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JIN Formula Inhibits Tumorigenesis Pathways in Human Lung Carcinoma Cells and Tumor Growth in Athymic Nude Mice

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1. Introduction

Lung cancer as the most common cancer in the world represents a major public health problem (1). Worldwide it has the highest rate of cancer mortality, exceeding the mortality rates of colorectal, breast and prostate cancers combined (2). Despite major advances in the treatment and management of lung cancer, most patients with lung cancer eventually die of this disease. Because conventional therapies have failed to make a major impact on survival, newer approaches are necessary in the battle against lung cancer. The poor lung cancer survival figures argue powerfully for new approaches to control this disease through chemoprevention, which has been defined as the use of agents that could reverse, suppress or completely halt tumor development. Developing novel mechanism-based chemopreventive approaches for lung cancer which humans can accept has become an important goal.

Many traditional Chinese medicine (TCM) formulas have been used in cancer therapy. JIN formula, an ancient herbal formula from classical book JIN KUI YAO LUE (Golden Chamber) for the treatment of lung cancer, which is composed of Ophiopogon japonicus 30g, Prepared Rhizoma Pinelliae15g, Ginseng radix 30g, Glycyrrhiza radix 12g, Peach Kernel 15g, Unprepared Coix lachryma jobi seed 30g, Chinese waxgourd seed 30g, and Phragmititis Caulis 30g. TCM theory regarded that lung cancer is related with both deficiency of Qi and Yin, or Qi insufficiency of the Spleen and Lung, as well as pathological changes of Qi stagnation, blood stasis, and accumulation of phlegm and toxin. Whereas, JIN formula could replenish both Qi and Yin, strengthen the Spleen and Lung, clear lung, resolve phlegm, activate blood circulation and remove stasis.

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We extracted JIN formula with different solvents, hereafter referred to as JIN formula extracts (JFE). It is hypothesized that JFE may afford chemopreventive as well as chemotherapeutic effects against lung cancer. In the present study, we first demonstrated the antiproliferative effects of JFE (including E1 to E8) in A549 cells. This involves the tumorigenesis network of cells. Next, we determined the chemopreventive potential of JFE on regulation of PI3K/Akt, MAPK, NF-kB pathways in A549 and H157 human lung carcinoma cells. Based on the results of our in vitro data, we next carried out in vivo study in mice. We found that oral administration of a human acceptable dose of JIN formula (3%, wt/vol) to athymic nude mice implanted with A549 and H157 cells resulted in significant inhibition of tumor growth.

2. Materials and methods

2.1 Materials

Akt, JNK, and p38 antibodies were obtained from Cell Signaling Technology. The polyclonal antibodies NF-kB/p65, ERK1/2 were procured from Santa Cruz Biotechnology Inc. Anti-mouse and anti-rabbit secondary antibody horseradish peroxidase (HRP) conjugate was obtained from Amersham Life Science Inc.

2.2 Methods

2.2.1 Preparation of JFE

Fresh JIN formula was decocted in distilled water. As shown in Figure 1, different solvents were applied to acquire the following eight extracts: Water extraction concentrate (E1), Ethanol precipitation (E2), Precipitates in ethanol recovery (E3), Ethanol concentration (E4), Cyclohexane extract (E5), Ethyl acetate extract (E6), N-butanol extract (E7) and Water solution (E8). The extracts were condensed and freeze dried. The freeze-dried extracts were stored at 4°C to be used for various treatments.

2.2.2 Cell culture and treatment

The human lung carcinoma A549 and H157 cells were obtained from American Type Culture Collection and cultured in DMEM medium, supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin (P-S) in a 5% CO_2 atmosphere at 37°C. The extracts were dissolved in dimethyl sulfoxide (DMSO) and were used for the treatment of cells. A total of 50–60% confluent cells were treated with the extracts (1–100 μg/ml) for 72 h in complete growth medium.

2.2.3 Cell viability (MTT assay)

The effect of JFE on the viability of cells was determined by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazoliumbromide assay. The cells were plated at 1×10^4cells/well in 200 μl of complete culture medium containing 1–100 μg/ml concentrations of JFE in 96-well microtiter plates for 72 h. After incubation for specified times at 37°C in a humidified incubator, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazoliumbromide (5 mg/ml in PBS) was added to each well and incubated for 4 h, after which the plate was centrifuged at 1800g for 5 min at 4°C. The absorbance was recorded on a microplate reader at the wavelength of 540 nm. The effect of JFE on growth inhibition was assessed as percent cell viability where DMSO-treated cells were taken as 100% viable. DMSO at the concentrations used was without any effect on cell viability.

