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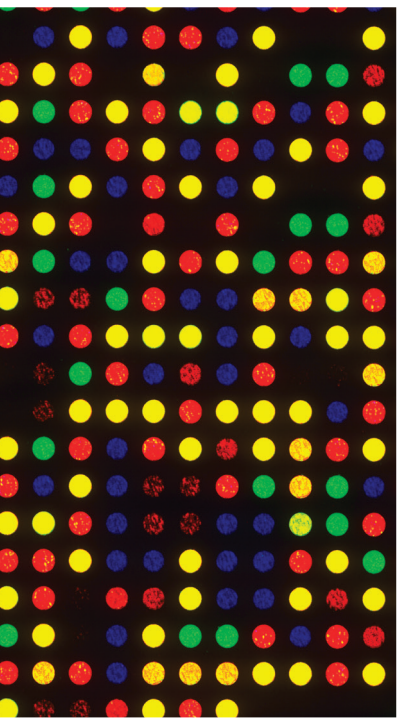
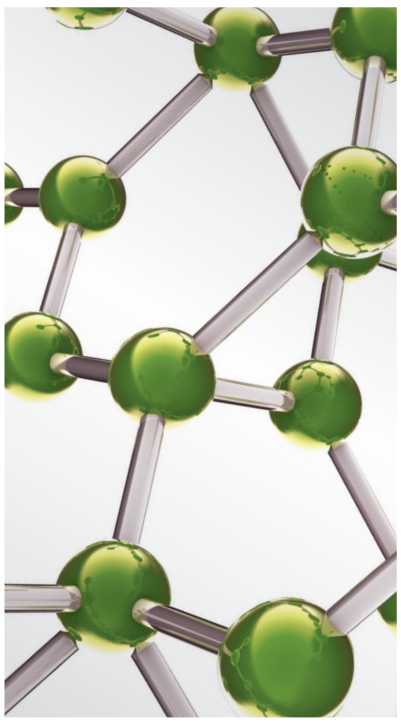
Complementary and Alternative Therapies for Liver Diseases 2014

Guest Editors: Yong-Song Guan, Qing He, and Mohammad Ahmad Al-Shatouri



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Editorial Board

Contents

Hindawi Publishing Corporation
Evidence-Based Complementary and Alternative Medicine
Volume 2015, Article ID 476431, 1 page
http://dx.doi.org/10.1155/2015/476431

Editorial

Complementary and Alternative Therapies for Liver Diseases 2014

Yong-Song Guan,[1] Qing He,[1] and Mohammad Ahmad Al-Shatouri[2]

[1]*Department of Oncology and Radiology, West China Hospital of Sichuan University, Chengdu, Sichuan 610041, China*
[2]*Suez Canal Medical School, Suez Canal University, Ismailia 41522, Egypt*

Correspondence should be addressed to Yong-Song Guan; yongsongguan@yahoo.com

Received 24 April 2015; Accepted 24 April 2015

Liver diseases are now increasing, especially the chronic ones. The therapy of liver diseases is often challengeable because conditions of the liver are complicated in both structure and function. Different pathological liver conditions are associated with reactive oxygen species, viruses, toxins, inflammation and infection, and others. The resultant liver fibrosis and liver cirrhosis are a problem that every year thousands of people suffer from and many doctors are puzzled by. Liver cirrhosis, a potential cause of liver cancer, has long been considered irreversible or extremely difficult to reverse.

There has been a good deal of interest in complementary and alternative therapies for the treatment of various liver diseases. Patients generally appreciate the treatment of liver diseases with natural agents because of their diverse effects and mild side effects. One natural agent commonly has multiple therapeutic effects including antioxidant, anti-inflammatory, antiviral, and antitoxic properties. The selection of a plant or plants as a medicine needs sufficient data and definite proof of the efficacy in liver diseases.

This special issue provides original research, review, and meta-analysis about the recent information on complementary and alternative therapies for liver diseases involving *ex vivo*, *in vivo,* and clinical studies. A number of natural agents are deeply investigated and analyzed for the treatment of liver diseases on the basis of evidence in the aspects of cell signal pathways and other molecular mechanisms and clinical practice.

Readers of this journal are expected to find in this special issue recent developments not only in treatment but also in prevention of liver diseases by complementary and alternative methods. The readers might be interested in studies on Fuzheng-Huayu Formula against liver fibrosis and cirrhosis by developing healthy liver cells to replace the damaged ones. The cure of a cirrhotic liver is no longer like getting blood out of a stone.

Yong-Song Guan
Qing He
Mohammad Ahmad Al-Shatouri

Hindawi Publishing Corporation
Evidence-Based Complementary and Alternative Medicine
Volume 2015, Article ID 824185, 10 pages
http://dx.doi.org/10.1155/2015/824185

Review Article

Plants Consumption and Liver Health

Yong-Song Guan[1,2] and Qing He[1,2]

[1]*Department of Oncology, West China Hospital, Sichuan University, Chengdu 610041, China*
[2]*State Key Laboratory of Biotherapy, West China Medical School, Sichuan University, Chengdu 610041, China*

Correspondence should be addressed to Yong-Song Guan; yongsongguan@yahoo.com

Received 29 August 2014; Accepted 13 October 2014

Academic Editor: Mohammad Ahmad Al-Shatouri

The liver is a very important organ with a lot of functions for the host to survive. Dietary components are essential for and can be beneficial or detrimental to the healthy or diseased liver. Plants food is an essential part of the human diet and comprises various compounds which are closely related to liver health. Selected food plants can provide nutritional and medicinal support for liver disease. At the present, the knowledge of the effects of plants on the liver is still incomplete. The most urgent task at the present time is to find the best dietary and medicinal plants for liver health in an endless list of candidates. This review article updates the knowledge about the effects of plants consumption on the health of the liver, putting particular emphasis on the potential beneficial and harmful impact of dietary and medicinal plants on liver function.

1. Introduction

As a proverb goes, "a closed mouth catches no flies." Selecting the best food for the mouth is essential for good health, especially the health of the liver. The liver is the largest digestive gland in the body playing a major role in metabolism of various substances. The liver is also under the great load of conducting various functions for the survival of the host, including detoxification, breakdown of red blood cells and substances, synthesis of proteins and hormones, and storing glycogen, as well as holding a reservoir of blood [1, 2]. Any damage that weakens the functioning of the liver is called liver disease including liver cancer [3, 4]. Currently food of plant origin is consumed more frequently for human health and leafy plants or plant parts are eaten usually as vegetables [5]. Generally speaking, all the plants, plant parts, and their ingredients which we ingest are related to the health of the liver because of the existence of enterohepatic circulation and hepatic detoxification [6].

Dietary and medicinal prevention or treatment of liver disease by plant-based stuff is an essential constituent of complementary and alternative medicine [7]. Human has a long history of consuming edible plants for food and survival and now still consumes a wide variety of wild and semidomesticated food plants, domesticated crops, vegetables, fruits, and plant food supplements, as well as plants for medicinal use [8–11]. In spite of the long history and wide distribution of use, the knowledge of the impact of these plants on the liver remains incomplete [12]. In addition, the known knowledge of botany-hepatology as a discipline can be used to learn new knowledge in the era of molecular medicine, and many of the traditional views and opinions gained through experience need to be confirmed by modern technology on the basis of evidence [13–16]. In a tremendous body of countless plants, finding the best ones with edible or potable elements for liver health presents too much a challenge to the researchers of liver disease. Based on the most recent literatures in the area, this paper reviews the impact of plants consumption on the health of the liver, with special emphasis on the positive and negative influence of dietary and medicinal plants on liver function.

2. Wild and Semidomesticated Food Plants Good for Liver

There is a very long history of consumption of domesticated and cultivated food plants including crops, fruits, and vegetables. People should have gained a lot of knowledge about the effects of these foods on human health. For example, the

Table 1: Simple grouping of antioxidants for liver health.

Group	Name	Characteristics	Effective in liver disease
Enzymatic	Catalase	Very common antioxidant	Alcoholic liver disease
	Superoxide dismutase	Both endo- and exogenous	Chronic liver injury
	Peroxidases	Neutralizing hepatotoxins	Hepatotoxicity
Nonenzymatic	Reduced glutathione	Neutralizing hepatotoxins	Hepatotoxicity
	Melatonin	Power very strong, versatile	Nonalcoholic fatty liver disease
	Ebselen	Glutathione peroxidase analog	Alcoholic liver disease
	Vitamin C	Cofactor in enzyme reactions	Viral hepatitis, cirrhosis
	Vitamin A	Enhancing immunity	Cirrhosis, steatohepatitis
	Vitamin E	Richest in flaxseed oil	Hepatitis B, nonalcoholic fatty liver disease
	4-Hydroxynonenal	Cell signal transduction	Alcoholic liver disease
	Malondialdehyde	Potentially mutagenic	Acute liver cell injury

earliest domestication of common millet in East Asia can be dated to the Neolithic era 10,000 years ago [8]. However, both animal and human studies on many of these plants, like berries, carrot, grapes, ginger, green tea, pistachio, pomegranate, tomato, and wheat, have yielded conflicting results; thus, it is now still very hard to recommend what is the best estimate of the amount of these plants a person consumes for liver health [1, 3, 4].

The wild and semidomesticated food plants are now consumed as supplements to the domesticated foods and as main foods to suppress hunger at times of food shortage in underdeveloped world [10]. For example, the Northeast region of Thailand is regarded as the largest and poorest portion of the country. In anthropogenic areas there, wild food plants are a fundamental part of the diet for vulnerable farmer households and acquisition of wild food plants improves the readiness of seasonal crop throughout the year [17]. Forms of wild food plants include aquatic herb, bamboo, climber, rattan, terrestrial herb and tree, and edible parts of these plants cover shoot, flower, fruit, whole plant, leaves, cladode, seed, tuber, rhizome, stalk of flower, and stem [17, 18].

Reduction-oxydation (redox) state represents a crucial background of various liver disorders. There is always the paradox of oxygen use in metabolism for the existence of life. On one hand, oxygen is fundamental for the organism to survive. On the other hand, oxygen as a strong reactive molecule devastates the organism by generating reactive oxygen species. The organism in turn develops an antioxidant network to prevent this damage. Oxidation reaction can generate free radicals which can damage cell membranes and cause diseases. The imbalance between production of reactive oxygen species and the system's defense represents oxidative stress which is one of the essential pathogenic factors in numerous liver diseases including inflammatory, metabolic and proliferative ones and antioxidants are chemicals rich in many food plants and can be used as prevention and treatment of such diseases [19]. Almost all chronic liver diseases are under the background of elevated oxidative stress. The organism maintains various systems of antioxidants which could be simply divided into enzymatic and nonenzymatic categories (Table 1).

Great sources of essential antioxidants are foods rich in vitamin C, vitamin E, and trace element selenium [1]. Some nonessential substances from plant food origin also have antioxidant activity, such as ascorbic acid, β-carotene, coenzyme Q10, curcumin, dong quai (*Angelica sinensis*), ebselen, ellagic acid, epigallocatechin gallate, lipoic acid, lycopene, mitoquinone, N-acetyl cysteine, quercetin, and resveratrol [20–23]. Minor dietary nonnutrients in plant foods also have notable activity in cancer prevention by their effects of suppression or block or both on carcinogenesis. Inhibitors with suppressing effect prevent development of tumorigenesis in cells that would become cancerous under other circumstances while those with blocking effect prevent cancer-causing agents from contacting or responding with all-important target sites [3, 24].

Redox state activates the innate immune system and elevates the discharge of proinflammatory cytokines and other mediators for the establishment of alcoholic liver disease and nonalcoholic fatty liver disease. And oxidative stress also encourages the progression from steatosis to steatohepatitis [25]. Redox state impacts on brain function in hepatic encephalopathy by overproduction of reactive oxygen and nitrogen oxide species [26]. Fibroproliferative liver diseases can result from disturbance of redox homeostasis by activation of myofibroblast-like, hepatic stellate cells, and other profibrogenic cells [27]. Animal and human studies have shown that both hepatitis B virus (HBV) and hepatitis C virus (HCV) stimulate oxidative stress in liver cells with elevated oxidative DNA damage. In addition, both viruses disturb the response of liver cells to oxidative DNA damage with consequent genome instability and cancer formation [28]. Several RNA viruses including HCV are reported to induce oxidative stress with changes in host defense modulated by antioxidants [29].

Phytoestrogens are dietary plant estrogens that are not produced in the endocrine system but obtained by consuming phytoestrogenic plants. These nonsteroidal plant compounds are naturally occurring and similar in structure to estradiol with estrogenic and/or nonestrogenic effects exerted by binding to estrogen receptors. A group of phytoestrogens in the Coumestan family has been proved to be anti-HCV

agents by inhibiting viral RNA replication [30]. One of the phytoestrogens that is called genistein falls into the category of isoflavones and has been demonstrated at the molecular level to have similar therapeutic effect to interferon-α on HBV infection [31].

Plant foods richest in phytoestrogens are oilseeds and nuts. The most frequently encountered sources of phytoestrogens are the plants of the legume family [32]. Such plant foods from wild and semidomesticated origin include alfalfa, anise, chaste-tree berry, *Dunbaria villosa*, fennel, fenugreek, ginkgo, ginseng, hops, kava, kudzu, lentil, licorice root, lupine, mint, psoralea, red clover, saw palmetto, and wild yam [33, 34]. Nevertheless, evidence is lacking for therapeutic use of these plants, and clinical trials are needed with concerns for long-term safety and efficacy.

Other hepatoprotective plant foods from wild and semidomesticated origin consist of amaranth, *Aralia elata Seem*, asparagus, balloonflower root (*Platycodon grandiflorus*), buckwheat, capillary wormwood, celery, chestnut, Chinese chive, Chinese small iris *(Iris lactea)*, Chinese toon, heartleaf (*Cordate houttuynia*), cress, dandelion, daylily, devil's tongue (*Lilium brownii*), hawthorn, hazelnut, kelp, kiwi fruit, longan, longstamen onion bulb, lotus root, mango, Manyflower Gueldenstaedtid herb (*Herba Gueldenstaedtiae*), olive, papaya, philippine violet herb (*Herba violae*), purslane, red date, rivier (*Rhizoma amorphophalli*), shepherd purse, sow thistle, spring bamboo shoots, summer squash, tangerine, tzu tsai (*Porphyra haitanensis*), wild bracken, and yam [35–39]. The list is still growing and in-depth studies on different phytonutrients are warranted for rationale consumption of these plant foods to improve liver health. Well-designed randomized clinical trials are needed.

3. Wild and Semidomesticated Food Plants Harmful to Liver

Phytoestrogens are now used for estrogen replacement as complementary and alternative therapy of several conditions. For example, black cohosh is widely advertised as bust-enhancing product and prescribed for menopausal symptoms and pain relief. However, this product has been associated with liver toxicity [40]. Many of the dietary plants associated with phytoestrogens are substrates for a genus of fungi called Fusarium to produce zearalenone which is a potent estrogen and has strong genotoxicity and liver toxicity [34, 40]. Excessive phytoestrogens have adverse effects not only on the reproductive system but also on the liver [41]. Use of food containing phytoestrogens is generally safe. However, estrogen-like effects are observed and increased with prolonged use [42]. A safety study on a phytoestrogen called genistein in Wistar rats demonstrated that very slight proliferation of bile duct, increased gamma glutamyl transferase, and hypertrophy of liver cells were observed at repeated doses of 500 mg/kg/day [43]. Another study of genistein effects on Wistar rats found that the phytoestrogen has strong impact on hepatic gene expression [44]. Phytoestrogens also have been shown to induce gene activation in human liver hepatocellular carcinoma cell line HepG2 cells [45]. So, researches are needed to acquire knowledge of avoiding particular components of plant-based foods for liver health.

