phyla nodiflora* Greene (Verbenaceae) is used as a folk medicine in Taiwanese for treating and preventing inflammatory diseases such as hepatitis and dermatitis. However, the antimelanogenesis activity and molecular biological mechanism underlying the activity of the methanolic extract of *P. nodiflora* (PNM) have not been investigated to date. Our results showed that PNM treatment was not cytotoxic and significantly reduced the cellular melanin content and tyrosinase activity in a dose-dependent manner ($P < 0.05$). Further, PNM exhibited a significant antimelanogenesis effect ($P < 0.05$) by reducing the levels of phospho-cAMP response element-binding protein and microphthalmia-associated transcription factor (MITF), inhibiting the synthesis of tyrosinase, tyrosinase-related protein-1 (TRP-1), and TRP-2, and decreasing the cellular melanin content. Moreover, PNM significantly activated the phosphorylation of mitogen-activated protein kinases, including phospho-extracellular signal-regulated kinase, c-Jun N-terminal kinase, and phospho-p38, and inhibited the synthesis of MITF, thus decreasing melanogenesis. These properties suggest that PNM could be used as a clinical and cosmetic skin-whitening agent to cure and/or prevent hyperpigmentation.

1. Introduction

Melanogenesis is a well-known mechanism for preventing skin damage caused by ultraviolet (UV) radiation [1]. However, overproduction of melanin may be caused by overexposure to UV rays, inflammation, and many skin injuries and may result in many disorders of hyperpigmentation such as melasma and freckles [2, 3]. Hyperpigmentation is commonly observed in patients with skin types IV and V, especially in Asian and Indian population. Hyperpigmentation not only causes aesthetic problems, such as skin discoloration, but also has a significant impact on the psychological status of an individual, for example, this condition may decrease social functioning, reduce productivity at work or school, and lower the self-esteem of patients [4–6].

Tyrosinase-related proteins (TRPs) such as tyrosinase (TYR), TRP-1 (5,6-dihydroxyindole-2-carboxylic acid oxidase), and TRP-2 (dopachrome tautomerase) are rate-limiting enzyme in the process of melanogenesis. TYR hydroxylates tyrosine to dihydroxyphenylalanine (DOPA) and oxidizes DOPA to the corresponding dopaquinone. In turn, TRP-2 catalyzes the conversion of dopachrome to 5,6-dihydroxyindole-2-carboxylic acid (DHICA) and TRP-1 oxidizes the DHICA to indole-5,6-quinone carboxylic acid and subsequently produces melanin [7]. Hence, overactivity of TRPs can cause abnormal accumulation of melanin pigments and lead to hyperpigmentation disorders such as melasma and lentigo senilis [8]. Moreover, microphthalmia-associated transcription factor (MITF), a basic helix-loop-helix leucine zipper transcription factor involved in the development of melanocytes, is a major regulator of the synthesis of TRPs [9, 10]. Previous studies have shown that cyclic adenosine monophosphate response element-binding protein (CREB) can bind to the cAMP response element motif of the MITF

promoter and increase the level of MITF protein. Therefore, phosphorylation of CREB can regulate the expression of tyrosinase and induce melanogenesis through MITF transcription [11, 12]. In addition, the phosphorylation of mitogen-activated protein kinases (MAPKs) such as extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38 and effectively modulates the transcription of MITF, thereby leading to antimelanogenesis [13–15].

Phyla nodiflora (L) Greene (Verbenaceae), a Taiwanese folk medicine, has been widely used as an herbal drink, and a nourishing agent and an immunomodulator and anti-inflammatory agent to prevent many diseases [16]. *P. nodiflora* and *Lippia nodiflora* are synonyms, and *P. nodiflora* possesses many pharmacological effects such as antiseptic, antitussive, antipyretic, and anti-inflammatory [17–19]. Abbasi et al. have also mentioned the ethnopharmacological application of *P. nodiflora* for skin diseases and in folk cosmetics, such as pimples, carbuncle, and skin burns [20]. The phytochemical ingredients of *P. nodiflora*, including flavonoids (hispidulin, eupafolin, Nodifloretin-A) [21, 22], flavone glycosides (lippiflorin A and B) [23], alkaloids, essential oils (methyl salicylate, eugenol), resin (α-copaene, β-bisabolene) [24], quinol (halleridone and hallerone) [25], and steroidal (4′,5′-dimethoxybenzoloxystigmasterol, γ-sitosterol) [26, 27], have been previously identified. These phytochemicals are suggested to be responsible for the pharmacological effects of *P. nodiflora*. In addition, the ethanol extract of *P. nodiflora* exerts antiurolithiatic activity by reducing the supersaturation of urine with calcium oxalate and has diuretic properties and antioxidant potential [28]. Balamurugan et al. have demonstrated that γ-sitosterol isolated from the methanol extract of *P. nodiflora* had antidiabetic activity to prevent the streptozotocin induced diabetic [27]. Ahmed et al. have also manifested that the methanol extract of *P. nodiflora* had anti-inflammatory and antinociceptive effects [29]. These reports indicated that the active phytochemicals are easily extracted with organic solvents such as methanol and ethanol. However, the antimelanogenesis activity and molecular biological mechanism of *P. nodiflora* has not been investigated to date. The present study was therefore used methanol to extract the *P. nodiflora* for evaluating its antimelanogenesis activity.