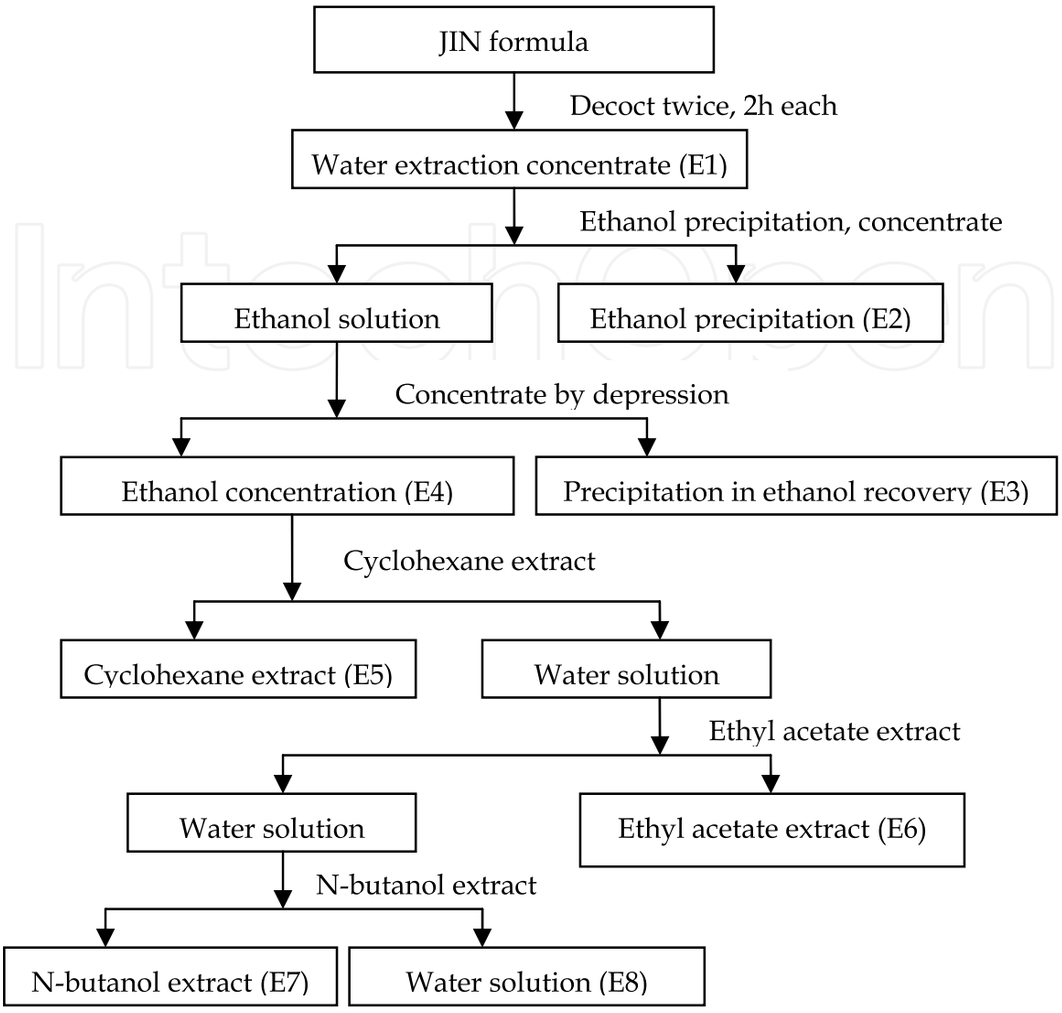


Fig. 1. The Extracting Procedure of JIN formula

2.2.4 Protein extraction and western blotting

Following the treatment of cells as described above, the media was aspirated, the cells were washed with cold PBS (pH 7.4), and ice-cold lysis buffer (50 mM Tris–HCl, 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, 20 mM NaF, 100 mM Na_3VO_4, 0.5% NP-40, 1%Triton X-100, 1 mM PMSF (pH 7.4) with freshly added protease inhibitor cocktail (Protease Inhibitor Cocktail Set III; Calbiochem) over ice for 30 min. The cells were scraped and the lysate was collected in a microfuge tube and passed through needle to break up the cell aggregates. The lysate was cleared by centrifugation at 14 000g for 15 min at 4°C and the supernatant (whole cell lysate) was used or immediately stored at -80°C.