Pesticide residue in wild plant foods is highly hepatotoxic and leads to change in metabolism and oxidative balance in the liver [46]. Fumigated or grilled foods are also harmful. A study showed that fumigation residues bound on seeds were highly bioavailable to experimentally fed animals with resultant hepatic injury [47]. Animals that were fed on a diet consisting of grilled foods showed with elevated serum levels of cholesterol, aspartate transaminase, creatinine, and urea and many kinds of chromosomal aberrations in examined cells [48]. Foods can go bad easily in summer. Deteriorated and rotten foods are full of molds and fungi and are dangerous to eat. One study examined the effects of decayed foods on the liver in rats. Male Wistar rats were fed with a diet containing gluten thermally processed with oil spontaneously for 11 weeks and damage in the liver occurred subsequently [49]. Rotten ginger is strongly poisonous. Rotting ginger produces a highly toxic substance safrole known as natural hepatoxin which leads to liver cell degeneration and necrosis and may induce liver cancer as tumorigenic effects in the liver were shown after long-term exposure of animals to other plants [50, 51]. Rotten potatoes as well as other food plants also have potent toxic effects on the liver through the intake of mycotoxins because rotting plants are frequently infected with *Fusarium spp.* [33, 40, 52].

Cassava is a woody shrub and is widely consumed for its starchy tuberous root as food in Latin American, Caribbean, African, and Asian countries. It is the third major source of dietary carbohydrates in the tropical zone, following rice and maize. However, people consume cassava excessively or incorrectly are at risk of poisoning. Goats fed with cassava leaves for 30 consecutive days showed toxic effects of cyanogenic glycosides with vacuolation of periportal hepatocytes [53]. Liver cancer has also been associated with this food plant [54, 55].

Currently literature is limited about liver damage induced by food plants but evidence will continue to accumulate for the effects of dietary components on the liver. The potential hazards of nightshades to liver health are described in the next section.

4. Medicinal Use of Food Plants for Liver Health

For thousands of years people have the belief of food as medicine and medicine as food [56]. A commonest English axiom reads, "an apple a day keeps the doctor away." Apples were one of the earliest foods that medical specialists accepted as beneficial and healthy. Apple polyphenol extract has been shown to have hepatoprotective effects on liver oxidative stress which was induced by aluminum chloride in the rat [57]. Tamoxifen is a nonsteroidal antiestrogen and has been used to induce oxidative stress in rats showing increase in aminotransferases. This effect was reduced significantly by a food product consisting of dried apple and mandarin juice [58]. Table 2 briefly lists medicinal use of common plant foods for liver health.

Table 2: Medicinal use of common plant foods for liver health.

Category	Common name	Botanical name	Special active elements	Benefits to liver
Vegetables	Beets	*Beta vulgaris*	Betaine	Chloretic
	Broccoli	*Brassica oleracea*	Diindolylmethane, glucoraphanin	Antiviral, anticancer
	Carrots	*Daucus carota*	Beta carotene and other carotenoids	Antioxidative activity
	Collard greens	*Brassica oleracea*	Diindolylmethane, sulforaphane	Anticancer, anti-inflammation
	Kale	*Brassica oleracea*	A group of resins	Lowering cholesterol and fat
	Sweet potato	*Ipomoea batatas*	Beta carotene, fiber	Attenuating liver injury
	Yams	*Dioscorea alata*	Diosgenin	Inhibiting hepatomegaly
	Cabbage	*Brassica oleracea*	Glucosinolates	Countering alcohol, hangover
Fruits	Avocado	*Persea americana*	Adiponectin	Hypolipidemic activity
	Banana	*Musa acuminata*	Pectin	Relieving cirrhosis
	Cherry	*Prunus avium*	Methyl jasmonate	Antioxidant activity
	Fig	*Moraceae ficus*	Fumaric acid, ficin	Antifatty liver action
	Lemon	*Citrus limon*	Naringin, citric acid	Decreasing liver damage
	Papaya	*Carica papaya*	Lycopene, danielone	Antioxidative activity
	Pomegranate	*Punica granatum*	Punicalagins (pomegranate ellagitannins)	Anticancer
	Watermelon	*Citrullus lanatus*	Citrulline, lycopene	Antitoxic, hypoglycemic
Grains	Barley	*Hordeum vulgare*	Caffeic acid, *p*-coumaric acid	Antifatty liver action
	Maize	*Zea mays*	Lutein, linolic acid	Antioxidative activity
	Brown rice	*Oryza sativa*	Anthocyanins, tocopherols	Anti-inflammatory effects
	Oat	*Avena sativa*	Ergothioneine	Antioxidative activity
	Wheat	*Triticum stivum*	Alkylresorcinols, ferulic acid	Increasing lipid metabolism
	Sorghum	*Sorghum bicolor*	*p*-Hydroxybenzaldehyde, methyl ferulate	Antioxidative activity

Green leaves are the best for liver health. There is the saying in traditional Chinese medicine: "the dark-green colored falls into liver meridian." A flavone glucoside named as saponarin has been extracted from young green barley leaves. This flavonoid gives the typical green color to the leaves and demonstrates powerful antioxidant potencies with therapeutic effects on various cancers and inflammations [59]. *In vivo* studies proved that green tea leaves have strong inhibitory activity for liver cancer. Camella sinensis is a common Chinese green tea. Alcoholic extract of the leaves of this plant was prepared and given by gavage to Wistar rats bearing Walker-256 liver cancer. Strong antitumor activity was achieved in rats that received the treatment with the green tea extract [60].

Several categories of food plants have chemopreventive effects on carcinogen-induced neoplasia. They are cruciferous vegetables, citrus fruits, caraway (*Carum carvi*) seed oils, and *Allium* species [4, 61]. Cruciferous vegetables are green leafy veggies including bok choy, broccoli, cabbage, cauliflower, and cress. A large integrated series of case-control studies consisting of 1468 cancers presented supporting evidence of favorable effect of these food plants on several common cancers [62]. Citrus is the general name for many flowering plants cultivated since ancient time. The well-known citrus fruits are the grapefruit, lemons, limes, mandarins, and oranges. Citrus fruit oil was reported to improve hepatotoxicity in chickens fed with a diet containing aflatoxin, a potent hepatocarcinogen, showing reduced lesions of hydropic degeneration and bile duct hyperplasia in the liver [63]. Caraway is also called meridian fennel and has long been used as a valuable aromatic herb and a spice in food to enhance flavors. This plant shows a large range of antimicrobial activities especially distinct inhibitory effects on growth of fungi and aflatoxin production. Caraway seed oils are commonly employed as household medicine for many ailments including hepatobiliary complications [64]. *Allium* is the term for garlic in Latin language and represented unofficially as the onion genus. Food plants in the *Allium* genus include different chives, garlics, leeks, onions and scallions. They present various flavors and mouthfeels and are consumed either cooked or raw all over the world in different delicacies. A number of studies both *in vitro* and *in vivo* have been published reporting that allium-genus plants have potent hepatoprotective activity and distinct effects on various liver conditions such as hypercholesterolemia-induced oxidative stress, cadmium liver accumulation, liver fibrosis, liver fluke, and alcoholic fatty liver [65–69].

Patients with liver disease are advised to avoid nightshade plants which are the common name for the Solanaceae family that consists of more than 2800 plants. Well-known nightshades include eggplant, ground cherries (any of the genus *Physalis*), mandrake (*Mandragora officinarum*), peppers, pimentos (*Capsicum annuum*), potatoes, tobacco, tomatillos (*Physalis ixocarpa*), and tomatoes. Animal study of several nightshades resulted in the conclusion that the plants are hepatotoxic showing amyloidosis and moderate necrosis in liver [70]. Another nightshade plant (*Solanum cernuum Vellozo*) was also involved in hepatic toxicity when it was used in high dose and significant increase in the activities of alanine aminotransferase and aspartate aminotransferase

was observed [71]. Jimson weed (*Datura stramonium*), also known as thorn apple, is a nightshade plant having spiny capsule fruits. Jimsonweed is ingested by some people to enjoy hallucinations that this plant can cause. However, jimsonweed is strongly poisonous and sometimes fatal. Jimsonweed intoxication can lead to fulminant hepatitis and acute liver failure requiring subsequent liver transplantation for salvage [72, 73].

Manufacturers of dietary supplements usually cannot provide clinical data supporting their claims of safety or efficacy [16]. Physicians and the general public must take care of drug-food interactions and potential adverse effects when plant-based foods are used for medicinal purpose [74]. A physician must know as more as possible pharmacokinetic interactions of phytochemicals with drugs although currently our knowledge about nutrient-drug interactions is still limited and efforts to elucidate them should be reinforced [75]. Laxative plants can be used to clear the ingested toxins away from the digestive system. Such plants include aloe vera, dandelion, rhubarb rhizome and senna leaf [76]. The mung bean or moong bean is also known as green gram and is regarded as a detoxification agent for thousands of years in both in Traditional and folk Chinese medicine. Mung bean accelerates metabolism and transformation of toxins in food and drug by special enzymes which involve in the biosynthesis of phenolic compounds. Mung bean sprout produces several kinds of hepatoprotective compounds such as flavonoid and chlorogenic acid [77]. An aldehyde reductase has been extracted from mung bean that detoxifies fungal toxins [78]. Radish is in the Cruciferae family and also known as "Laifu" or "Luobo" in Chinese that has a history of being used for medicinal purpose for more than a thousand years. Spanish black radish comprises unique glucoraphasatin which has been proved to be a potent inducer of detoxification enzymes in liver cancer cell [79]. The degradation products of glucoraphasatin, such as sulforaphene, raphasatin and glucoraphenin, are also liver detoxification enzymes although not as potent as glucoraphasatin [80].

5. Liver Disease Herbs for Specific Therapy

Phyllanthus urinaria is an herbal medicine with potential antioxidative properties and has been proved to improve steatohepatitis both in cell cultures and in mice, perhaps via decreasing oxidative stress, relieving inflammation, and reducing lipid accumulation [14]. A trial of 1145 participants examined the effects of silymarin, an herbal product extracted from milk thistle (*Silybum marianum*), on patients with advanced chronic hepatitis C. The results showed that use of silymarin had no effects on hepatitis C virus RNA levels or serum alanine aminotransferase, but that better quality-of-life indices and fewer hepatic symptoms were observed in the silymarin users [15]. Curcuminoids have been proved to safeguard DNA against reactive oxygen species and protect liver cells in the time of liver damage and cirrhosis [81].

Simaroubaceae (*Picrasma quassioides*) is a family of tropical trees and shrubs and has been shown to be protective for carbon tetrachloride-induced liver injury and effective in treating liver cancer in moderate and late stage by comprehensive Chinese medicine with extended pain-relieving sustained time, improved quality-of-life, prolonged survival, and less adverse effects [82].

There are a great variety of live disease herbs all over the world and about 80% of the world population use herbal medicine. Elucidation of medicinal properties and hepatoprotective compounds of these herbs is of principal significance [83]. Herb induced adverse effects on liver functions are called herbal hepatotoxicity and there have been public concerns of the use of herbs. Although a large number of herbs and plant products have been involved in the causation of liver injury, the majority of the issues in causality is not yet validated and lack of enough evidence [13]. The establishment of the definite diagnosis for toxic liver disease needs hard evidence or at least sufficient supporting evidence. These reports often fall short of necessary diagnostic details and the methods for evaluating causality are nonspecific. Many external factors, such as difference in batches, adulterants, impurities, and misidentification of plant species, lead to the negative results of assessment [13]. The judgment system of herbal hepatotoxicity has to be improved for future research.

6. Amount and Methods of Consumption and Combination Use of Plants

It is of paramount importance to eat a well-balanced diet for liver health. A recent study conducted in Japan investigated the effects of well-balanced lunches on liver function. The lunch was low in animal protein and high in vegetables. This diet was provided to 10 subjects once a day for 1 month. At last, serum alanine aminotransferase status of the subjects reduced by 20.3% [84]. Although we are talking about plants consumption here, the role of animal protein and fat in improving liver health cannot be neglected and the amount of plant components in the food should be well regulated as a diet rich in sucrose was shown to cause inflammation and liver damage in mice [85]. The oxidative efficiency declines along with the age growth of the individual and consequently some chemical substances that need oxidation might accumulate to cause toxicity [86]. Simultaneous use of drugs with herbs may imitate, intensify, or counter the effect of drugs resulting in herb-drug interactions, and prediction and identification of such interactions present challenges for health professionals involved in the management of liver disease [50, 87].

Coleus forskohlii extract is a natural herbal product commonly used to offset obesity and induces hepatic drug metabolizing enzymes [12]. This induction is enhanced in mice by the amount of dietary starch, implying that the combination of *coleus forskohlii* extract and food rich in starch further increases enzyme activity of the liver. Hepatotoxicity of this plant is dose-related and hepatic cytochrome P450 enzyme is induced significantly [88].

The processing method of plant food is usually the key point for liver health [89]. For example, brown rice is well known as a healthy food. However, the therapeutic effect will be highly decreased when brown rice is simply steamed

or cooked over for a patient with liver disease to take. The best way of processing it is preparing rice gruel, a very thin porridge that has been known as "congee" and applied in China for thousands of years to the treatment of digestive diseases. Brown rice congee is easy to digest and helpful for the liver to recover naturally [90]. But this procedure demands time, patience, and skill. Laba porridge, also known as babao gruel, is highly nutritious and famous meal eaten on the day that by folk legend Prince Siddhartha attained the top of enlightenment and became Buddha after eating this congee [91]. Babao means eight treasures and is made of eight elements. Today there are dozens of recipes for this dish using various nutritious and therapeutic ingredients such as glutinous rice, red bean, mung bean, black soybean, peanut kernel, sorghum, foxtail millet, brown rice, red date, Job's tears, lotus seed, lily bulb, and raisin. Each of the components represents a different medicinal use and many of them have hepatoprotective activities [92, 93]. There are several different ways in which plants are prepared and used for therapeutic purpose like eating fresh raw plant, boiling, steaming, sauteing, pickling, oven curing, country curing, solar drying, shade drying and mechanical drying. There are remarkable differences between the methods of preparing plants in effects on liver health. For example, broccoli is best cooked by lightly steaming or stir-frying to preserve the most of its natural nutrients, while boiling or brewing will let the most important nutrients to come into the cooking fluid [21, 75, 89].

Although nightshades show antitumor activity, the mild hepatotoxicity is of concern as stated above. The effects of concomitant consumption of several nightshades on the liver need investigating. Poisoning by black nightshades is of particular concern because a recent investigation from New Zealand listed this plant as the commonest one in the 15 common poisonous plants [94].

The liver can be detoxified and cleansed by drinking more water. Good hydration is important for most basic physiological functions of the liver. Sufficient hydration is required to promote blood circulation and to dissolve nutrients. An adequate intake of water encourages metabolism and facilitates biliary secretion of bile, the process of digestion, absorption, and excretion of wastes to reduce the impairment of the liver by poisoning metabolites and toxins [95]. However, drinking too much water has negative impact on the liver and can be lethal. Acute hypoosmolarity leads to protein conservation related to impaired insulin sensitivity to glucose metabolism, increased lipid oxidation, lipolysis, and ketogenesis [96].

A well life-style is the best medicine. A balanced diet is necessary and binge overeating or frequent starvation should be avoided. Imbalanced food habits result in abnormal secretion of digestive juices and hepatic dysfunction [97]. Good sleep is also very important for liver health. Patients with cirrhosis often have sleep-wake abnormalities and they are observed sleeping significantly less well than healthy subjects [98]. A recent study from Seoul investigated the association of sleep quality and duration with nonalcoholic fatty liver disease in middle-aged males and females. Confounding factors such as alcohol drink and smoking were excluded from the cohort. Poor sleep quality and short sleep duration significantly increased the incidence of this liver disease [99]. Obstructive sleep apnea is now associated with liver injury that is caused by intermittent hypoxia. So sleep on the left or right side may be better for liver health than on the back [100]. Good temper is another great medicine for liver disease. The Traditional Chinese Medicine believes that anger leads to troubles of the liver. Acute stress showed significant impacts on gene expression and function of the liver in the rat, proving "raged impairing liver" [101]. Findings of rage experiences were obtained in HCV patients with evidence showing greater rage was in relation to poorer quality-of-life as negative feedback of the disease [102]. Patients with liver disease are suggested to take limited spicy food. Although curcumin is regarded as a great hepatoprotective product and has now been used in many countries as a supplement, and dietary spice turmeric has been consumed for medicative use for thousands of years, the natural turmeric has been found to contain up to 200 compounds of which the most are toxigenic. In addition, curcumin and its by-products may generate dose dependent hepatotoxicity [103].