This study aimed to determine the effect on melanin production and biological mechanisms underlying antimelanogenesis of methanol extract of *P. nodiflora* (PNM) in B16F10 cells. We determined cell viability, cellular melanin content, and tyrosinase activity for estimating the decrease in melanin production. In addition, western blotting was performed to determine the expression of tyrosinase regulator (p-CREB and MITF); TRPs (TYR, TRP-1, and TRP-2); and agents responsible for MITF degradation (p-ERK, p-JNK, and p-p38) for elucidating the biological mechanism of antimelanogenesis.

2. Materials and Methods

2.1. Chemicals and Reagents. B16F10 melanoma and 3T3 (mouse embryonic fibroblast) cells were purchased from Bioresource Collection and Research Center (BCRC, Hsinchu, Taiwan). Dimethyl sulfoxide (DMSO), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and L-DOPA were purchased from Sigma-Aldrich Chemicals Co. (St. Louis, MO, USA). The antibodies for phospho-ERK (p-ERK) (Thr202/Tyr204), p-p38 (Thr180/Tyr182), p-JNK (Thr183/Tyr185), and p-CREB (Ser 133) were purchased from Cell Signaling Technology (USA). MITF, TYR, TRP1, TRP-2, GAPDH, anti-mouse, anti-goat, and anti-rabbit horseradish peroxidase-conjugated immunoglobulin G (IgG) antibodies were purchased from Santa Cruz Biotechnology (USA).

2.2. Preparation of PNM. We collected *P. nodiflora* from a local farm (Tainan, Taiwan) in April 2007. The authenticity of the plant species was identified by a pharmacognosist, Professor Chen, and stored as a voucher specimen (2007-02-PNM) in the Herbarium of the Department of Fragrance and Cosmetic Science, Kaohsiung Medical University, Kaohsiung, Taiwan. We powdered 200 g of the dried aerial part of *P. nodiflora* and immersed them in a flask with 1 L of methanol and then decocted (boiled under reflux) this mixture for 2 h; this extraction procedure was repeated 3 times. The entire methanolic extract of *P. nodiflora* was blended and filtered using filter paper. Then, the filtrate was concentrated by rotary vacuum evaporation and then lyophilized in a freeze dryer and calculated. The method can obtain 28.2 grams of freeze-dried methanol extract and the yield calculated for crude methanol extract was 14.1% with respect to the initial dry material. The dry powders of PNM were placed at −20°C until use.

2.3. Cell Viability after Treatment with PNM. The viability of cells treated with PNM was determined according to the method of Ye et al. [30]. B16F10 and 3T3 (mouse embryonic fibroblast) cells were, respectively, cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco Life Technologies, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum, 100 units/mL penicillin G, 100 μg/mL streptomycin, and 0.25 μg/mL amphotericin and then were incubated at 37°C with 5% CO_2. The viability of cells treated with PNM was determined by MTT assay. Briefly, 1×10^4 B16F10 or 3T3 cells were seeded and adhered in 96-well plates. After 24 h, DMEM was removed and 90 μL of fresh DMEM and 10 μL of different concentrations of PNM were added, and the cells were incubated for 48 h. We used 1% DMSO as a control for comparing the viability of cells treated with PNM. After 48 h incubation, the medium was removed, and the cells were washed twice with phosphate-buffered saline; further, 150 μL of MTT in DMEM solution (0.5 mg/mL) was added to each well, and the cells were incubated for 4 h at 37°C. Subsequently, the MTT solution was removed and 100 μL of DMSO was added into each well, and the plate was gently shaken for dissolving the formazan crystals. The absorbance of each well was measured at 550 nm using a microplate spectrophotometer (BIOTEK, μQuant). The absorbance of 1% DMSO was used as a control to compare the absorbance of cells treated with different concentrations of PNM. All determinations were performed in triplicate.

2.4. Determination of Cellular Melanin Content. Cellular melanin content was determined as described previously [30]. Briefly, 1×10^5 B16F10 cells were seeded in 6-well plates and cultured at 37°C for 24 h. Subsequently, the cells were treated with 1% DMSO and different concentrations of PNM for 48 h. We used 1% DMSO as control. Then, the cells were washed with PBS and lysed in 150 μL of 1 M NaO at 95°C. We added 100 μL of the lysate in a well of a 96-well microplate and quickly measured the absorbance at 490 nm using a microplate spectrophotometer (BIOTEK, μQuant). All determinations were performed in triplicate.