For western blotting, 30-50 mg protein was resolved over 8-12% polyacrylamide gels and transferred to a nitrocellulose membrane. The blot was blocked in blocking buffer (5% non-fat dry milk/1% Tween 20; in 20 mM TBS, pH 7.6) for 1 h at room temperature, incubated with appropriate monoclonal or polyclonal primary antibody in blocking buffer for 1.5 hours to overnight at 4°C, followed by incubation with anti-mouse or anti-rabbit secondary antibody HRP conjugate obtained from Amersham Life Science Inc. Densitometric

measurements of the band detected by chemiluminescence in western blot analysis were performed using digitalized scientific software program Quantity One.

2.2.5 In vivo tumor xenograft model

Balb/c athymic (nude) mice (male, 6−8 weeks) weighing 21−25 g were purchased from Animal Center, Academy of Military Medical Sciences, (Beijing, China) and were housed under specific pathogen-free conditions according to the guidelines of Animal Care, Nanjing University of Chinese Medicine. The animal room was controlled for temperature (22 ±2°C), light (12 hour light/dark cycle) and humidity (50±10%). All laboratory feed pellets and bedding was autoclaved.

The tumor regression model in nude mice has been successfully applied to evaluate antitumor activity. This model was therefore used to evaluate suppression of solid tumor growth in JIN Formula (JIN). A total of 1×10^7 A549, H157 cells in 0.2 ml culture medium were injected subcutaneously into the flank of each mouse using a 26-gauge needle. After 7 days observation, an apparently solid tumor mass was excised from mice inoculated with lung cancer cells. When the tumor volume reached about 50 mm^3 in the nude mice, xenografted tumor model were randomly distributed into normal group, model group, Jin group, after sacrifice of animals with oral administration of JIN formula (3%, wt/vol) for 16 days, tumor inhibiting rate was recorded to show the effects of drugs on tumor growth.

Tumor inhibiting rate= (average of tumor weight in model group-average of tumor weight in JIN group) /average of tumor weight in model group ×100%.

2.3 Statistical analysis

Results were analyzed using a two-tailed Student's t-test to assess statistical significance and P-values<0.05 were considered significant.

3. Result

3.1 Inhibition of cell growth by JFE in A549 cells

This study was designed to show the chemopreventive/chemotherapeutic potential of JIN formula against lung cancer. Initially in our study, we investigated the antiproliferative effects of JFE treatment on human lung carcinoma A549 cells. Therefore, using A549 cells, we first evaluated the effect of JFE on the growth of these cells by MTT assay. We compared the antiproliferative effects of JFE (including E1 to E8) on A549 cells. As shown in Figure 2, treatment of JFE (1–100 μg/ml) for 72 h was found that E6 and E7 could decrease the viability of A549 cells.

3.2 Inhibition of tumorigenesis pathways by JFE in A549 and H157 cells

To explore the mechanism of antiproliferative effects of JIN formula against lung cancer, we investigated the involvement of Akt, ERK1/2, JNK1/2, p38 and NF-κB machinery during the inhibition of tumorigenesis by JFE in A549 and H157 cells.

Akt, also known as protein kinase B, which is a serine or threonine kinase, has been identified as an important component of tumorigenesis signaling pathway. The PI3K/Akt promotes cell survival by activating the NF-kB signaling pathway (3). Studies have shown that Akt plays an important role in carcinogenesis. We first investigated the effect of JFE on Akt protein expression in human lung carcinoma cells. In our study, we have demonstrated that the treatment of A549 and H157 cells with JFE (E1–E8) resulted in different degree inhibition of Akt pathways on tumor promotion processes as shown in Figure 3.