7. Conclusions

First, do no harm. Various plants are consumed for dietary and medicinal use as wild, semidomesticated, and cultivated crops, vegetables, fruits, and herbs. It is necessary to increase availability of plants safety data to the general public and medical professionals. Safety concerns for plant consumption are rationale because of lack of evidence obtained from well-designed randomized clinical trials for long-term and large amount use. Herbal hepatotoxicity has been reported many a time involving a large number of herbs and plant products, but the most of the causal relationships are not confirmed and lack of convincing evidence.

A well-balanced diet is critically important for liver health. A healthy life-style includes additionally rejoicing with a merry mind, keeping smoke-free and alcohol-free, having good sleep, and drinking adequate water. The intake amount of a certain plant, method of consumption, and combination of plants could be either hepatoprotective or hepatotoxic. Older age should be considered as a risk factor for accumulated toxicity caused by plant chemicals. Concomitant use of drugs and herbs sometimes leads to herb-drug interactions.

Hepatoprotective plants contain substances with antioxidant activities. Plant sources of antioxidants are essential nutrients, such as vitamins and trace elements, and some nonessential substances. Plant-based antioxidants have preventive and therapeutic effects on various liver diseases including alcoholic liver disease, nonalcoholic fatty liver disease, fibroproliferative liver disease, viral hepatitis, and liver cancer. Cruciferous vegetables, citrus fruits, caraway seed oils, and *Allium* species are chemopreventive for liver cancer. Dietary plant estrogens are very effective in treating viral hepatitis but some of them are associated with liver toxicity caused by fungi contamination. Fumigated, grilled, pesticide-contaminated, or rotten plant foods should be avoided. Excessive or incorrect consumption of cassava is harmful.

Nightshades show mild hepatotoxicity, and poisoning by black nightshades can be lethal and is of particular concern.

Nearly all the foods we consume are associated with the health of the liver. Diets rich in plant ingredients are getting popular now for human health and people are taking supplements from plant origin both in over-the-counter and in prescription form to detoxify and cleanse the liver. Prevention and treatment of liver disease by dietary or herbal method is one of the important components of complementary and alternative medicine. The knowledge of effects of various food plants on liver health is still insufficient. Traditional methods and experiences about use of food plants for treatment of liver disease must be validated by admissible evidence obtained on the basis of modern technology. The immediate challenge is to find the best dietary and medicinal plants for liver health in an infinite list of candidates. All of these topics require further assessment.

Conflict of Interests

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

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Hindawi Publishing Corporation
Evidence-Based Complementary and Alternative Medicine
Volume 2015, Article ID 675270, 9 pages
http://dx.doi.org/10.1155/2015/675270

Research Article

Integrated Treatment of Aqueous Extract of *Solanum nigrum*-Potentiated Cisplatin- and Doxorubicin-Induced Cytotoxicity in Human Hepatocellular Carcinoma Cells

Chien-Kai Wang,[1,2,3,4] Yi-Feng Lin,[5] Cheng-Jeng Tai,[2,3] Chia-Wowi Wang,[1,6] Yu-Jia Chang,[7,8,9,10] Chen-Yen Choong,[1,6] Chi-Shian Lin,[11] Chen-Jei Tai,[1,4,12] and Chun-Chao Chang[3,13]

[1] *Department of Obstetrics and Gynecology, School of Medicine, College of Medicine, Taipei Medical University, Taipei 110, Taiwan*
[2] *Division of Hematology and Oncology, Department of Internal Medicine, Taipei Medical University Hospital, Taipei 110, Taiwan*
[3] *Department of Internal Medicine, School of Medicine, College of Medicine, Taipei Medical University, Taipei 110, Taiwan*
[4] *Department of Chinese Medicine, Taipei Medical University Hospital, Taipei 110, Taiwan*
[5] *Division of General Surgery, Department of Surgery, Chi-Mei Medical Center, Tainan 710, Taiwan*
[6] *Department of Obstetrics and Gynecology, Taipei Medical University Hospital, Taipei 110, Taiwan*
[7] *Cancer Research Center, Taipei Medical University and Hospital, Taipei 110, Taiwan*
[8] *Department of Surgery, Taipei Medical University and Hospital, Taipei 110, Taiwan*
[9] *Division of General Surgery, Department of Surgery, Taipei Medical University Hospital, Taipei Medical University, Taipei 110, Taiwan*
[10] *Graduate Institute of Clinical Medicine, College of Medicine, Taipei Medical University, Taipei 110, Taiwan*
[11] *Graduate Institute of Medical Sciences, College of Medicine, Taipei Medical University, Taipei 110, Taiwan*
[12] *Traditional Herbal Medicine Research Center, Taipei Medical University Hospital, Taipei 110, Taiwan*
[13] *Division of Gastroenterology and Hepatology, Department of Internal Medicine, Taipei Medical University Hospital, Taipei 110, Taiwan*

Correspondence should be addressed to Chen-Jei Tai; chenjtai@tmu.edu.tw and Chun-Chao Chang; chunchao@tmu.edu.tw

Received 26 September 2014; Revised 26 December 2014; Accepted 26 December 2014

Academic Editor: Mohammad Ahmad Al-Shatouri

Chemotherapy is the main approach for treating advanced and recurrent hepatocellular carcinoma (HCC), but the clinical performance of chemotherapy is limited by a relatively low response rate, drug resistance, and adverse effects that severely affect the quality of life of patients. The aqueous extract of *Solanum nigrum* (AE-SN) is a crucial ingredient in some traditional Chinese medicine (TCM) formulas for treating cancer patients and exhibits antitumor effects in human HCC cells. Therefore, this study examined the tumor-suppression efficiency of AE-SN integrated with a standard chemotherapeutic drug, namely, cisplatin or doxorubicin, in human HCC cells, namely, Hep3B and HepJ5. The results suggested that the integrated treatment with AE-SN-potentiated cisplatin and doxorubicin induced cytotoxicity through the cleavage of caspase-7 and accumulation of microtubule-associated protein-1 light chain-3 A/1B II (LC-3 A/B II), which were associated with apoptotic and autophagic cell death, respectively, in both the Hep3B and HepJ5 cells. In conclusion, AE-SN can potentially be used in novel integrated chemotherapy with cisplatin or doxorubicin to treat HCC patients.

1. Introduction

Liver cancer is one of the most common malignant diseases worldwide, particularly in eastern Asia and sub-Saharan Africa, and hepatocellular carcinoma (HCC) is the most prevalent type of liver cancer [1]. A major challenge in treating HCC is the poor prognosis for advanced and recurrent cases.

Although chemotherapy is the main approach used to treat advanced and recurrent HCC cases, its clinical performance is largely limited by various factors such as a relatively low response rate, drug resistance, and various adverse effects that substantially impact the quality of life (QOL) of HCC patients [2]. The development of complementary and alternative medicines for improving the tumor-suppression efficiency of current chemotherapeutic drugs and managing the QOL of HCC patients has become an accepted optional approach worldwide [3, 4]. Traditional Chinese medicine (TCM) has long been employed in treating various cancers through the use of numerous herb-based formulas; however, most of these formulas lack sufficient, basic clinical medical evidence verifying their antitumor efficacy.

TCM formulas are normally prepared using mixed extracts, with the composition and dose of the ingredients sometimes varying among individual cases. The varying composition and dosage cause difficulty in clarifying the antitumor efficacy of the formulas in clinical trials and experimental studies [2]. An alternative approach is examining the individual ingredients from specific TCM formulas that may contribute to the tumor-suppression efficacy. For instance, recent studies have suggested that certain crude extracts in TCM formulas, such as extracts of *Semen Coicis*, *Scutellaria barbata*, and *Solanum nigrum*, exhibited tumor-suppression efficacy in human HCC cells [5–7]. In recent studies, total flavonoids extracted from *Scutellaria barbata* inhibited cell proliferation and invasion of hepatocarcinoma via mediation of matrix metalloproteinases and metalloproteinases [8], and saikosaponin-D extracted from *Bupleurum chinense* DC also was reported to enhance radio sensitivity on hepatoma cells by adjusting cell cycle or hypoxic conditions [9, 10]. These findings suggested that components and crude extracts of some TCM herbs may inhibit hepatocarcinoma cells via various mechanisms. The crude extracts of *Solanum nigrum* have demonstrated antitumor effects in various types of cancer, including human melanoma and colorectal, endometrial, cervical, and breast cancers [11–15]. Previous studies have indicated that the aqueous extract of *Solanum nigrum* leaves (AE-SN) mainly suppressed tumor cell growth by activating programmed cell death associated with caspase-3-dependent apoptosis [7] and LC-3 A/B-related autophagy [7, 11, 12, 14]. In addition, AE-SN is capable of enhancing the cytotoxicity induced by various chemotherapeutic drugs, including cisplatin, doxorubicin, and docetaxel, in human endometrial and colorectal cancer cells [11, 12], suggesting that AE-SN is a potential ingredient to develop for integrated chemotherapy with standard chemotherapeutic drugs. Because cisplatin and doxorubicin are the standard therapeutic drugs for treating HCC cases, knowing the antitumor effects of AE-SN in combination with either cisplatin or doxorubicin in human HCC cells would be beneficial.

To understand the potential of AE-SN for use in integrated chemotherapy with cisplatin or doxorubicin in human HCC cells, the main aim of the present study was to clarify whether AE-SN enhances the cytotoxicity induced by cisplatin and doxorubicin in human HCC cells. The results showed that a single treatment with AE-SN activated programmed cell death and provides insight into the efficacy of integrating AE-SN with chemotherapeutic drugs in treating HCC cells. The study results provide experimental evidence for supporting further application of AE-SN in HCC therapy.

2. Materials and Methods

2.1. Cell Lines and Regents. Two human HCC cell lines, namely, Hep3B and HepJ5, and one normal human pulmonary fibroblast, namely, WI-38, were purchased from the Bioresource Collection and Research Center (Hsinchu, Taiwan). Hep3B is an HCC cell line commonly used to examine antitumor components, and, by comparison, HepJ5 is a more malignant and resistant cell line that exhibits high expression of survivin [16]. This study used these two HCC cell lines to examine the antitumor effects of AE-SN and used WI-38 cells to examine the cytotoxicity of AE-SN in normal human cells. All of the cell lines were maintained in Dulbecco's modified Eagle's medium (Gibco, Grand Island, NY, USA) in addition to 100 U/mL of penicillin and 100 μg/mL of streptomycin (Invitrogen Life Technologies, Carlsbad, CA, USA) at 37°C in a 5% CO_2 humidified incubator. Cisplatin and doxorubicin were purchased from Sigma-Aldrich (St. Louis, MO, USA). The cell lysis buffer was prepared using a solution of 150 mM NaCL, 50 mM Tris-HCL (pH 7.5), 1% NP-40, 0.5% deoxycholate, 0.1% SDS, 1 mM PMSF, 10 μg/mL of leupeptin, and 100 μg/mL of aprotinin. The primary antibodies used to detect protein expression and activation were LC-3 A/B, caspase-3, and caspase-7 (Cell Signaling Technology, Danvers, MA, USA) as well as glyceraldehyde 3-phosphate dehydrogenase (GAPDH, AbFrontier, Seoul, South Korea). A secondary antibody, donkey anti-rabbit horseradish peroxidase conjugate, was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). HPLC-grade acetonitrile (ACN) and methanol were purchased from JT Baker (Phillipsburg, NJ, USA). The purified solamargine was purchased from Tauto Biotech (Shanghai, China).

AE-SN was prepared using the TCM processing method to simulate the AE-SN administered to patients in TCM clinical practice. In brief, 50 g of dried *Solanum nigrum* leaves was immersed in 750 mL of distilled water, gradually heated to 100°C, and maintained at 100°C for 1 h. The AE-SN solution was further condensed to a final concentration of 1 g of raw material/mL through heating at 100°C. The final AE-SN stock solution (1 g/mL) was filtered using a 0.22 μm filter before the experiment.

2.2. Quantitative Analysis of Solamargine in AE-SN. Solamargine is considered as a crucial antitumor component that existed in *Solanum nigrum* and can be a marker component for AE-SN [17]. The concentration of solamargine was therefore determined in the AE-SN stock solution by using liquid chromatography-mass spectrometry (LC-MS/MS) analysis. Before analysis, 100 μL AE-SN stock solution was mixed with 900 μL mixture of water : methanol (30 : 70, v/v). LC-MS/MS analysis was performed in the mobile phase of HPLC-grade acetonitrile (ACN) and deionized water with a Syncronis C18 column (Thermo Scientific, MA, USA). The flow rate is 0.5 mL/min with splitted 0.25 mL/min to mass. The ionization mode of the mass spectrometry condition was

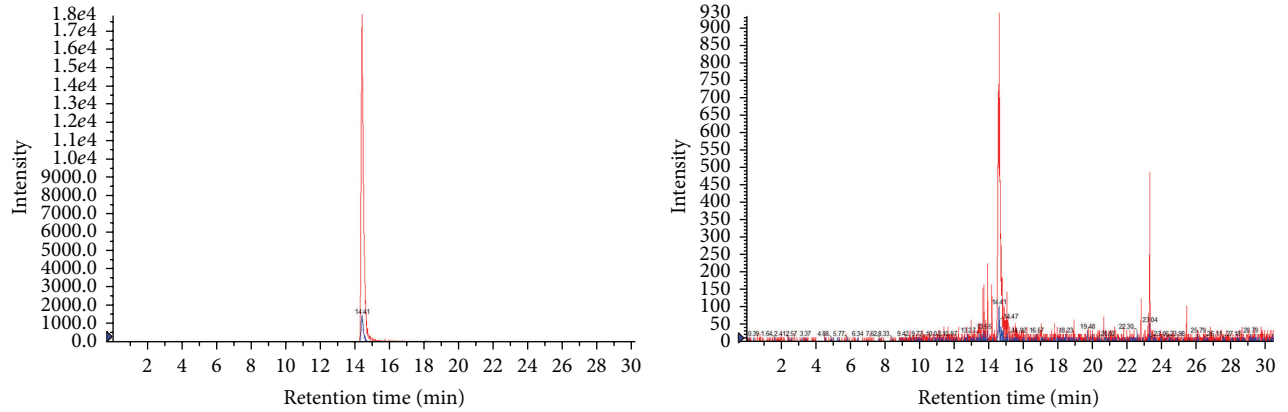

Figure 1: Extracted ion chromatography of solamargine. (a) The parent ion of purified solamargine was fragmented into two daughter ions (blue and red peaks). (b) Fragmented ions presented in AE-SN.

set as electrospray/positive ionization, and the mass scanning mode was multiple reaction monitor (MRM). The parent ion of purified solamargine is 867.5 m/z and fragmented into two daughter ions: 722.5 and 396.4 m/z, respectively (Figure 1(a)). These daughter ions were used to determine the solamargine concentration in AE-SN (Figure 1(b)). The concentration of solamargine in AE-SN stock solution was 77 μg/mL by using MRM analysis.

2.3. Cell Viability Assay and Morphological Observation. Hep3B and HepJ5 cells were plated onto 96-well plates, with 5×10^3 cells per well, and cultured overnight before treatment. To evaluate the antitumor effects of AE-SN, the cells were treated with 0 to 2.0 mg/mL of AE-SN for 48 h. To evaluate the antitumor effects of integrated treatment with the chemotherapeutic drugs and AE-SN, the cells were treated with 0 to 20 μM cisplatin or 0 to 10 μM and 0, 0.5, or 1.0 mg/mL of AE-SN for 48 h. In this study, cell viability was determined using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Table 1).

Two approaches, namely, microscopic observation and measurement of the cell size distribution, were performed to inspect the morphological changes in AE-SN-treated HCC cells. General morphological changes were observed using a Nikon Eclipse TS100 optical microscope (Nikon Instruments, Melville, NY, USA) and photographed at 100x magnification, whereas the distribution of cell diameter was measured using a Scepter cell counter (Merck Millipore Billerica, MA, USA), which divided surviving cells from cell fragments and debris in a borderline of 12 μm [18].