2.5. Determination of Cellular Tyrosinase Activity. Cellular tyrosinase activity was measured by the method of Tsang et al. with some modification [31]. Briefly, the culture method for determining cellular tyrosinase assay was similar to that for determining melanin content. After treatment with different concentration of PNM 48 h, the cells were collected after treatment with trypsin-EDTA and centrifuged at 12,000 rpm for 10 min to obtain cell pellets. The pellets were lysed with 100 μL 1% Triton X-100 and 100 μL of 0.1 mM PBS (pH 6.8) containing phenylmethylsulfonyl fluoride. The pellet solutions were frozen and thawed twice and then centrifuged at 12,000 rpm for 10 min. We added 80 μL of the supernatant in a 96-well plate and mixed with 20 μL of 0.2% L-DOPA. After incubation for 1 h, the optical densities were measured at 475 nm using a microplate spectrophotometer (BIOTEK, μQuant). The inhibitory activity of the PNM-treated cells was presented as percentage against that of the untreated cells.

2.6. Analysis of the Expression of Proteins Regulating Melanogenesis by Western Blotting. B16F10 cells were treated with different concentrations of PNM. Cells were collected and lysed in a sample buffer containing 4% sodium dodecyl sulfate (SDS), 20% glycerol, 10% 2-mercaptoethanol, 0.004% bromphenol blue, and 0.125 M Tris HCl. The lysates as protein samples were denatured at 95°C for western blot assay. Proteins were separated using 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) running gel. Subsequently, the resolved proteins were transferred to nitrocellulose membranes and then were blocked using 5% dried milk in Tris HCl buffer. Membranes were incubated with different primary antibodies for 24 h, including MITF, p-CREB, TYR, TRP1, TRP-2, p-ERK, p-p38, p-JNK, and GAPDH, and were further incubated with anti-mouse or anti-rabbit horseradish peroxidase antibody for 1 h. The bands of bound antibodies were detected by enhanced chemiluminescence reagents, and the images of protein expression were obtained using an AlphaImager HP High Resolution Imaging System. All determinations were performed in triplicate.

2.7. Statistical Analysis. All data are expressed as means ± standard deviations of the indicated number of experiments. Data were analyzed by one-way ANOVA followed with Tukey's post-hoc test to calculate statistical significance using the SPSS software (Version 19). $P < 0.05$ was considered to indicate a significant difference.

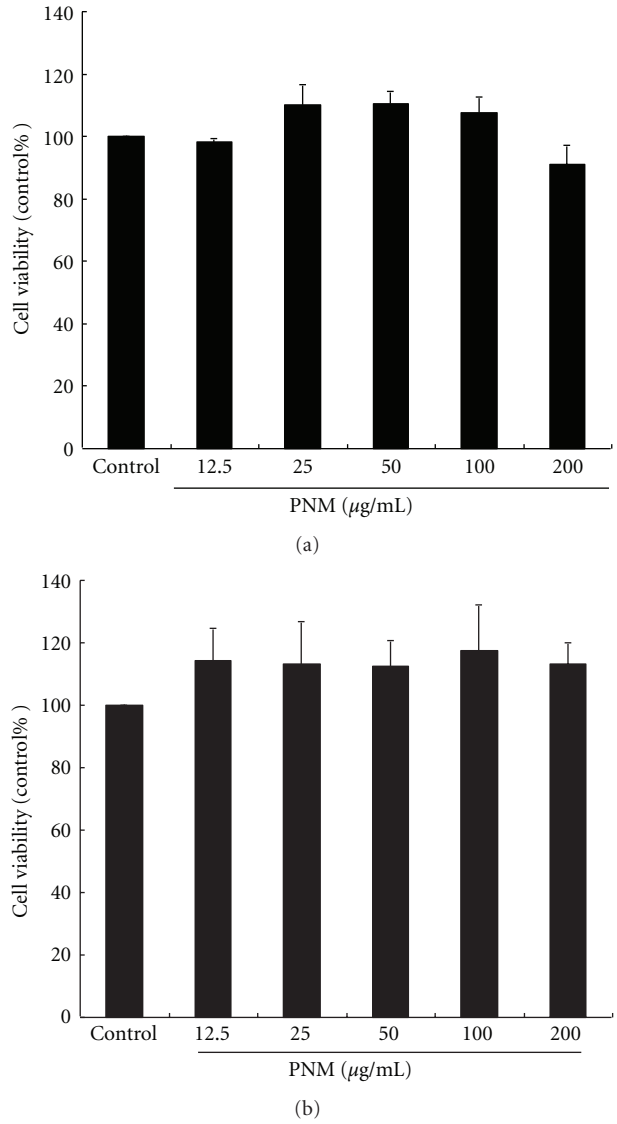

Figure 1: The viability of B16F10 (a) and 3T3 (b) cells treated with a methanolic extract of *Phyla nodiflora*.