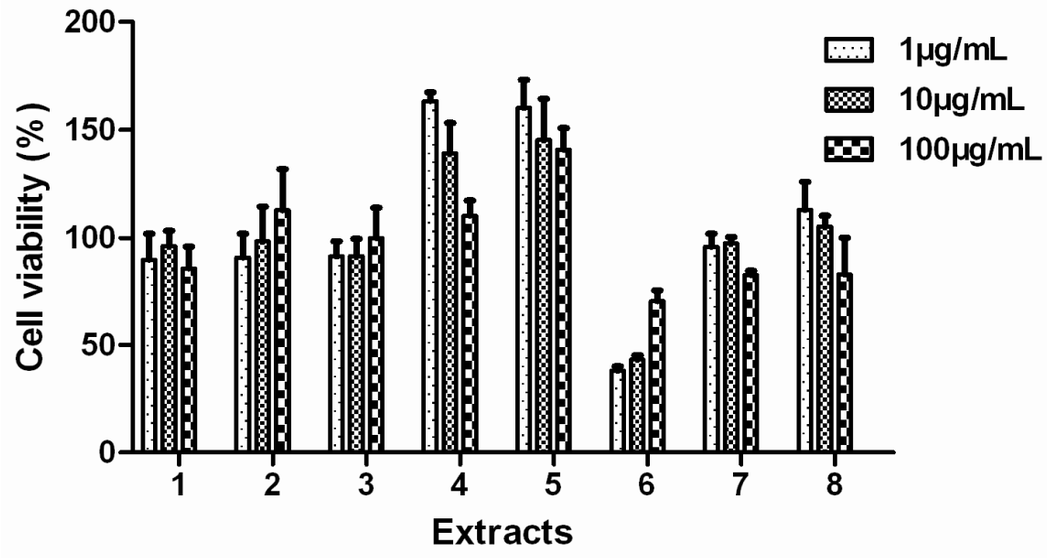


Fig. 2. The effects of JFE (including E1 to E8) on A549 cells growth

The MAP kinase superfamily (MAPKs) has been characterized into three groups which include extracellular signal-regulated kinase p44/42 (ERK), JNK/SAPK (c-jun N-terminal kinase/stress activated protein kinase) and p38 MAP kinase. MAPKs, a group of serine/threonine-specific, proline-directed protein kinases are known to modulate transcription factor activities. (4, 5). The involvement of the MAPK pathway in tumor proliferation is well documented. Transient activation of ERK is responsible for proliferation and differentiation and has also been shown to be involved in tumor promotion processes (3). Stimulation of JNK/SAPK and p38 can mediate differentiation, inflammatory responses and cell death (6). In the present study, we assessed the effect of JFE on MAPK pathway in A549 and H157 human lung carcinoma cells. The immunoblot analysis demonstrated that the treatment of cells with JFE inhibited ERK1/2, JNK1/2 and p38 proteins, and this inhibition of JNK1/2 and p38 in A549 cells was stronger than in H157 (Figure 3). Several studies have shown that JNK pathway plays a major role in cellular function, such as cell proliferation and transformation, whereas the ERK pathway suppresses apoptosis and enhances cell survival or tumorigenesis (4). ERK1/2 and p38 are also involved in the transcriptional activation of NF-kB (7, 8).

NF-kB is a sequence specific transcription factor that is known to be involved in the inflammatory and innate immune responses (9, 10). NF-kB is sequestered in the cytoplasm in an inactive form through interaction with IkB. Phosphorylation of IkB by IkB kinase (IKK) causes ubiquitination and degradation of IkB, thus releasing NF-kB which then translocates to the nucleus, where it binds to specific kB binding sites in the promoter regions of several genes (11). Studies have shown that NF-kB activation plays an important role in cell survival, by its ability to block or reduce apoptosis (12). In the present study, we further investigated the effect of JFE on the pattern of NF-kB activation and whether treatment with JFE inhibits nuclear translocation of NF-kB/p65 in A549 and H157 cells. As is evident from western blot analysis data and the relative density of bands, we found that JFE (E2, E3, E7) treatment of A549 cells resulted in inhibition of translocation of NF-kB/p65, meanwhile, we demonstrated that NF-kB is activated in H157 human lung carcinoma cells treated with JFE (E2, E3, E7) and is translocated to the nucleus when measured by western blot analysis (Figure 3).