2.4. Western Blotting Analysis. Hep3B and HepJ5 cells were planted into 6 cm dishes with 5×10^5 cells per dish and cultured overnight. The cells were treated with a control medium, namely, 5 μM of cisplatin or 2 μM of doxorubicin, and 0 or 1.0 mg/mL of AE-SN for 48 h. After 48 h of incubation, the cells were collected using a cell lysis buffer. The protein concentrations of the cell lysates were determined using a Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA) and equalized before western blotting analysis. The cell lysates were separated using 12.5% sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred into a polyvinylidene fluoride membrane (Pall Corp, Port Washington, NY, USA).

The expression and activation of the selected protein markers, namely, LC-3 A/B, caspase-3, and caspase-7, as well as the internal control, GAPDH, were then determined using corresponding primary and secondary antibodies. The immunoreactivity was detected using an electrochemiluminescence western blotting detection kit (Western Lightning Plus-ECL, PerkinElmer Inc., Waltham, MA, USA).

Table 1: IC_{50} values in HCC cells treated with AE-SN alone or in combination with AE-SN and either chemotherapeutic drug. The IC_{50} values were analyzed using cell viability data determined after 48 h treatment with AE-SN (Figure 2) and with AE-SN integrated with either chemotherapeutic drug (Figure 3).

	Hep3B		Hep5J	
AE-SN (mg/mL)	0.96		0.97	
	Combination of AE-SN (mg/mL)			
	0	0.5	0	0.5
Cisplatin (μM)	6.75	2.74	8.71	2.84
Doxorubicin (μM)	4.65	1.31	6.39	1.42

2.5. Statistical Analysis. Analyses of the half maximum inhibitory concentration (IC_{50}) were performed using CalcuSyn software (Biosoft, Cambridge, UK), whereas Student's t test and one-way ANOVA were performed using SPSS software (SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Cytotoxicity of AE-SN Alone and with the Chemotherapeutic Drugs in Hep3B and Hep5J Cells. In this study,

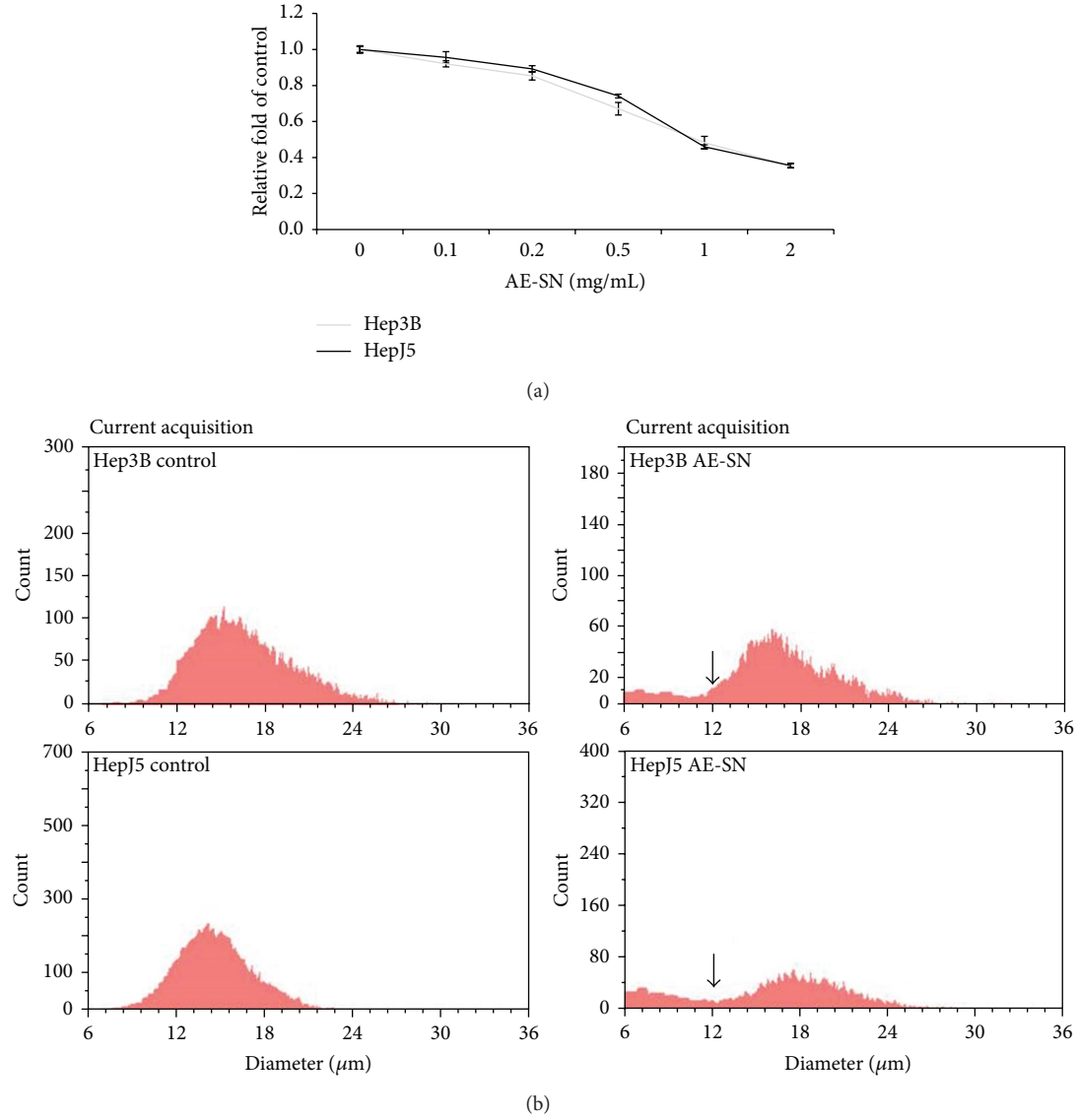

Figure 2: AE-SN treatment inhibited Hep3B and HepJ5 cell growth. (a) Hep3B and HepJ5 cells were treated with 0.1 to 2 mg/mL of AE-SN for 48 h, and the cell viability was determined using an MTT assay. The data are presented as the mean ± standard deviation. (b) Hep3B and HepJ5 cells were treated with 1.0 mg/mL of AE-SN for 48 h, and the cell size distribution was determined according to the cell diameter by using a Scepter cell counter. Arrows indicate a cell diameter of 12 μm.

48 h treatment with 0 to 2.0 mg/mL of AE-SN gradually inhibited the growth of Hep3B and HepJ5 cells in a concentration-dependent manner (one-way ANOVA, $P < 0.01$, Figure 2(a)). The IC_{50} values of AE-SN for the Hep3B and HepJ5 cells were 0.96 and 0.97 mg/mL, respectively. AE-SN treatment also resulted in the production of cell fragments and debris, which were less than 12 μm in diameter in comparison with cells treated with the control medium in both Hep3B and HepJ5 cells (Figure 2(b)). Collectively, these results suggested that AE-SN inhibited cell growth and demonstrated cytotoxicity in Hep3B and HepJ5 cells.

To evaluate cytotoxicity on the integrated treatment with chemotherapeutic drugs and AE-SN, cisplatin (1 to 20 μM) or doxorubicin (1 to 10 μM) was used to treat the Hep3B and HepJ5 cells, respectively, with 0, 0.5, or 1.0 mg/mL of AE-SN for 48 h (Figure 3). The IC_{50} values of the cisplatin integrated with 0.5 mg/mL of AE-SN in the Hep3B and HepJ5 cells were reduced to 2.74 μM and 2.84 μM, respectively, whereas those of doxorubicin were reduced to 1.31 μM and 1.42 μM. In other words, the IC_{50} values for cisplatin and doxorubicin integrated with 0.5 mg/mL of AE-SN were reduced to 40% and 30% of the values of the drugs used alone in the Hep3B and J5 cells, respectively. Human pulmonary fibroblast cells, WI-38, were also treated with 0, 0.5, or 1.0 mg/mL of AE-SN and cisplatin or doxorubicin to identify the cytotoxicity of the combined treatment (Figures 3(c) and 3(f)). AE-SN was not likely to enhance cisplatin- or doxorubicin-induced cytotoxicity in the WI-38 cells in comparison with that in the

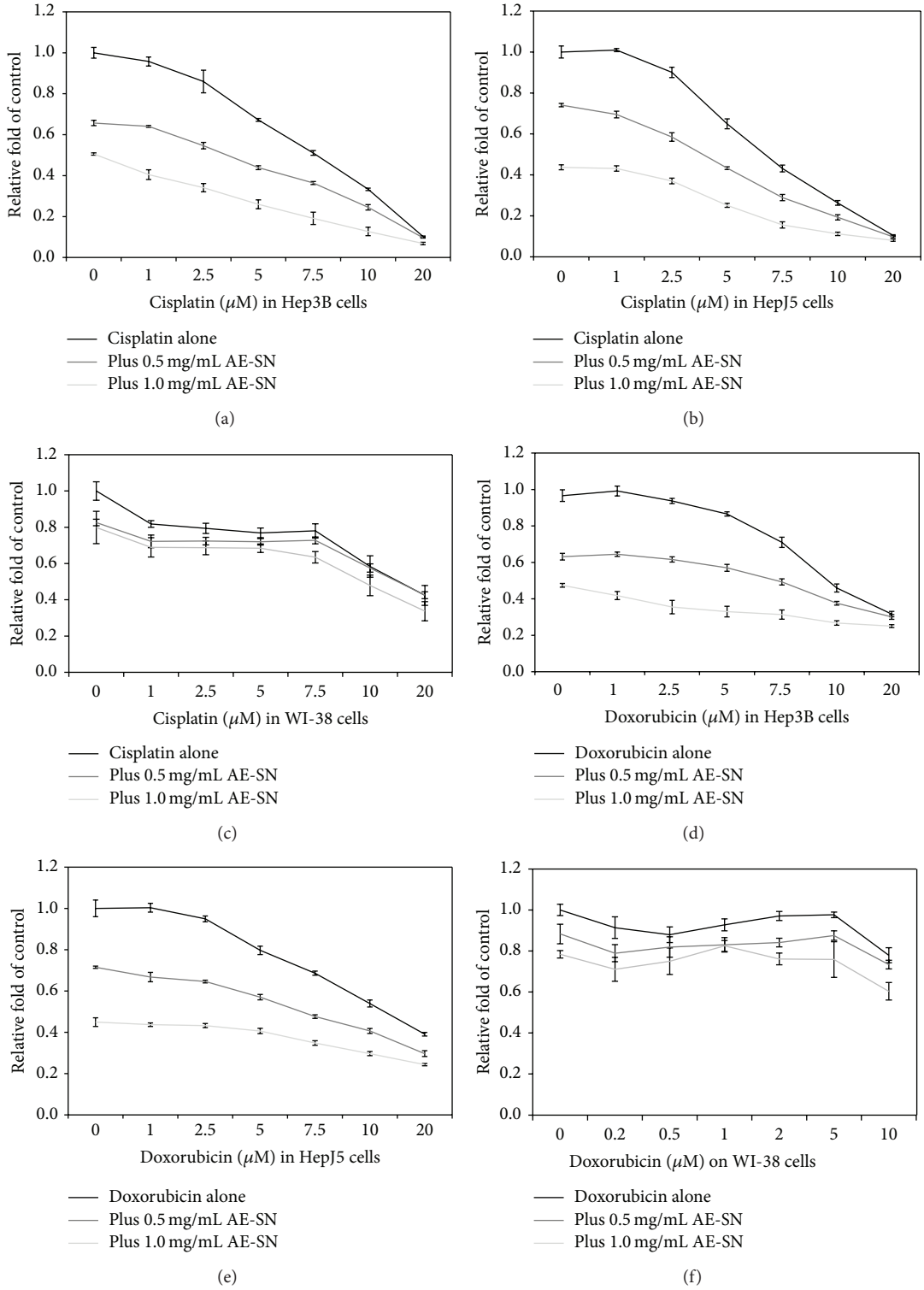

Figure 3: AE-SN-potentiated cisplatin and doxorubicin induced cytotoxicity in Hep3B and HepJ5 cells but had no effect on normal human pulmonary fibroblasts (WI-38 cells). (a–c) Cells were treated with 0 to 20 μM cisplatin and 0, 0.5, or 1.0 mg/mL of AE-SN for 48 h. (d–f) Cells were treated with 0 to 10 μM doxorubicin and 0, 0.5, or 1.0 mg/mL of AE-SN for 48 h. The cell viability was determined using an MTT assay; the data are presented as the mean ± standard deviation.

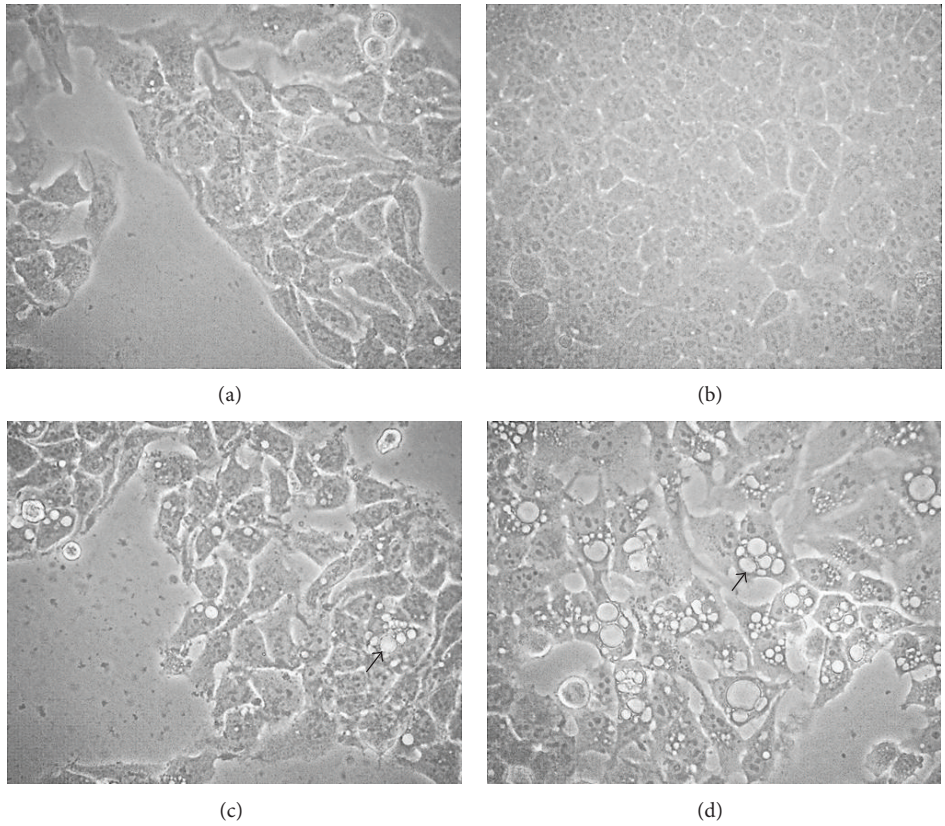

Figure 4: AE-SN-activated programmed cell death in Hep3B and HepJ5 cells. (a) Hep3B and (b) HepJ5 cells were treated with a control medium for 48 h. (c) Hep3B and (d) HepJ5 cells were treated with 1.0 mg/mL of AE-SN for 48 h. Arrows indicate the morphological changes present in the AE-SN-treated cells (100x magnification).

Hep3B and HepJ5 cells. Together, these results suggested that the AE-SN treatment potentiated cisplatin- and doxorubicin-induced cytotoxicity in both Hep3B and HepJ5 cells but not in WI-38 normal human cells.

3.2. Activation of Programmed-Cell-Death-Related Protein in AE-SN and Chemotherapeutic-Drug-Treated Cancer Cells. Hep3B and HepJ5 cells treated with AE-SN demonstrated a clear morphological change similar to the formation of phagolysosome-like vacuoles (Figures 4(a) to 4(d)). This morphological change was also observed in AE-SN-treated human endometrial and colorectal carcinoma cells [11] and related to the activation of autophagy.