3. Results

3.1. Viability of Cells after Treatment with PNM. In vitro safety of the extract or pure compound is the first consideration in formulating an agent as a health food and/or cosmetic. We determined the cytotoxicity of PNM in B16F10 cells by an MTT assay. The viability of B16F10 cells treated with different concentrations of PNM is shown in Figure 1(a). B16F10 cells were treated with a serial dose of PNM (12.5 to 200 μg/mL), and their viability was more than 90%. In addition, the viability of 3T3 cells treated with the same concentration of PNM was not less than 90% (Figure 1(b)). These results indicated that PNM is a safe ingredient for determining the antimelanogenesis effect of PNM. Therefore, we used PNM at doses of 12.5–100 μg/mL to determine the cellular melanin synthesis and tyrosinase activity in B16F10 cells.

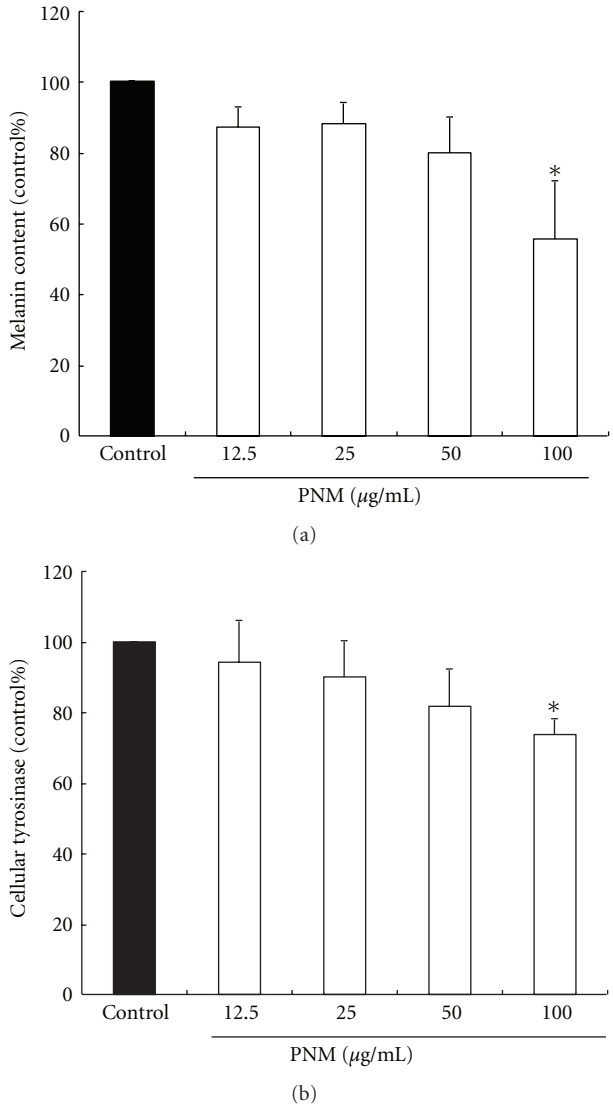

Figure 2: The cellular melanin content (a) and tyrosinase activity (b) in B16F10 cells treated with a methanolic extract of *Phyla nodiflora*. The different superscript letters indicate significant difference at $P < 0.05$ using ANOVA followed with Tukey's post-hoc test. *Significantly different from control.

3.2. Cellular Melanin Synthesis and Inhibition of Tyrosinase Activity by PNM. Cellular tyrosinase activity is the major factor that stimulates melanin synthesis and ultimately induces melanogenesis [7]. We determined the cellular tyrosinase activity and melanin content for investigating the antimelanogenesis activity of PNM on B16F10 cells. B16F10 cells were pretreated with PNM at dose of 12.5–100 μg/mL (Figure 2(a)). PNM treatment significantly decreased the cellular melanin content in a dose-dependent manner compared to that in the control group ($P < 0.05$). In addition, PNM treatment significantly reduced the cellular tyrosinase activity in a dose-dependent manner compared to the control ($P < 0.05$) (Figure 2(b)). Further, we determined the protein level of TYR, TRP-1, and TRP-2 by western blotting. PNM treatments effectively reduced the synthesis of TYR (Figure 3(a)), TRP-1 (Figure 3(b)), and TRP-2 (Figure 3(c)), thereby inhibiting melanogenesis in a dose-dependent manner. These results indicate that PNM treatment reduced the melanin content and inhibited cellular tyrosinase activity in B16F10 cells.

3.3. PNM Reduces the Synthesis of Tyrosinase by Inhibiting MITF and p-CREB Proteins. Synthesis of the protein tyrosinase is closely regulated by MITF and p-CREB proteins, including TYR, TRP-1, and TRP-2, which leads to melanogenesis [12–15]. Therefore, we performed western blotting to determine the expression levels of MITF and p-CREB in B16F10 cells treated with serial doses of PNM (12.5–100 μg/mL). PNM treatments significantly reduced the expression levels of MITF (Figure 4(a)) and p-CREB (Figure 4(b)) in B16F10 cells in a dose-dependent manner. These findings clearly indicated that the antimelanogenesis effect of PNM is directly related to reduced synthesis of tyrosinase by downregulation of the expression of MITF and p-CREB.