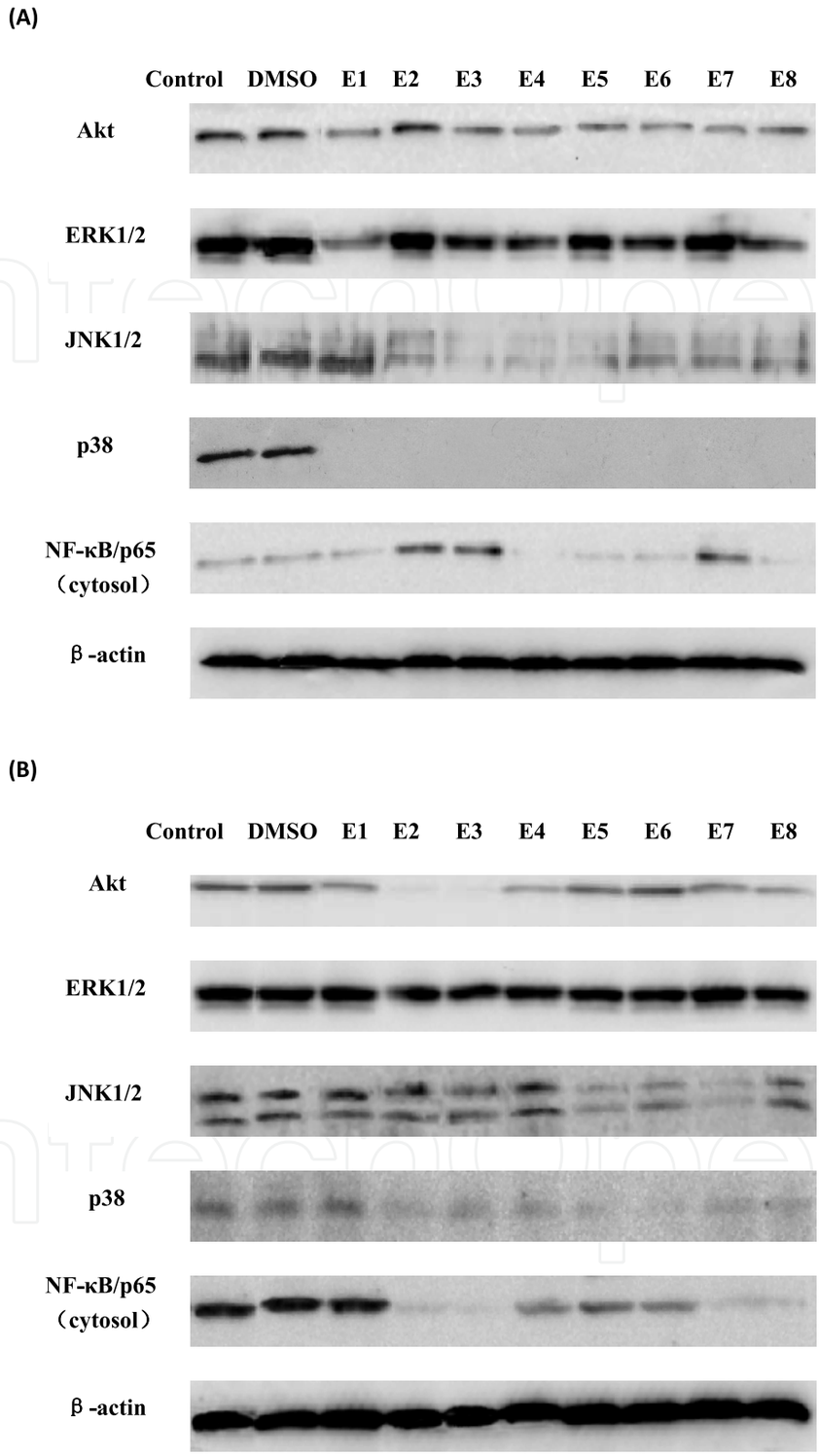


Fig. 3. (A) Inhibitory effects of JFE on tumorigenesis pathways in human lung carcinoma A549 cells (B) Inhibitory effects of JFE on tumorigenesis pathways in human lung carcinoma H157cells

The immunoblot analysis demonstrated that JFE (E1–E8) treatment could induce (i) inhibition of AKt, (ii) inhibition of ERK1/2 (p44 and p42), (iii) inhibition of JNK1/2 (p54 and p46), (iv) inhibition of p38 protein and (v) regulation of NF-kB.

3.3 Inhibition on the growth of human lung carcinoma A549 and H157 cells by JIN formula in nude mice

JFE inhibits the development of lung tumorigenesis by modulating and inhibiting PI3K/Akt, MAPK, and NF-kB signaling. To establish the relevance of these in vitro findings to in vivo situation, athymic nude mice were implanted with human lung carcinoma A549 and H157 cells. Compared with model group, Jin formula could significantly inhibit volume growth of A549 and H157 xenografted tumors. The inhibiting rate could reach to 28.5% and 25% in A549 and H157 tumor-bearing mice respectively ($p < 0.05$). We found that the oral administration of JIN formula significantly slowed the progression of A549 and H157 tumor growth in nude mice.