To further understand whether AE-SN increases the activation of cell death protein when combined with either cisplatin or doxorubicin in the two HCC cell lines, the activation of LC-3 A/B and caspase-7 was observed in HCC cells treated with 1 mg/mL of AE-SN and 5 μM cisplatin or 2 μM doxorubicin for 48 h. As shown in Figure 5, the results suggested that, in both Hep3B and HepJ5 cells, AE-SN induced LC-3 A/B II accumulation in all of the treatment groups. The cleavage of caspase-7 was also enhanced by AE-SN cotreatment in cisplatin-treated Hep3B and HepJ5 cells and in doxorubicin-treated HepJ5 cells, but not in doxorubicin-treated Hep3B cells. Doxorubicin cotreatment seemed to eliminate the AE-SN-induced caspase-7 cleavage in the Hep3B cells.

4. Discussion

In this study, the impact of AE-SN on tumor-suppression efficiency was evaluated by employing the preparation of AE-SN that is used in TCM clinical practice. The IC_{50} values of AE-SN for Hep3B and HepJ5 cells indicated a moderate anti-tumor effect through direct exposure. Our previous studies showed that administering AE-SN at approximately 0.5 to 1 mg/mL for 48 h yielded similar IC_{50} values in human endometrial and colorectal cancer cells [11, 12]. In comparison with the tumor-suppression efficiency observed in previous studies using AE-SN extracts prepared from dried powder through water extraction [7, 13, 14, 19, 20], the tumor-suppression efficiency of AE-SN in this study was similar. The results of these experimental studies collectively suggest that cancer treatment may be a reasonable application for the preparation of AE-SN used in TCM practice. In addition, integrated treatment of AE-SN with cisplatin and doxorubicin may substantially reduce the required dose of cisplatin and doxorubicin required to achieve the same tumor-suppression efficiency, thereby improving the QOL of HCC patients during chemotherapy.

AE-SN was suggested to activate both autophagic and apoptotic cell death in many human cancer cell lines by inducing the cleavage of caspase-3 and accumulation of LC-3 A/B II [7]. By contrast, this study indicated that AE-SN

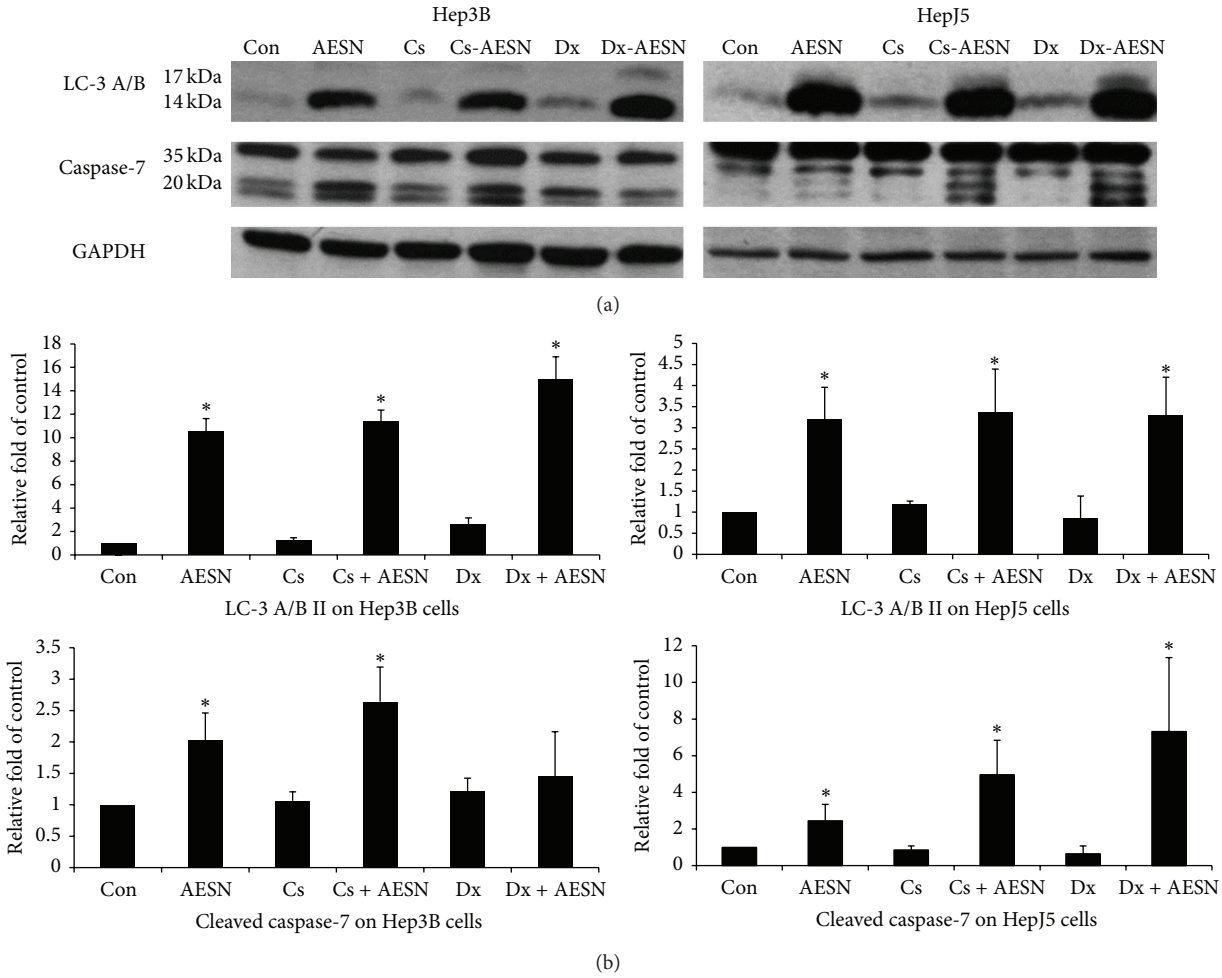

Figure 5: Activation of selected protein markers in HCC cells treated with AE-SN and either cisplatin or doxorubicin. (a) Cells were treated with a control medium, namely, 5 μM cisplatin or 2 μM doxorubicin with 0 or 1.0 mg/mL of AE-SN for 48 h. The activation of LC-3 A/B and caspase-7 was determined using western blotting analysis. GAPDH served as an internal control. (b) Semiquantitation of the LC-3 A/B II and cleaved caspase-7 in the Hep3B and HepJ5 cells. The data are presented as the mean ± standard deviation. ∗ indicates statistical significance in comparison with the previous group, namely, Con versus AESN (vehicle control versus AE-SN), Cs versus Cs + AESN, and Dx versus Dx + AESN (two-tailed Student's t-test, $P < 0.05$).

treatment failed to induce the cleavage of caspase-3 in both Hep3B and HepJ5 cells (data not shown). This result coincided with observations of human colorectal and endometrial cancer cells [11, 12] that suggested that resistance to AE-SN-induced caspase-3 cleavage may vary among cancer cell types. Cleaved caspase-7 is another biomarker for apoptotic cell death and was found to be induced in Hep3B and HepJ5 cancer cells after a single treatment with AE-SN. The cleavage of caspase-7 can be induced through mitochondria-mediated and extracellular signal-induced apoptosis pathways [21]. AE-SN-induced caspase-7 cleavage may occur through an alternative signal pathway, independent of caspase-3 cleavage, and activates apoptosis in caspase-3 resistant cancer cells. In addition, AE-SN induces LC-3 A/B II accumulation, which is associated with autophagy activation in Hep3B and HepJ5 cells. These results have been a common feature among AE-SN studies [7, 11, 12, 14]. In integrated treatment, AE-SN still induced both LC-3 A/B II accumulation and caspase-7 cleavage in both cisplatin- and doxorubicin-treated cells, suggesting that AE-SN may enhance cisplatin- and doxorubicin-induced cytotoxicity through the activation of autophagy and caspase-7-related apoptosis.

In comparison with Hep3B cells, HepJ5 cells are more malignant and resistant and exhibit a higher expression of survivin [16]. In the present study, the IC_{50} values for cisplatin and doxorubicin in HepJ5 cells were higher than those in Hep3B cells (8.71 versus 6.75 and 6.39 versus 4.65 μM, resp.). This result confirmed that HepJ5 cells are more resistant to cisplatin- and doxorubicin-induced cytotoxicity than Hep3B cells were. By contrast, the IC_{50} values for AE-SN were similar in HepJ5 and Hep3B cells (0.97 versus 0.96 mg/mL), and combined treatment with cisplatin or doxorubicin and 0.5 mg/mL of AE-SN further reduced the IC_{50} values to the same levels (2.84 versus 2.74 μM in the cisplatin combination and 1.31 versus 1.42 μM in the doxorubicin combination). These results collectively suggested that HepJ5 cells were

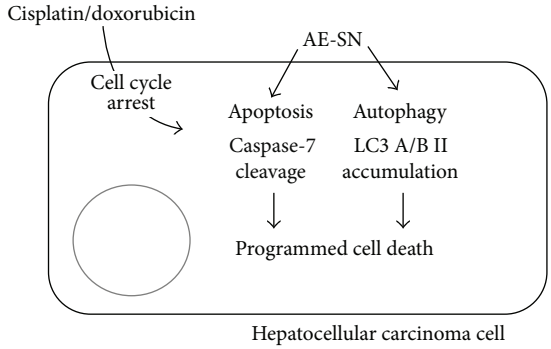

Figure 6: Illustration of the integrated-cell-death mechanism activated by AE-SN and either cisplatin or doxorubicin in HCC cells.

unable to resist AE-SN-induced cytotoxicity and tended to be more vulnerable to cisplatin- and doxorubicin-induced cytotoxicity in combined treatment with AE-SN. The molecular mechanisms involved in this AE-SN-mediated HCC cytotoxicity require further investigation.

Some steroidal alkaloid glycosides and glycoproteins are considered the major active substances responsible for AE-SN-induced programmed cell death. Solamargine is one of the major compounds present in AE-SN that can activate caspase-3-related apoptosis and LC-3-A/B-II-related autophagy in human leukemia cells [22], and a 150 kDa glycoprotein isolated from AE-SN was reported to be another effective antitumor compound for activating caspase-3-related apoptosis in human colorectal, cervical, and HCC cells [23–25]. However, these isolated compounds do not seem to completely explain the tumor-suppression mechanism of AE-SN because the specific compound that activates the cleavage of caspase-7 remains unknown. Further investigation is therefore required to identify the exact composition of the AE-SN substance that contributes to the antitumor effects.

This study clarified the antitumor effects of AE-SN and the potential of AE-SN for enhancing cytotoxicity induced by cisplatin and doxorubicin in human HCC cells in vitro. In consideration of the absorption rate of AE-SN through the gastrointestinal tracts and its metabolism in vivo, the real tumor-suppression efficiency of AE-SN with chemotherapeutic drugs as well as the optimal dosage, preparation, and administrative approach of AE-SN remains to be evaluated using an animal cancer model. In addition, any unexpected adverse effects of integrated treatment with AE-SN and chemotherapeutic drugs in vivo should be carefully examined before clinical trials are conducted.

5. Conclusion

Clear experimental evidence obtained in this study indicates that the AE-SN cotreatment potentiated cisplatin- and doxorubicin-induced cytotoxicity in human HCC cells. This AE-SN-potentiated cytotoxicity may occur through the accumulation of LC-3 A/B II and cleavage of caspase-7 to activate apoptosis and autophagic cell death in human HCC cells (Figure 6). Collectively, our results suggest that AE-SN can be used in novel integrated chemotherapy with cisplatin and doxorubicin to improve tumor-suppression efficiency in HCC treatment.

Conflict of Interests

The authors declare that there is no conflict of interests.

Authors' Contribution

Chien-Kai Wang and Yi-Feng Lin contributed equally to this study.

Acknowledgments

This study was supported in part by Taipei Medical University and Taipei Medical University Hospital (102-TMU-TMUH-19).

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Hindawi Publishing Corporation
Evidence-Based Complementary and Alternative Medicine
Volume 2015, Article ID 715638, 10 pages
http://dx.doi.org/10.1155/2015/715638

Research Article

Intervening TNF-α via PPARγ with Gegenqinlian Decoction in Experimental Nonalcoholic Fatty Liver Disease

Yun-liang Wang, Li-juan Liu, Wei-han Zhao, and Jun-xiang Li

Gastroenterology Department, Dongfang Hospital, Beijing University of Chinese Medicine, Beijing 100078, China

Correspondence should be addressed to Jun-xiang Li; lijunxiang1226@163.com

Received 2 August 2014; Revised 16 November 2014; Accepted 7 December 2014

Academic Editor: Mohammad Ahmad Al-Shatouri

This paper is to explore the effect and mechanism of Gegenqinlian decoction on experimental nonalcoholic fatty liver disease (NAFLD) *in vivo* and *in vitro*. The final aim is to make clear whether Gegenqinlian decoction would impact NAFLD through improving PPARγ to suppress inflammation and regulate lipid. The data in this research suggested that Gegenqinlian decoction is a potent way to manage NAFLD through improving PPARγ to regulate lipid and suppress inflammation.

1. Background

Nonalcoholic fatty liver disease (NAFLD) is a kind of disease ranging from simple hepatic steatosis to nonalcoholic steatohepatitis (NASH) and irreversible cirrhosis [1]. The prevalence of NAFLD has risen rapidly in parallel with dramatic rise in obesity, diabetes [2, 3], hypertension, and dyslipidaemia and NAFLD is now regarded as the liver manifestation of metabolic syndrome (Mets). Up till now, the pathogenesis and progression of NAFLD are not yet fully understood; the proven therapeutic trails are still in improving [4, 5]. Some researchers approve the "2-hit" hypothesis on the NAFLD pathogenesis. Briefly, the "first hit" involves hepatic triglyceride accumulation, or steatosis. The "second hit" usually links inflammatory cytokines and oxidative stress and so forth. As concerned, hepatic triglyceride accumulation can occur as a result of increased fat synthesis, increased fat delivery, decreased fat export, and/or decreased fat oxidation [6]. Inflammatory cytokines, such as TNF-α, IL-6, and IL-8, are involved in the second hit stage, mediate steatohepatitis in patients with NAFLD [7], and can be regulated by PPARγ, which is not only related to anti-inflammatory effects, but also close to improving insulin resistance [8].

Giving an effective therapeutic method for NAFLD/NASH to prevent future disease is of priority. Till now, intervention for NAFLD remains mainly through lifestyle modifications and nonpharmacological treatment. Hence, finding a new approach to NAFLD is in urgent need.

Chinese herbal medicine (CHM) has been traditionally used in China and other parts of Asian countries for thousands of years and is now spreading worldwide. A special and basic feature of Chinese medicine is the use of a formula containing several herbs (mixed as a cocktail) to ameliorate a series of abnormalities syndrome related to a certain disease. Herbal extracts contain multiple natural compounds that can aim to different targets via various pathological pathways involved in the disease, providing therapeutic effects via a spectrum of actions. Gegenqinlian decoction (GGQLD), a classical formula from *Treatise on Febrile Diseases,* is one of the well-known traditional Chinese medicines, which consists of *Kudzu root*, *Rhizoma coptidis, Scutellaria baicalensis* Georgi, and *Main licorice*. It is widely used to treat diarrhea. In previous studies, we have found that Gegenqinlian decoction can both improve diabetes [9, 10] and play an anti-inflammatory effect based on the compounds included [11–13], in which glucose and lipid metabolism disorder and inflammatory reaction play vital roles as in NAFLD. Hence, under the guidance of traditional Chinese medicine theory "same treatment for different diseases," we propose a hypothesis that Gegenqinlian decoction could intervene and improve NAFLD by a multitargets solution.

2. Materials and Methods

2.1. Preparation of GGQLD and Rosiglitazone. GGQLD granules were provided by Pharmacy Department of Dongfang Hospital, Beijing University of Chinese Medicine (Beijing, China). The GGQLD granules contain equal weights of the ingredients of the Gegenqinlian formula: *Kudzu root* 24 grams, *Rhizoma coptidis* 9 grams, *Scutellaria baicalensis* Georgi 9 grams, and *Main licorice* 6 grams. Rosiglitazone was purchased from Sigma, USA.