3.4. PNM Inhibits Melanogenesis by Degradation of MITF via MAPK Signaling Pathway. Previous studies have shown that phosphorylation of MAPKs such as ERK, JNK, and p38 mainly inhibit the synthesis of MITF and thus reduce the levels of tyrosinase, thereby inhibiting melanogenesis [13–15]. Therefore, western blotting was performed to determine the effect of 100 μg/mL of PNM on the expression level of p-ERK, p-JNK, and p-p38 in a time-course experiment. Our results showed a marked increase in the expression of p-ERK (Figure 5(a)), p-JNK (Figure 5(b)), and p-p38 (Figure 5(c)) after treatment with 100 μg/mL of PNM at 12, 6, and 6 h, respectively, ($P < 0.05$). These data indicate that PNM may induce the phosphorylation of 3 MAPKs and subsequently change the degradation of levels of MITF protein. In addition, we further investigated whether the effect of PNM treatment on melanin synthesis is prevented by the addition of 10 μM of U0126 (a selective inhibitor of MAPK/ERK), SB202190 (a selective inhibitor of p38), SP600125 (a selective inhibitor of JNK), and a combination of all inhibitors. Addition of each inhibitor increased the melanin production and showed a significant difference ($P < 0.05$) compared to that in the control group (Figure 6). The treatment with a combination of all inhibitors significantly increased the melanin production compared to that in the control group and in the group treated with a single inhibitor ($P < 0.05$). Our results showed that the melanin production in cells treated with U0126, SB202190, and a combination of all inhibitors in the presence of 100 μg/mL of PNM was significantly lower ($P < 0.05$) than that in cells treated only with the inhibitor; however, this effect is not significant in the case of cells treated with SP600125. Thus, these results suggest that PNM exerts antimelanogenesis effects through phosphorylation of both ERK and p38 but not of JNK.

4. Discussion

Safety is the first and foremost consideration while developing therapeutic or cosmetic agents using active ingredients