4. Discussion

Lung cancer as the most common cancer in the world represents a major public health problem. Worldwide it accounts for 1.18 million cancer-related deaths and is the most common cause of cancer death in both men and women (13). Lung carcinogenesis is a chronic and multistep process resulting in malignant lung tumors. This progression from normal to neoplastic pulmonary cells or tissues could be arrested or reversed through pharmacological treatments. These therapeutic interventions should reduce or avoid the clinical consequences of lung cancer by treating early neoplastic lesions before the development of clinically evident signs or symptoms of malignancy. Preclinical, clinical and epidemiological findings relating to different classes of candidate chemopreventive agents provide strong support for lung cancer prevention as a therapeutic strategy (14). Cancer chemoprevention is an attractive approach to reduce lung cancers by treating early steps in lung carcinogenesis. There is a convergence of basic scientific and clinical findings in lung cancer chemoprevention. Pharmacological interventions also can be used to reverse or arrest the progression of lung carcinogenesis. For this reason, additional clinical trials are needed that emphasize a mechanistic approach in which mechanisms identified in vitro can be validated in vivo.

This study was designed to show the chemopreventive/chemotherapeutic potential of JIN formula against lung cancer. we assessed the efficacy of the JIN formula, which is an ancient herbal formula from classical book JIN KUI YAO LUE (Golden Chamber) for the treatment of lung cancer and explored its probable molecular mechanisms. Initially, employing human lung carcinoma A549 cells, we compared the growth inhibitory effects of JIN formula extracts (JFE, including E1 to E8). The results showed that E6 and E7 could decrease the viability of A549 cells by the MTT assays. To explore the mechanism of antiproliferative effects of JIN formula against lung cancer, we investigated the involvement of Akt, ERK1/2, JNK1/2, p38 and NF-κB pathway during the inhibition of tumorigenesis by JFE in A549 and H157 cells. The immunoblot analysis demonstrated that JFE treatment could result in different degree inhibition of above signaling pathways on tumor promotion processes. Oral administration of JIN formula (3%, wt/vol) to athymic nude mice implanted with A549 and H157 cells resulted in a significant inhibition in tumor growth.

In conclusion, the present study demonstrates that human non small cell lung cancer A549 and H157 cells are highly sensitive to JIN formula both in vitro and in vivo experimental models. JIN formula inhibits the development of lung tumorigenesis by modulating the imbalance of

proliferative and apoptotic signaling network. Based on the present study it is tempting to suggest that JIN formula and its effective extracts have strong potential for development as a chemopreventive and possibly as a chemotherapeutic agent against lung cancer.

5. Acknowledgements

This study was supported by National Science & Technology Pillar Program in the 11th Five year Plan of China (2006BAI11B08－01) and the Priority Academic Program Development (PAPD) of Jiangsu Higher Education Institutions.

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Recent Advances in Theories and Practice of Chinese Medicine

Edited by Prof. Haixue Kuang

ISBN 978-953-307-903-5
Hard cover, 504 pages
Publisher InTech
Published online 18, January, 2012
Published in print edition January, 2012

During the recent years, traditional Chinese medicine (TCM) has attracted the attention of researchers all over the world. It is looked upon not only as a bright pearl, but also a treasure house of ancient Chinese culture. Nowadays, TCM has become a subject area with high potential and the possibility for original innovation. This book titled Recent Advances in Theories and Practice of Chinese Medicine provides an authoritative and cutting-edge insight into TCM research, including its basic theories, diagnostic approach, current clinical applications, latest advances, and more. It discusses many often neglected important issues, such as the theory of TCM property, and how to carry out TCM research in the direction of TCM property theory using modern scientific technology. The authors of this book comprise an international group of recognized researchers who possess abundant clinical knowledge and research background due to their years of practicing TCM. Hopefully, this book will help our readers gain a deeper understanding of the unique characteristics of Chinese medicine.

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Yuhui Zhou, Fei Xiong, Zhenzhou Huang, Luyu Zheng, Miao Jiang, Ming Jiang, Yuping Tang, Jian Ma, Zhen Zhan, Jinao Duan and Xu Zhang (2012). JIN Formula Inhibits Tumorigenesis Pathways in Human Lung Carcinoma Cells and Tumor Growth in Athymic Nude Mice, Recent Advances in Theories and Practice of Chinese Medicine, Prof. Haixue Kuang (Ed.), ISBN: 978-953-307-903-5, InTech, Available from: http://www.intechopen.com/books/recent-advances-in-theories-and-practice-of-chinese-medicine/jin-formula-inhibits-tumorigenesis-pathways-in-human-lung-carcinoma-cells-and-tumor-growth-in-athymi

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