2.2. Animals and Treatment. Male Sprague-Dawley (SD) rats at 7 weeks of age were supplied by Vital River Laboratory Animal Technology Co. Ltd. (Beijing, China). All experimental procedures were approved by the Animal Ethics Committee of Beijing University of Chinese Medicine, following guidelines issued by Regulations of Beijing Laboratory Animal Management. SD rats were maintained on a 12 h light/dark cycle under constant temperature (22 ± 2°C) with *ad libitum* access to standard chow diet ($n = 6$) or a high-fat diet (HFD, 34% fat, 19% protein, and 47% carbohydrate by energy composition) ($n = 6$) for 8 weeks to induce NAFLD. The granules and rosiglitazone were dissolved in 100 mL of distilled water and kept at 2–8°C until use. The animals in GGQLD low dose (GGQLDL, 5.04 g/kg/day, *p.o.*, $n = 6$), GGQLD high dose (GGQLDH, 10.08 g/kg/day, *p.o.*, $n = 6$), and rosiglitazone (ROS, 20 mg/kg/day, *p.o.*, $n = 6$) were given at the beginning of HFD feeding; saline (10 mL/kg/day, *p.o.*, $n = 6$) was administrated to chow fed rats (chow, $n = 6$) as the model control. All of groups were given the drugs or saline for 8 weeks.

2.3. Cell Model Establishment and Intervention. HepG2 cells were cultured in Dulbecco's Modified Eagle's Medium (Gibco, USA) supplemented with 10% fetal bovine serum (FBS), penicillin (100 units/mL), and streptomycin (100 μg/mL) at 37°C under 5% CO_2, in a 95% humidified atmosphere. For the cell model establishing [14], palmitic and oleic acid 0.1 M stock solutions were prepared by dissolving free fat acid (FFA) in DMSO (with a final concentration less than 0.1%). The cells were exposed to a fresh mixture of exogenous 500 μM FFA in molar ratio 1 : 2 palmitic : oleic for 24 h. Since albumin concentration is an important factor in determining the concentration of available FFA, 1%BSA for the final concentration was considered. For the intervention, different concentrations of GW9662 and PPARγ selective agonist rosiglitazone [15] (Sigma, USA) were prepared and intervened together with FFA.

2.4. Determination of Metabolic Parameters: Liver Enzymes in Rats. At the end of treatment, animals were anesthetized using 4% chloral hydrate after a 12 h overnight fast; blood samples were collected from the abdominal aorta of rats. Fasting serum triglycerides (TGs), high-density lipoprotein cholesterol (HDL-C), and low-density lipoprotein cholesterol (LDL-C) were analyzed using Enzyme-linked immunoabsorbent assay (ELISA) (Bio Sino, Beijing, China). Fasting serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were also determined by ELISA (Bio Sino, Beijing, China).

2.5. Histological Analysis in Rats. A fresh liver tissue was fixed with 10% formaldehyde solution. Paraffin sections were used to Haematoxylin and Eosin (H&E) staining (Zeping, Beijing, China). Frozen sections were stained with Oil Red O (Sigma-Aldrich). Both staining methods were used to investigate architecture of the liver and hepatic lipid droplets. Stained H&E and Oil Red O (ORO) slides were visualized with the Olympus microscope, and images were captured with Olympus digital camera (BX40, Beijing, China) using NIS Element SF 4.00.06 software (Beijing, China). For each group, liver samples from 3 to 5 rats were prepared and stained.

2.6. ELISA for Tumor Necrosis Factor-α (TNF-α) in Serum, Liver Tissue, and Cell Culture Media. After blood samples were collected, serum tumor necrosis factor-α (TNF-α) was tested by using rat ELISA kits (Boster Bio-Engineering, Wuhan, China).

Liver was collected and washed by ice-cold PBS and then stored at −80°C for subsequent histological and molecular assays.

Frozen liver tissue (0.5 g) was homogenized in 1 mL lysis buffer containing 150 mM NaCl, 1 mM PMSF, 10% glycerol, and the complete protease inhibitor cocktail (Roche, USA). The supernatant was collected for analysis of TNF-α using rat ELISA kits (Boster Bio-Engineering, Wuhan, China).

The TNF-α released in the culture media was determined by ELISA (Boster Bio-Engineering, Wuhan, China) according to manufacturer's instructions. Cells were cultured and treated as previously described. The culture media were collected after 24 h, possible contamination of cellular fractions was eliminated by centrifugation, and the test was performed in cell-free supernatant.

2.7. Western Blotting to Detect PPARγ. Proteins in liver tissue homogenate were extracted using ice-cold tissue lysis buffer. Protein concentration was determined using a BCA protein assay kit. Samples were separated by 10% SDS-PAGE and transferred onto polyvinylidene difluoride membranes. The membranes were immunoblotted with primary antibodies that recognize PPARγ (1 : 2000) and GAPDH (1 : 20000) (TDY Biotech, Beijing, China). Peroxidase-conjugated secondary antibodies and an ECL detection system were used according to routine methods. The intensities of the protein bands were analyzed using Gel-Pro 3.2 software. GAPDH protein was used as the internal control to normalize the protein loading.

2.8. Real-Time Polymerase Chain Reaction for PPARγ mRNA Expression. Reverse transcription was performed with 1 μg of total RNA per 12 μL reaction using a standard cDNA Synthesis Kit (Takara, Japan). The real-time PCR primer

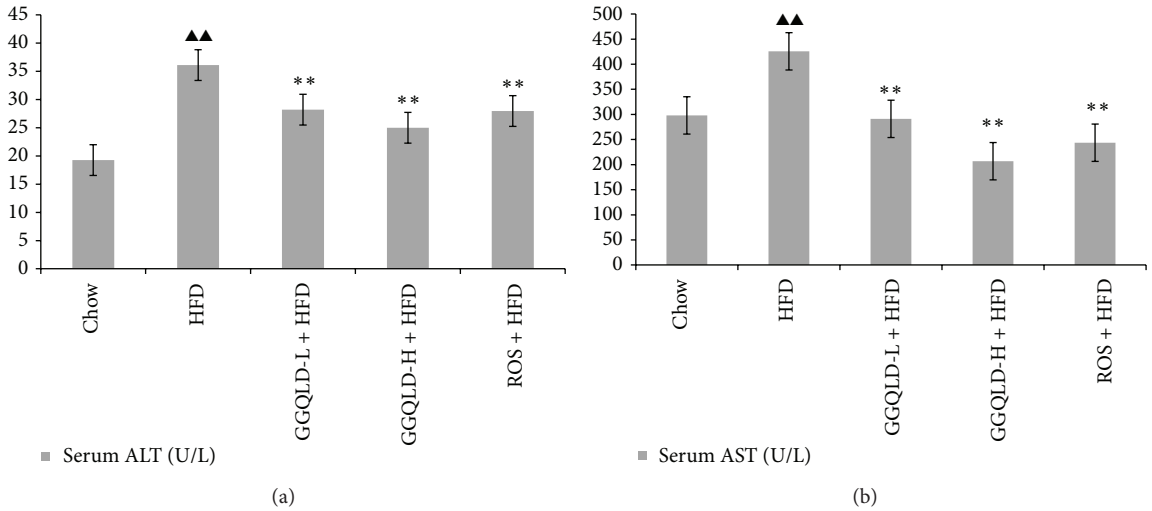

Figure 1: Effect of GGQLD on the concentration of ALT and AST. (a) ALT levels in serum; (b) AST levels in serum. *In vivo* study, data are means ± SD; $n = 6$ rats/group. $^{\blacktriangle}P < 0.05$, $^{\blacktriangle\blacktriangle}P < 0.01$ versus chow; $^{*}P < 0.05$, $^{**}P < 0.01$ versus HFD.

sequences for target genes were as follows: PPARγ, forward 5′-TACCACGGTTGATTTCTC-3′ and reverse 5′-GCTCTACTTTGATCGCACT-3′; GAPDH, forward 5′-TGGAGTCTACTGGCGTCTT-3′ and reverse 5′-TGTCATATTTCTCGTGGTTCA-3′ (CWbio, Beijing, China). For each real-time PCR, the typical thermal cycling conditions included an initial activation step at 95°C for 5 min, 45 cycles of amplification, and a final melting curve (55–95°C). For comparison of the PPARγ mRNA levels, the cDNA concentrations were normalized with GAPDH PCR products. The data were analyzed using the ΔΔCt method.

2.9. Determination of Intracellular Fat Content: Oil Red O Staining and Quantifying. Cells were fixed and stained with Oil Red O and then washed by distilled water until the extracellular oil red is rinsed clean. The intracellular fats then trickle out after the rupture of cell membrane with 100% isopropyl alcohol. After shocks, OD values were read and analyzed by ELISA.

2.10. Data Analysis. All data are expressed as the mean ± SD unless otherwise indicated. Data were analyzed by using one-way ANOVA and a post hoc test. Differences were considered to be statistically significant at $P < 0.05$.

3. Results

3.1. Effect of Gegenqinlian Decoction on Lipid Metabolism and Liver Enzymes in HFD Rats. All animals tolerated the experimental procedures well, and no deaths occurred during the study. Serum ALT [(36.10 ± 3.92 U/L)] and AST [(425.85 ± 13.84 U/L)] concentrations in HFD fed rats were significantly higher than those in chow fed rats [(19.27 ± 3.36) U/L in ALT, (298.10 ± 5.13) U/L in AST, $P < 0.01$, resp.]. Both the GGQLDL [(28.20 ± 3.01) U/L in ALT, (291.24 ± 22.36) in AST] and GGQLDH [(24.98 ± 1.55) U/L in ALT, (206.68 ± 20.48) in AST] treatments significantly attenuated the elevated ALT and AST ($P < 0.01$), together with rosiglitazone [(27.98 ± 3.14) U/L in ALT, (243.48 ± 26.52) in AST, versus HFD, $P < 0.01$, resp.] (Figure 1). HDL-C levels rose in GGQLDH [(1.90 ± 0.10 mmol/L)] group compared to HFD model group [(0.95 ± 0.04) mmol/L, $P < 0.01$]. Meanwhile, GGQLDH [(0.98 ± 0.15 mmol/L)] also decrease LDL-C concentrations, but the statistical significance was not achieved ($P > 0.01$), whereas GGQLDL [(0.79 ± 0.09 mmol/L)] and rosiglitazone [(0.87 ± 0.22 mmol/L)] groups significantly decreased LDL-C level compared to HFD model group [(1.07 ± 0.20 mmol/L, $P < 0.01$, resp.)]. Both GGQLDL [(1.94 ± 0.39 mmol/L)] and GGQLDH [(1.53 ± 0.11 mmol/L)] treatments significantly attenuated the elevated TC [(2.81 ± 0.79) mmol/L in HFD, $P < 0.01$, resp.)]. Rosiglitazone not only decreased serum TC [(1.94 ± 0.12 mmol/L)], but also TG [(0.59 ± 0.02) mmol/L versus (0.67 ± 0.04) mmol/L in HFD, $P < 0.01$] (Figure 2).

3.2. GGQLD Improved Histopathology in Rats. The photomicrographs of the H&E stain showed that HFD feeding increased hepatic fat deposits, evidenced by the majority of the hepatocytes of HFD rats that were distended by fat in comparison to the chow group (Figures 3(b) and 3(a)). The images of H&E stain also displayed steatosis and ballooning degeneration and evident infiltration with inflammatory cells in the intercellular substance, causing conspicuous swelling of the cell and cytoplasmic vacuolation as shown in Figure 3(b). The treatment of HFD rats with GGQLDs and rosiglitazone reduced fat deposits in liver (Figures 3(c), 3(d), and 3(e)) and the GGQLDs showed histological features similar to the chow group with little steatosis as shown in Figures 3(c) and 3(d).

Oil Red O staining on frozen liver sections showed few lipid droplets were detected in the liver sections from the chow group (Figure 4(a)). Compared with that of HFD fed model rats (Figure 4(b)), both GGQLDs and rosiglitazone

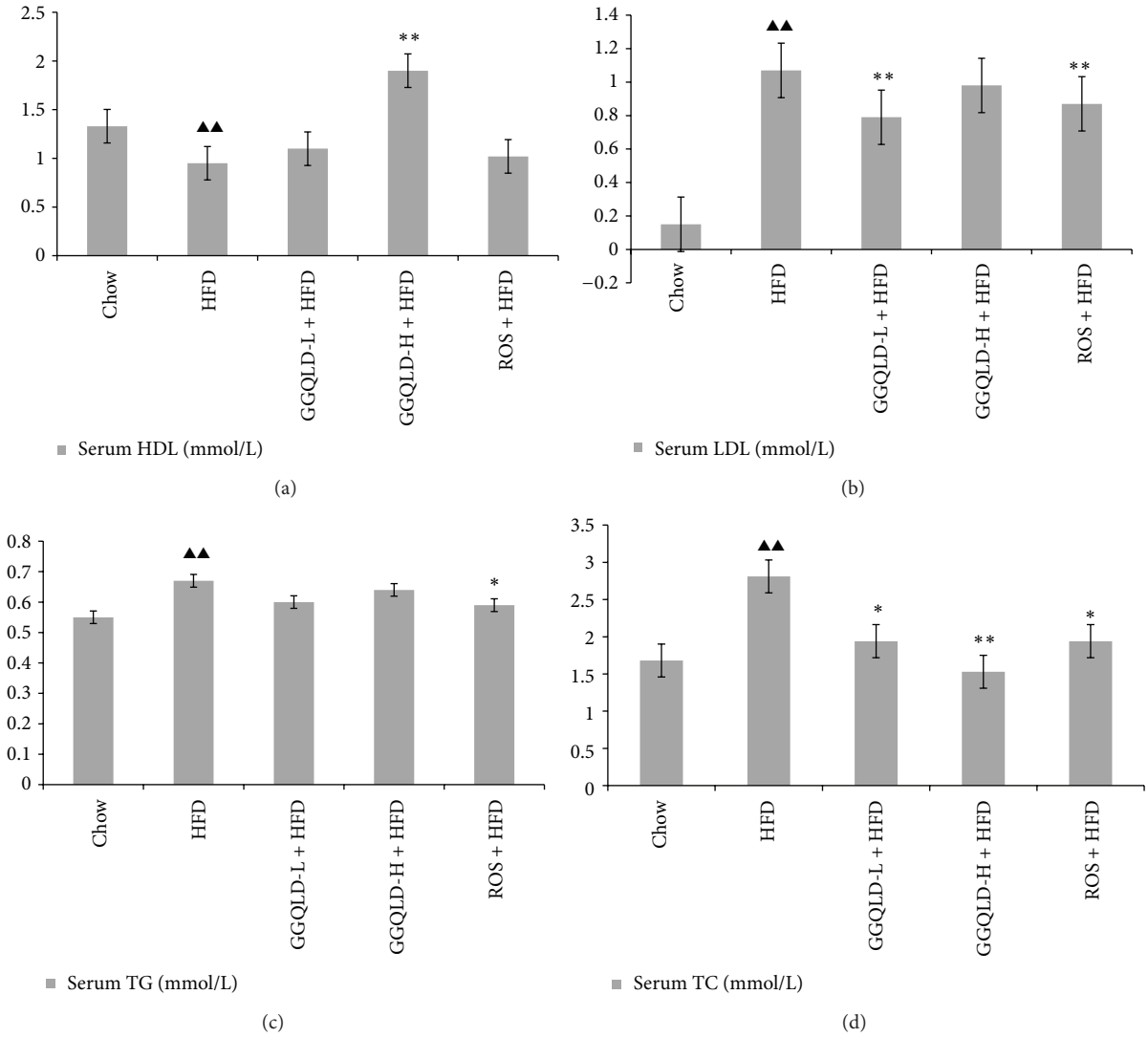

Figure 2: Effect of GGQLD on the concentration of triglyceride (TG), total cholesterol (TC), high-density lipoprotein (HDL), and low-density lipoprotein (LDL). (a) HDL levels in serum; (b) LDL levels in serum; (c) TG levels in serum; (d) TC levels in serum. In the *in vivo* study, data are means ± SD; $n = 6$ rats/group. $^{\blacktriangle}P < 0.05$, $^{\blacktriangle\blacktriangle}P < 0.01$ versus chow; $^{*}P < 0.05$, $^{**}P < 0.01$ versus HFD.

remarkably restrained lipid droplets deposited in hepatocytes (Figures 4(c), 4(d), and 4(e)).