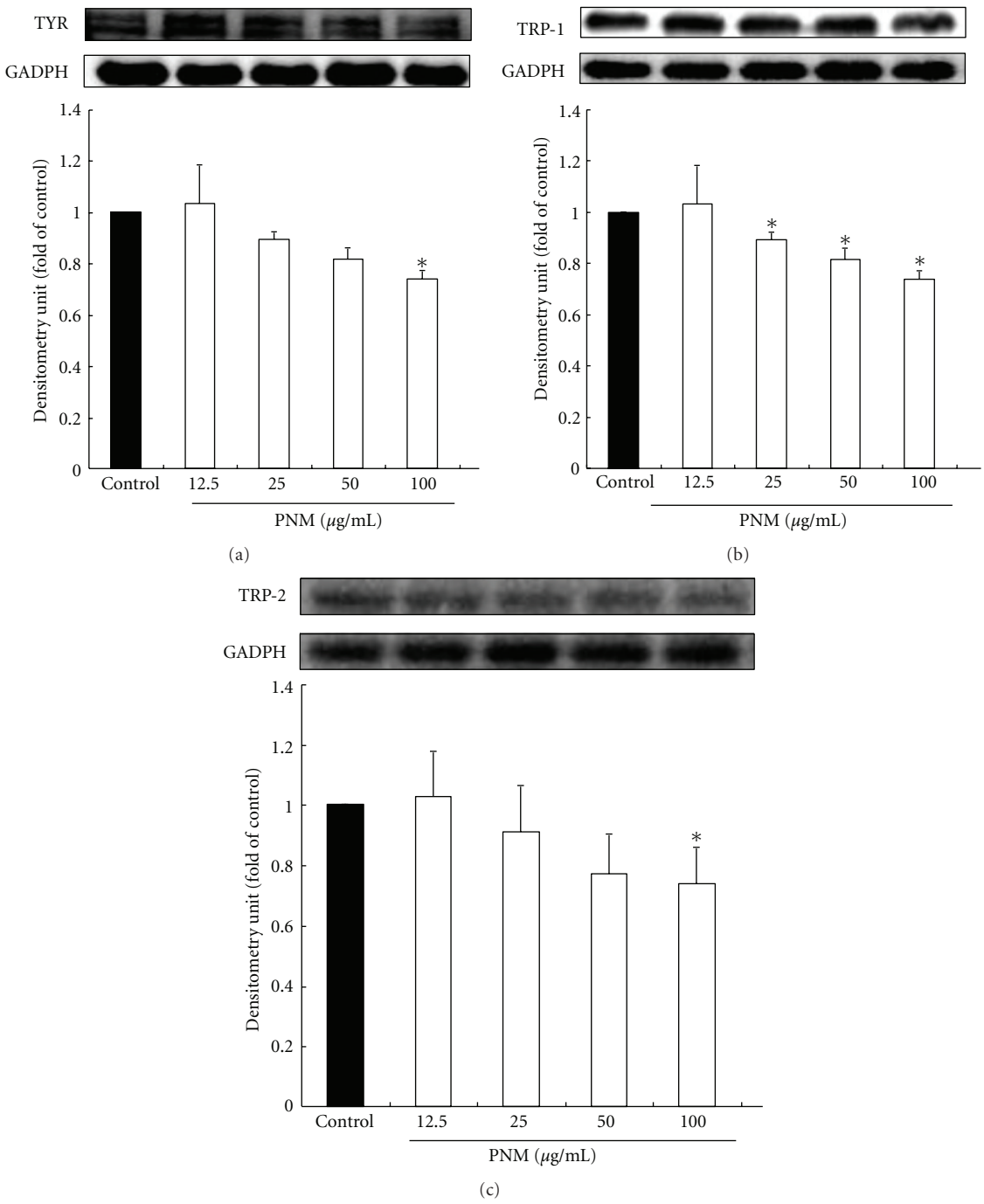

Figure 3: Methanolic extract of *Phlya nodiflora* decreases the melanin production by inhibiting the levels of tyrosinase-related proteins (TRPs) (a) tyrosinase (TYR), (b) TRP-1, and (c) TRP-2. The different superscript letters indicate significant difference at $P < 0.05$ using ANOVA followed with Tukey's post-hoc test. *Significantly different from control.

obtained from plants. Hydroquinone is a commonly used skin-whitening agent for treating and preventing hyperpigmentation disorders such as melasma and freckles. However, previous studies have shown that hydroquinone itself can cause a depigmentation of the skin, for example, vitiligo, as a side effect because of melanocyte cytotoxicity. Therefore, use of hydroquinone as an additive in a cosmetic or skin care product is prohibited [32, 33]. According to that, we performed the cell viability of PNM for estimating the in vitro safety, including B16F10 and 3T3. Our data showed that PNM at a dose of 12.5–200 μg/mL did not have any significant cytotoxicity in mouse embryonic fibroblast cells. These results indicated that PNM is a safe component and therefore the doses of PNM in the above range are safe for determining cellular melanin content, tyrosinase activity, and antimelanogenesis effect.

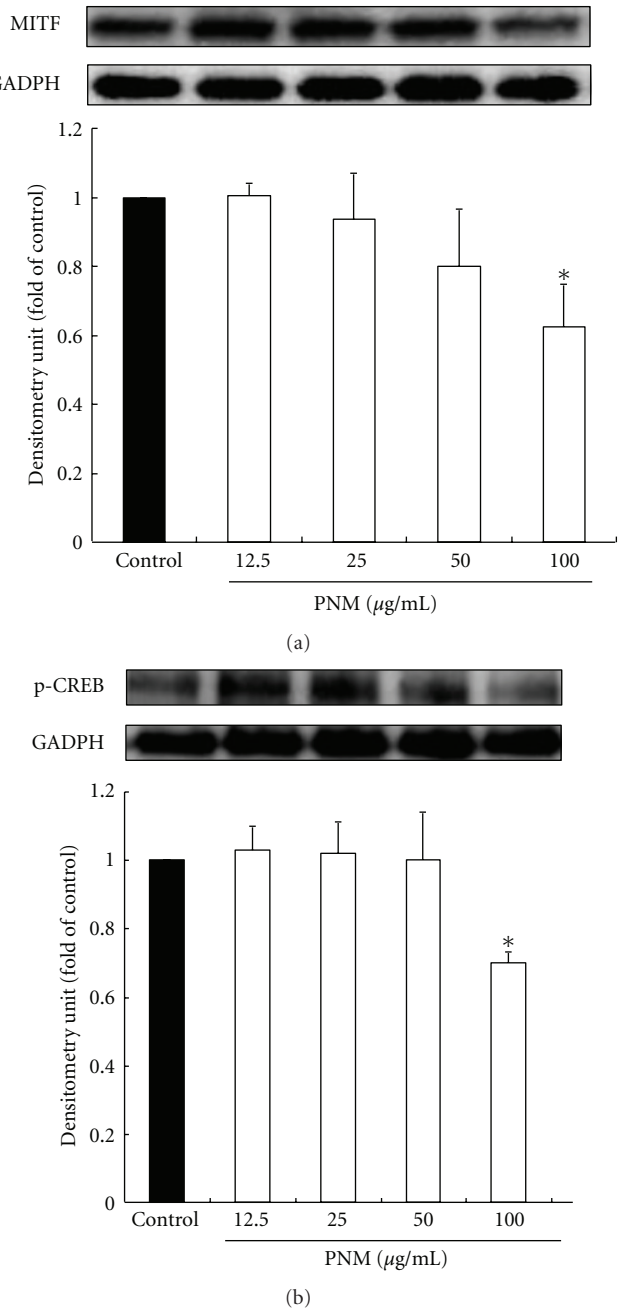

Figure 4: Methanolic extract of *Phyla nodiflora* downregulates the expression of (a) microphthalmia-associated transcription factor (MITF) and phospho-cyclic adenosine monophosphate response element-binding protein (p-CREB) (b). The different superscript letters indicate significant difference at $P < 0.05$ using ANOVA followed with Tukey's post-hoc test. *Significantly different from control.

TRPs are rate-limiting enzymes in the melanogenesis process that increase the conversion of tyrosine to dopaquinone, the rearrangement of dopachrome to DHICA and thus cause overproduction and accumulation of melanin pigments in the skin. In our study, PNM treatment inhibited the cellular tyrosinase activity in a dose-dependent manner and thus reduced the melanin content in B16F10 cells, especially at a dose of 100 μg/mL of PNM. In addition, 200 μM of kojic acid did not decrease the melanin content in B16F10 cells (data not shown). Hyperpigmentation is by overactivity of TRPs, but not all skin-whitening agents can simultaneously inhibit TYR, TRP-1, and TRP-2, such as *Viola mandshurica* [34] and nicotinic acid hydroxamate [35]. However, our western blotting assay showed that PNM treatment reduced the expression of all rate-limiting enzymes, including TYR, TRP-1, and TRP-2 protein, and prevented abnormal accumulation of melanin in the process of melanogenesis. These data suggested that the decrease in melanin content by PNM treatment was because of inhibition of TRPs; therefore, PNM could be used as a skin-whitening agent against hyperpigmentation.

MITF is the major regulator of the synthesis of TRPs such as TYR, TRP-1, and TRP-2 during the process of melanogenesis in mammalian cells [16–18]. Our result showed that cells treated with PNM showed degradation of the MITF protein in a dose-dependent manner compared to the cells in the control group ($P < 0.05$); therefore, PNM treatment can inhibit the synthesis of the proteins TYR, TRP-1, and TRP-2 through upregulation of MITF degradation. Moreover, phosphorylation of CREB is a prime factor that interacts with the cAMP response element motif of the MITF in the cAMP pathway, which stimulates tyrosinase synthesis and in turn the synthesis of melanin [10, 11]. Our results indicated that PNM treatment showed a dose-dependent reduction in the expression level of p-CREB level to inhibit the tyrosinase synthesis and melanin production. A recent study showed that a traditional Chinese medicine, Qian-wang-hong-bai-san, downregulated the expression level of p-CREB and MITF to inhibit tyrosinase synthesis and melanin production in B16 cells [31]. These results indicate that PNM is a good skin-whitening agent, and it inhibits tyrosinase synthesis and decreases the production of melanin by inhibiting phosphorylation of CREB and degradation of MITF.

On the other hand, activation of MAPK signaling pathway plays a role in the phosphorylation of MITF at serine-73 and subsequently leads to ubiquitination of MITF followed by proteasome-mediated degradation and thus inhibits tyrosinase synthesis and melanin production [12–15]. Previous studies have shown that skin-whitening agents such as *Cuscuta japonica* [36] and quercetin [37] activate the phosphorylation of MAPKs and downregulate the expression of MITF and subsequently inhibit the synthesis of TRPs and melanin production. Our study indicated that PNM treatment significantly phosphorylated the MAPK proteins, including ERK, JNK, and p38, to degrade the MITF protein and further diminished tyrosinase, TRP-1, and TRP-2 synthesis for decreasing melanin production. Furthermore, the inhibitors of ERK, JNK, and p38 were added in B16F10 cells treated with PNM to confirm the intracellular signaling pathway regulating melanin production. Our results indicated that PNM could reduce the overproduction of melanin after addition of specific inhibitors of ERK and p38; therefore, PNM-mediated decrease in melanin production was thought to occur via activation of ERK and p38 pathways