3.3. Effects of GGQLD on Serum and Liver TNF-α. Serum and liver TNF-α were determined in chow fed rats and HFD rats treated with saline, GGQLDL, GGQLDH, or rosiglitazone, respectively. As shown in Figures 5(a) and 5(b), HFD feeding significantly increased TNF-α compared with chow fed rats [(228.05 ± 26.85) pg/mL in HFD versus (87.59 ± 53.23) pg/mL in chow, in serum, $P < 0.01$; (603.68 ± 45.57) pg/mL in HFD versus (459.50 ± 12.53) pg/mL in chow, in liver, $P < 0.01$]. GGQLDL [(81.41 ± 15.39) pg/mL in serum, (525.55 ± 19.77) pg/mL in liver], GGQLDH [(98.42 ± 26.03) pg/mL in serum, (525.05 ± 58.87) pg/mL in liver], and rosiglitazone [(171.23 ± 18.63) pg/mL in serum, (533.30 ± 24.14) pg/mL in liver] were capable of significantly inhibiting HFD-induced elevated TNF-α both in serum and in liver (versus HFD, $P < 0.01$, resp.) (Figures 5(a) and 5(b)).

3.4. GGQLD Regulated PPARγ Expression in HFD-Induced NAFLD Rats. We investigated whether GGQLD had a regulatory effect on PPARγ expression. As shown in Figure 6, the PPARγ level was significantly decreased on HFD group ($P < 0.01$, versus chow group). More remarkably, GGQLDs and rosiglitazone produced a dramatically improved effect on PPARγ level (versus HFD group, $P < 0.01$, resp., Figure 6).

3.5. Validation of GGQLD on PPARγ Gene Expression with Real-Time PCR. In order to confirm the results of effects of GGQLD on liver PPARγ protein expression, the expression of PPARγ gene was measured. The gene expression in HFD rat group decreased compared to chow group, but without a statistical significance (Figure 7). Remarkably, GGQLDs

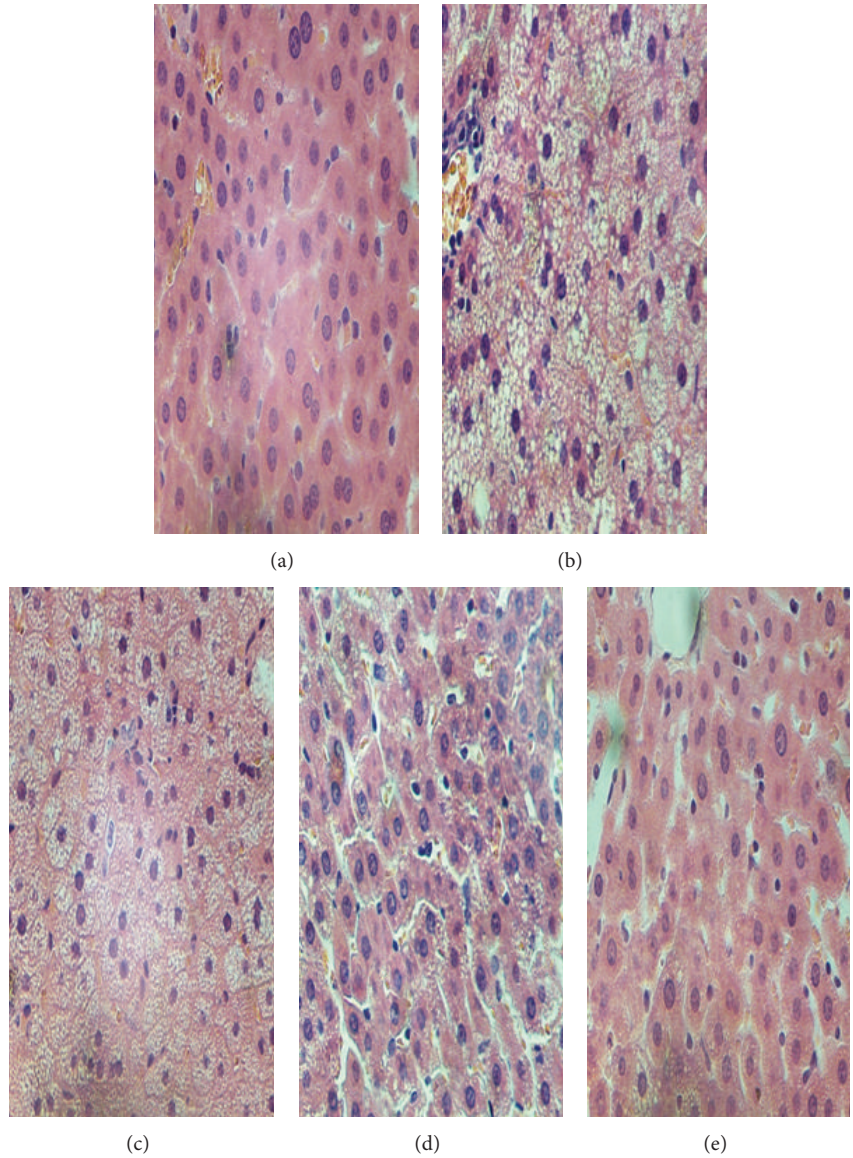

Figure 3: The results of paraffin section and H&E staining *in vivo*, ×400. (a) Chow fed rat, (b) HFD model rats, (c) GGQLDL treatment rats, (d) GGQLDH treatment rats, and (e) ROS treatment rats.

and rosiglitazone made a great contribution to increasing PPARγ gene expression (versus HFD group, $P < 0.01$, resp., Figure 7).

3.6. Cell Release of TNF-α in the Culture Medium. Compared with the model group [(203.83 ± 39.64) pg/mL], the production of TNF-α was evaluated in GW9662 10 mmol/L group [(212.17 ± 40.59) pg/mL, $P > 0.05$], while the TNF-α decreased in rosiglitazone 2 mmol/L group [(97.17 ± 2.93) pg/mL, $P < 0.01$] (Figure 8).

3.7. Determination of Intracellular Fat Content In Vitro. The intracellular lipid droplets content was analyzed by reading OD values; lipid droplets levels were increased significantly in groups with different concentrations of GW9662 compared with the model group ($P < 0.05$) (Figure 9).

4. Discussion

Nonalcoholic fatty liver disease (NAFLD), a multifactorial disorder with contribution of a variety of genetic and environmental factors, is considered to be closely associated with hepatic metabolic disorders, resulting in overaccumulation of fatty acids/triglycerides and cholesterol. The presence of steatosis is tightly associated with chronic hepatic inflammation [16], an effect in part mediated by activation of the Ikκ-b/NF-κB signaling pathway. In murine models of high-fat diet induced steatosis, increased NF-κB activity is associated with elevated hepatic expression of inflammatory cytokines such as TNF-α and activation of Kupffer cells [16]. TNF-α, a proinflammatory cytokine that is activated by the reactive oxygen species created by lipid peroxidation, not only promotes insulin resistance [17], but also mediates

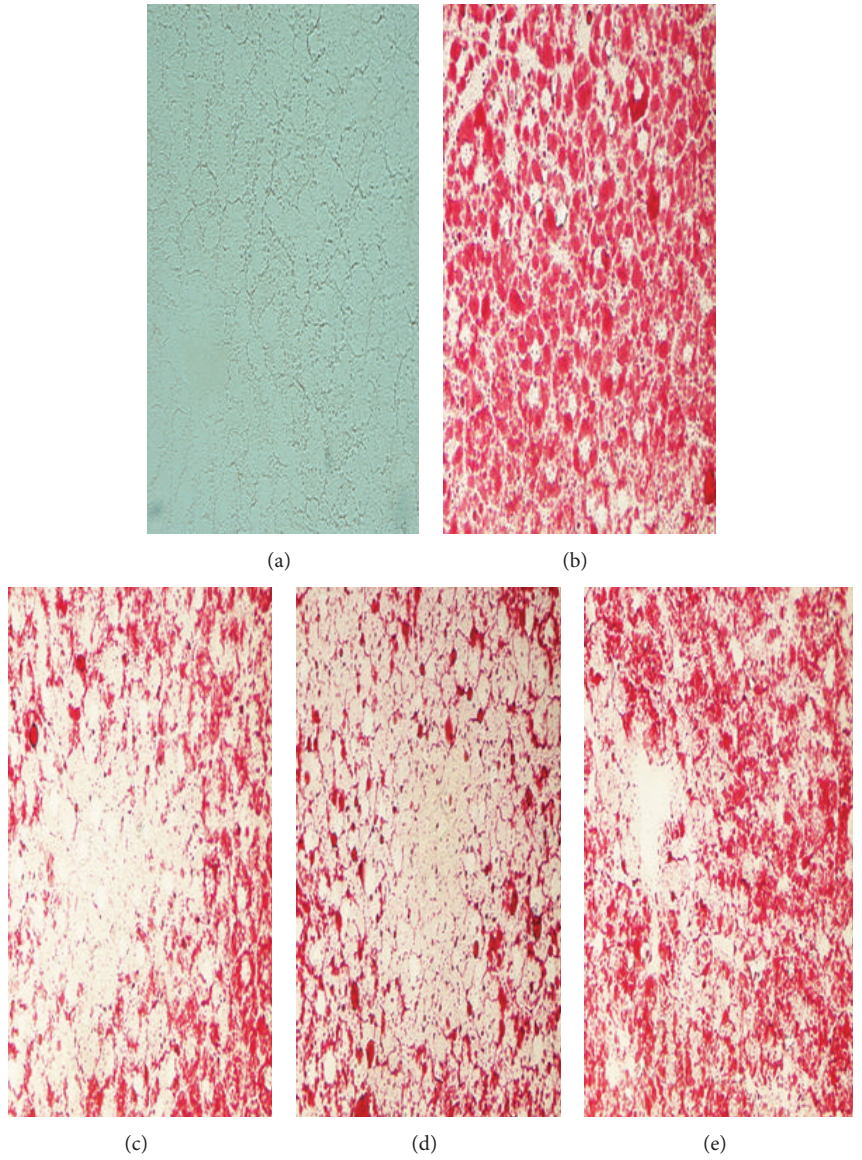

Figure 4: The results of frozen section and Oil Red O staining *in vivo*, ×400. The red blots show lipid drops in hepatocytes. (a) Chow fed rat, (b) HFD model rats, (c) GGQLDL treatment rats, (d) GGQLDH treatment rats, and (e) ROS treatment rats.

cholesterol and triglyceride metabolism [18]. It promotes necroinflammation, fibrogenesis, hepatic insulin resistance, and apoptosis [19]. Both serum and hepatic level of TNF-α are elevated in patients with NAFLD [20, 21], and levels correlate with histological severity [22]. Conversely, inhibition of TNF-α signaling improves IR and histological parameters of NAFLD [23, 24].

PPARγ is one subtype of the three PPARs; the others are PPARβ/δ. PPARγ agonists can promote adipose tissue to absorb and store FFAs and meanwhile inhibit liver fatty acid synthesis [25] via activating the AMP activated protein kinase (AMPK), of which the synthetic PPARγ ligand (TZDS) has been used in the treatment of diabetes for many years. PPARγ not only has anti-inflammatory effects, but also can effectively improve insulin resistance [8]. The activation of PPARγ in immune system modulates inflammatory response [26]. In recent years, scholars have found that there were obvious changes in liver fat, increased adiponectin, and decreased TNF-α, IL-6, and resistin in patients with NAFLD after giving PPARγ agonist treatment [27]. PPARγ has been an important endogenous regulator and potential therapeutic target for nutritional NAFLD [28], of which the selective agonist [15] and an insulin-sensitizing agent [29] rosiglitazone has been a focus of treating NAFLD [30, 31].

High-fat diet induced animal model of NAFLD has been widely used to identify the pathogenesis and evaluate new treatments for NAFLD [32, 33]. In the present study, we have demonstrated that eight weeks of high-fat diet feeding induced fatty liver disease in SD rats, revealing key biochemical features of NAFLD, including increased elevation of hepatic enzymes, hyperinsulinaemia, hyperlipidaemia associated with increased TG accumulation in the liver, and

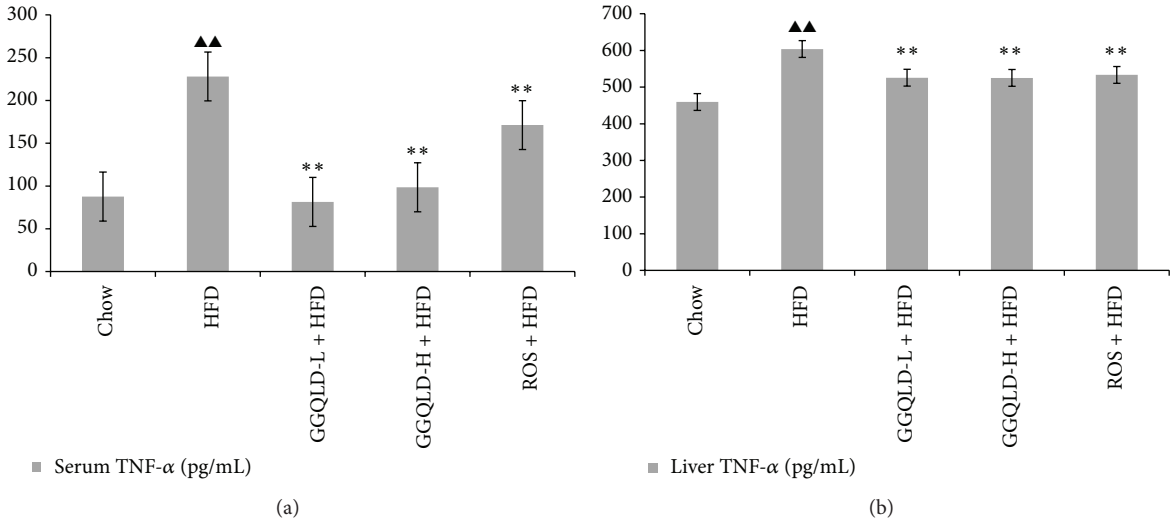

FIGURE 5: The effect of GGQLD on TNF-α level in serum and liver tissue. (a) TNF-α levels in serum; (b) TNF-α levels in liver tissue. In the *in vivo* study, data are means ± SD; $n = 6$ rats/group. $^{\blacktriangle}P < 0.05$, $^{\blacktriangle\blacktriangle}P < 0.01$ versus chow; $^{*}P < 0.05$, $^{**}P < 0.01$ versus HFD.

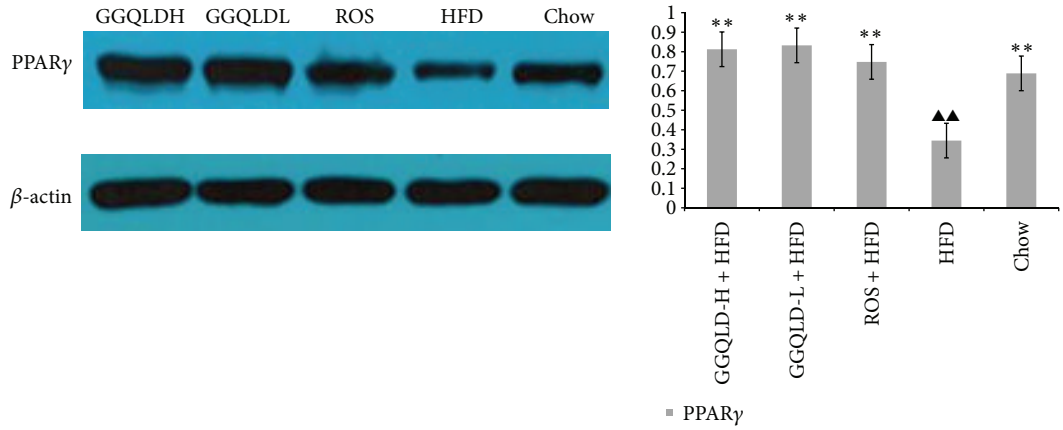

FIGURE 6: Western blotting for PPARγ in liver tissue. In the *in vivo* study, data are means ± SD; $n = 6$ rats/group. $^{\blacktriangle}P < 0.05$, $^{\blacktriangle\blacktriangle}P < 0.01$ versus chow; $^{*}P < 0.05$, $^{**}P < 0.01$ versus HFD.

histological changes containing steatosis, lobular and portal inflammation, and hepatocyte injury as ballooning, all about which reflected the characteristics of MetS [34]. Meanwhile, the histological abnormalities by H&E and ORO stains on the liver samples in the HFD rats of this study were consistent with existing reports [32, 35].