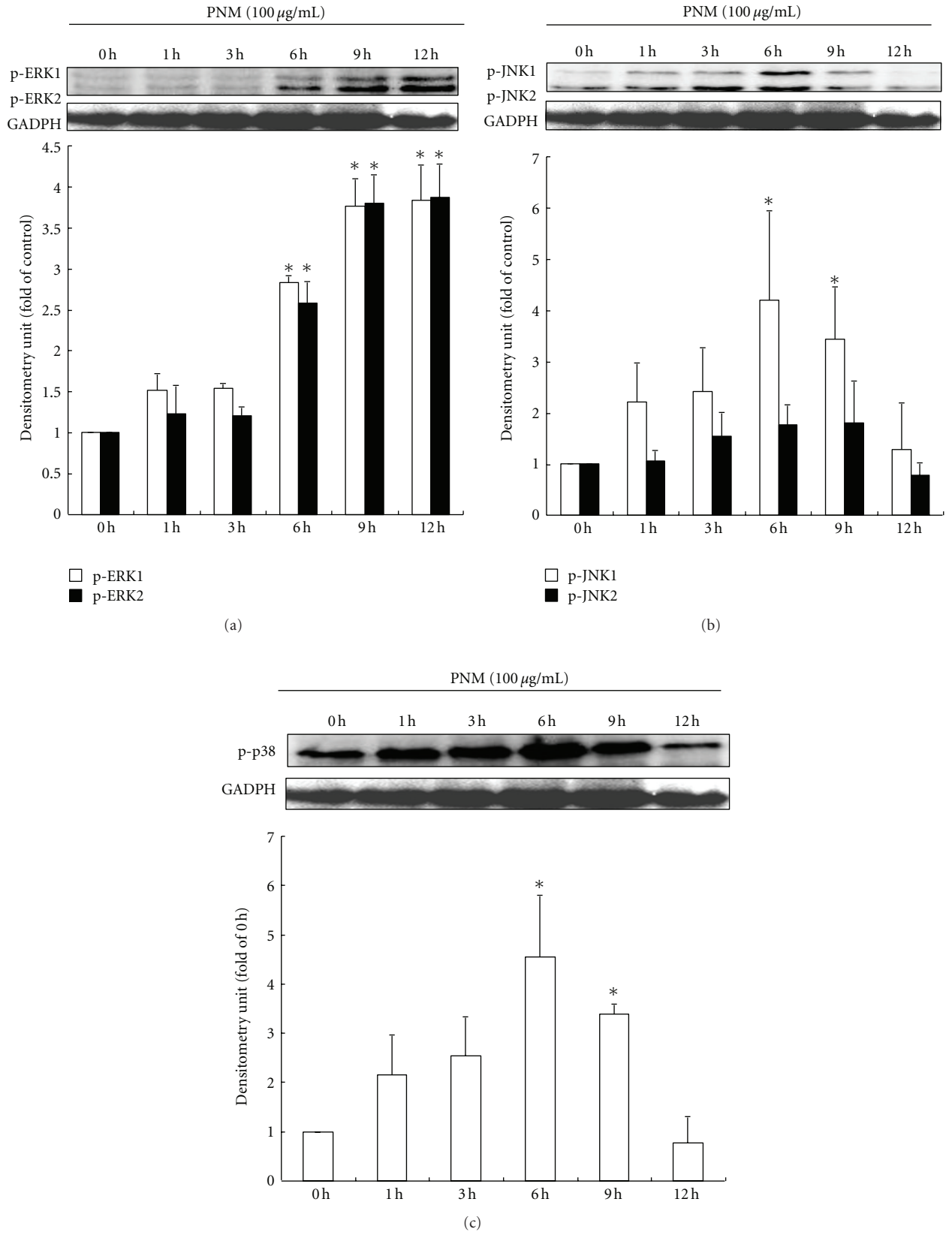

Figure 5: Methanolic extract of *Phyla nodiflora* degrades the MITF protein by activating phosphorylation of mitogen-activated protein kinases (MAPKs), (a) phospho-extracellular signal-regulated kinase (ERK), (b) phosphor-c-Jun N-terminal kinase (p-JNK), and (c) p-p38. The different superscript letters indicate significant difference at $P < 0.05$ using ANOVA followed with Tukey's post-hoc test. *Significantly different from 0 h.

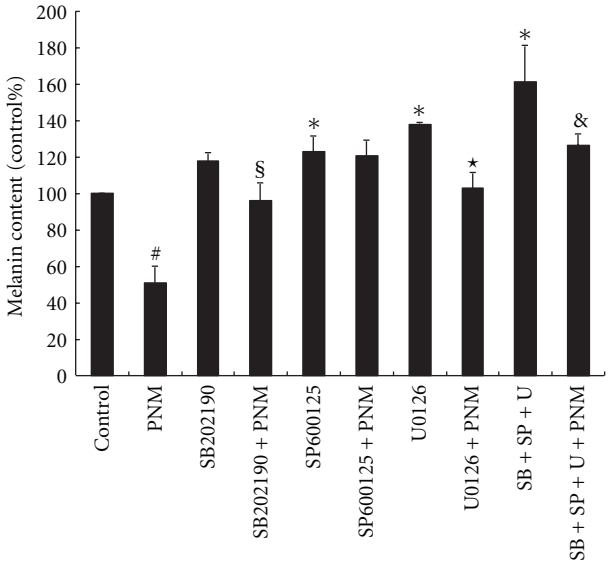

Figure 6: Methanolic extract of *P. nodiflora* decreased the melanin production induced by mitogen-activated protein kinase (MAPK)-specific inhibitors. The different superscript letters indicate significant difference at $P < 0.05$ using ANOVA followed with Tukey's post-hoc test. #Significant different from control; *U0126, SB202190, SP600125, and treatment with a combination of all 3 inhibitors compared to control; §PNM treated with SB202190 inhibitor compared to SB202190 only. ⋆PNM treated with U0126 inhibitor compared to U0126 only. &PNM treated with a combination of all 3 inhibitors compared to treatment with all 3 inhibitors.

and subsequent degradation of MITF protein to inhibit tyrosinase synthesis in B16F10 cells.

In conclusion, the present study is firstly demonstrated that PNM treatment inhibits melanogenesis by activating ERK and p38 signaling pathways, which lead to a downregulation of the MITF protein and finally reduces the synthesis of tyrosinase and production of melanin. Thus, on the basis of the molecular biological mechanism of PNM, we suggest that PNM can be safely used as be a skin-whitening agent, and we will perform clinical trials in the future to establish this treatment as evidence-based medicine and/or cosmetic.

Conflict of Interests

The authors declare that there is no conflict of interests in this paper and no competing financial interests exist.

Acknowledgments

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