There are no pharmacological agents that have been approved for the treatment of NAFLD. Therefore, most clinical efforts have been directed focusing on the components of metabolic syndrome, namely, obesity, diabetes, dyslipidemia, and hypertension. Other interventions are directed at specific pathways potentially involved in the pathogenesis of NAFLD, such as insulin resistance, oxidative stress, and proinflammatory cytokines [36].

Chinese herbal medicine has been proven to play an anti-inflammatory effect in many fields of diseases [37, 38] and has a broad scope of drug safety. In the present study, treatment with GGQLD for 8 weeks has been proven to significantly normalize the liver hepatic aminotransferase (ALT and AST) to a level as nearly normal as chow control group. What is more, it was effective in impeding fat infiltration, which is evidenced by decreased hepatic TG contents and lipid droplets. Although the pathogenic mechanisms of NAELD are still under investigation, fat accumulation, especially triglycerides filtration into hepatocytes, is considered in the first step in development of NAFLD [4]. Hence, lipid accumulation plays a vital role with no doubt in NAFLD. The results from biochemistry and histology assays showed that GGQLDL and rosiglitazone produced an effect of reducing serums LDL-C and TC, respectively. Meanwhile, HDL-C level rose and TC decreased in GGQLDH group. What is more, histological stains showed GGQLDs and rosiglitazone decreased lipids droplets in hepatocytes and normalized steatosis in HFD rats. Overexpression of TNF-α has been identified and can mediate macrophage infiltration locally and at distant sites, such as liver. What is more, hepatic inflammation resulting from adipose proinflammatory cytokines does play an important role in the development of NAFLD [39].

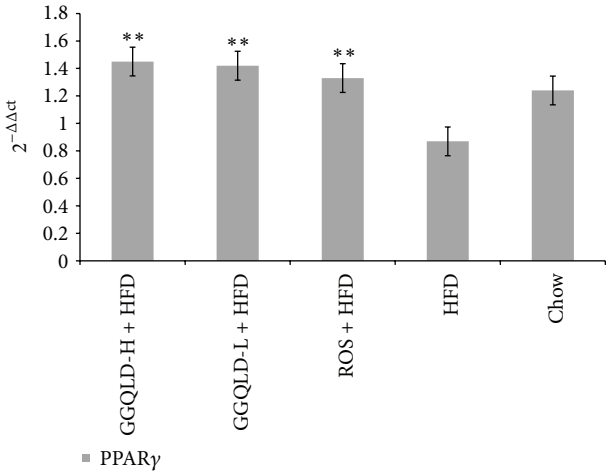

Figure 7: RT-PCR for PPARγ expression in liver tissue. In the *in vivo* study, data are means ± SD; $n = 6$ rats/group. $^{\blacktriangle}P < 0.05$, $^{\blacktriangle\blacktriangle}P < 0.01$ versus chow; $^{*}P < 0.05$, $^{**}P < 0.01$ versus HFD.

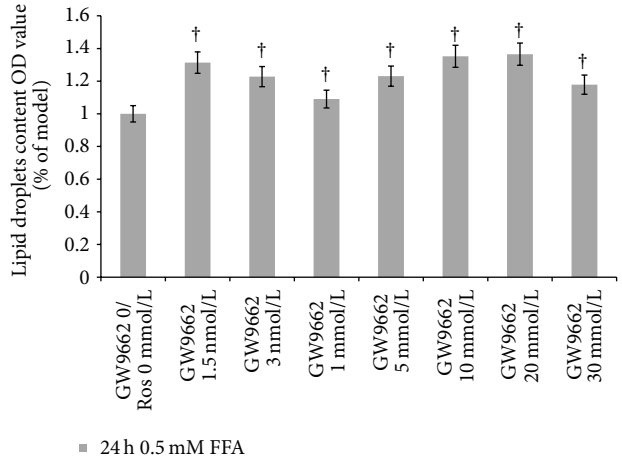

Figure 9: Lipid droplets content OD value (% of model) in NAFLD cell model. In the *in vitro* study, data are means ± SD; $n = 5$. $^{\dagger}P < 0.05$ versus model (GW9662 0/Ros 0 mmol/L); $^{\Delta}P < 0.05$ versus GW9662 10 mmol/L.

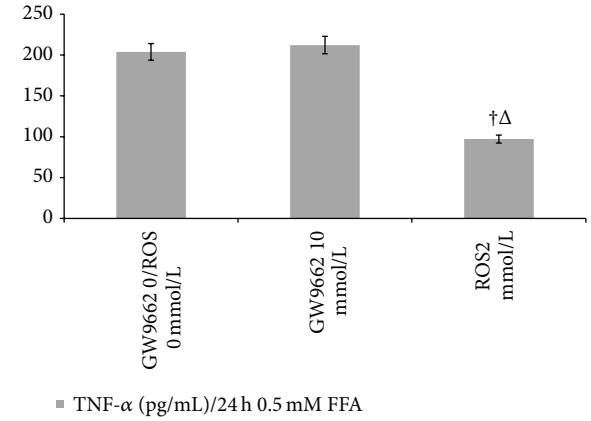

Figure 8: TNF-α level in supernatant in NAFLD cell model with GW9662 10 mmol/L and Ros 2 mmol/L. In the *in vitro* study, data are means ± SD; $n = 5$. $^{\dagger}P < 0.05$ versus model (GW9662 0/Ros 0 mmol/L); $^{\Delta}P < 0.05$ versus GW9662 10 mmol/L.

The present study detected serum and liver TNF-α by biochemical method. The serum TNF-α level elevated in HFD group was shown in the present study, while decreasing in GGQLD and rosiglitazone groups. Besides, the expression of PPARγ protein and gene was also measured. The protein and gene expression in HFD rats decreased compared to chow group. Remarkably, GGQLD made a great contribution to increasing PPARγ protein and gene expression, with the equal effects in rosiglitazone group, which approved the effect of GGQLD on PPARγ. In order to fully explore the PPARγ/TNF-α pathway, we also clarified the relation between PPARγ and TNF-α using PPARγ antagonist GW9662 in a NAFLD cell model induced by free fat acid [14], of which the results showed that the production of TNF-α was evaluated in GW9662 10 mmol/L group, while the TNF-α decreased in rosiglitazone 2 mmol/L group, suggesting TNF-α is strongly related to PPARγ. Meanwhile, lipid droplets levels were increased significantly in groups with different concentrations of GW9662 compared with the model group *in vitro*.

Numerous herbal products have been believed to have therapeutic benefit on NAFLD, such as silymarin (*milk thistle*), glycyrrhizin (*Main licorice*), curcumin (*Rhizoma curcumae longae*), berberine (*Rhizoma coptidis*), and puerarin (*Kudzu root*) [40–42], of which glycyrrhizin, berberine, and puerarin may give a great contribution to GGQLD on treating NAFLD. Puerarin, for example, has antioxidative and anti-inflammatory activity [43] and can exhibit therapeutic effect on NAFLD by antioxidation, lowering cholesterol, and improving leptin signal transduction [44, 45], while berberine improves glucose metabolism by inhibition of hepatic gluconeogenesis [46] and reduces methylation of the microsomal triglyceride transfer protein promoter in fatty liver [42].

Thus, based on the data in the present study, GGQLD had a positive intervention on NAFLD through improving lipid regulation and inflammation, which gave more lab data to support its clinical use. Because the medicine herbals in GGQLD have been used in traditional Chinese medicine (TCM) for thousand years, GGQLD has been considered for its safety and tolerability. This study explored that GGQLD is an optimal approach to NAFLD through managing lipid metabolic, inflammatory, and histological abnormalities via PPARγ/TNF-α pathway. Further experiments would focus on systematic molecular mechanisms of GGQLD, using different blocking agents and advanced experimental techniques.

Abbreviations

GGQLD: Gegenqinlian decoction
NAFLD: Nonalcoholic fatty liver disease
NASH: Nonalcoholic steatohepatitis
H&E: Haematoxylin and Eosin
ORO: Oil Red O

ROS: Rosiglitazone
TNF-α: Tumor necrosis factor-α
PPARγ: Peroxisome proliferator activated receptor γ.

Conflict of Interests

The authors declare that they have no conflict of interests.

Authors' Contribution

Yun-liang Wang and Li-juan Liu conducted the experiments. Yun-liang Wang performed the statistical analysis. Yun-liang Wang and Wei-han Zhao conducted H&E and ORO staining. Yun-liang Wang, Li-juan Liu, and Wei-han Zhao performed all the ELISA, Western blotting, and RT-PCR studies and drafted the paper. Jun-xiang Li performed the statistical analysis, drafting, and critical revision of the paper. All authors read and approved the final paper.

Acknowledgments

This paper is supported by Innovation Team of Beijing University of Chinese Medicine (no. 2011-CXTD-24); Self-Selected Subject of Beijing University of Chinese Medicine (no. 2013-JYBZZ-XS-153); Wang Bao-En Liver Fibrosis Research Fund (no. 2013-xjs).

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Hindawi Publishing Corporation
Evidence-Based Complementary and Alternative Medicine
Volume 2015, Article ID 171356, 11 pages
http://dx.doi.org/10.1155/2015/171356

Research Article

Huaier Aqueous Extract Induces Hepatocellular Carcinoma Cells Arrest in S Phase via JNK Signaling Pathway

Chengshuo Zhang,[1] Jialin Zhang,[1] Xin Li,[2] Ning Sun,[1] Rui Yu,[1] Bochao Zhao,[1] Dongyang Yu,[3] Ying Cheng,[1] and Yongfeng Liu[1]

[1]*Department of Hepatobiliary Surgery and Unit of Organ Transplantation, First Hospital of China Medical University, 155 Nanjingbei Street, Heping District, Shenyang 110001, China*
[2]*Department of General Surgery, Fourth Hospital of China Medical University, 4 Chongshandong Road, Huanggu District, Shenyang 110032, China*
[3]*Department of Gastrointestinal Surgery, First Hospital of China Medical University, 155 Nanjingbei Street, Heping District, Shenyang 110001, China*

Correspondence should be addressed to Jialin Zhang; jlz2000@yeah.net

Received 21 August 2014; Revised 12 November 2014; Accepted 12 November 2014

Academic Editor: Qing He

Huaier aqueous extract, the main active constituent of Huaier proteoglycan, has antihepatocarcinoma activity in experimental and clinical settings. However, the potential and associated antihepatoma mechanisms of Huaier extract are not yet fully understood. Therefore, in this study, we aimed to elucidate the inhibitory proliferation effect of Huaier extract on apoptosis and cycle of HepG2 and Bel-7402 cells. Our data demonstrated that incubation with Huaier extract resulted in a marked decrease in cell viability dose-dependently. Flow cytometric analysis showed that a 48 h treatment of Huaier extract caused cell apoptosis. Typical apoptotic nucleus alterations were observed with fluorescence microscope after Hoechst staining. Immunoblot analysis further demonstrated that Huaier extract activated caspase 3 and PARP. Additionally, Huaier extract inhibited the activity of p-ERK, p-p38, and p-JNK in terms of MAPK. Furthermore, Huaier extract induced HCC cells arrest in S phase and decreased the cycle related protein expression of β-catenin and cyclin D1. Studies with JNK specific inhibitor, SP600125, showed that Huaier extract induced S phase arrest and decreased β-catenin and cyclin D1 expression via JNK signaling pathway. In conclusion, we verify that Huaier extract causes cell apoptosis and induces hepatocellular carcinoma cells arrest in S phase via JNK pathway, which advances our understanding on the molecular mechanisms of Huaier extract in hepatocarcinoma management.

1. Introduction

Hepatocellular carcinoma (HCC) is the fifth most frequent malignancy and the second leading fatal disease worldwide [1]. There are several canonical strategies for hepatocellular carcinoma treatment, including surgical resection, local ablation, liver transplantation, radiotherapy, chemotherapy, and molecular targeted therapy [2–4]. However, these treatments cause serious side effects sometimes; they are not applicable to all of the patients and demonstrate to be of limited efficacy [5, 6]. Therefore, identification of novel antitumor agents from natural products with better effectiveness is an alternative choice for management of hepatocellular carcinoma.

In recent years, many Chinese herbs have been gradually discovered to be potential sources of antitumor drugs for its role in killing tumor cells less intensively and more naturally [7, 8]. *Trametes robiniophila* Murr. (Huaier) has been applied in traditional Chinese medicine for approximately 1600 years [9]; however, its antitumor properties are found and used as a complementary therapy only in recent decades. The main effective ingredient of this officinal fungi has been identified as proteoglycan which contains 41.53% polysaccharides, 12.93% amino acids, and 8.72% water [10]. A number of studies have demonstrated that Huaier extract inhibited proliferation and induced apoptosis in pulmonary cancer, breast cancer, melanoma, and colorectal cancer [11–14]. In

addition, Huaier extract has also been indicated as a suppressant in angiogenesis and cell motility of ovarian cancer [6, 15]. The accumulating evidences have demonstrated that Huaier extract dose-dependently inhibited the proliferation, adhesion, migration, invasion, and angiogenesis and induced apoptosis of hepatoma cells [16, 17]. However, the underlying molecular mechanisms of Huaier extract activities in hepatocellular carcinoma cells are not yet fully understood.

Cell cycle deregulation, resulting in uncontrolled cell proliferation, is one of the most common alterations that occur during tumor development. Therefore, cell cycle arrest is considered to be an effective strategy for eliminating cancer cells [18]. Two major checkpoints, one at the G1/S transition and the other at the G2/M transition, regulate the cell cycle and, therefore, the modulated expression of cell cycle regulatory molecules on antiproliferation has been investigated in numerous cell types [19]. A general critical event associated with DNA damage is the activation of cell cycle checkpoints and cycling and cyclic-dependent kinases (cdks) are evolutionarily conserved proteins that are essential for cell cycle control [20]. Distinct pairs of cyclins and cdks regulate the progression through the various stages of the cell cycle; cdk activity is regulated by cyclins, which bind to and activate cdks [21]. Among these cyclins, cyclin D1 is regarded as an oncogene and is a major driver of multiple types of human tumors including breast and squamous cell cancers, B-cell lymphoma, myeloma, and parathyroid adenoma [22]. In addition to cyclin D1 and its upstream effector β-catenin [23], the mitogen-activated protein kinase (MAPK) superfamily members are also associated with increased migration, invasion, proliferation, survival, and apoptosis, thus serving different roles in cellular responses [23]. ERK1/2, p38 MAPK, and JNK/SAPK have been reported to play a central role in the regulation of β-catenin and cyclin D1 expression [24].

Little is known about the molecular mechanisms responsible for the proliferative properties of Huaier extract, and no studies have investigated the potential role of Huaier in cell proliferation of human HCC cells. Understanding the mechanism of action of Huaier extract should provide useful information for their possible application in cancer therapy and cancer prevention. Therefore, in this study we mainly aim to explore the antiproliferation mechanisms of Huaier extract in HCC cells.

2. Materials and Methods

2.1. Preparation of Huaier Aqueous Extract. Huaier electuary ointment was a gift from Gaitianli Pharmaceutical Co., Ltd. (Qidong, Jiangsu, China). Two grams of the electuary ointment was dissolved in 20 mL of complete medium and was sterilized with 0.22 μm filter to get the 100 mg/mL stock solution for long storage at −20°C [25].

2.2. Cell Culture. HCC cell lines, including HepG2 and Bel-7402, were grown in RPMI 1640 medium (Gibco, USA) in the presence of 10% fetal bovine serum (Gibco, USA) and incubated in a humidified atmosphere containing 5% CO_2 at 37°C. Immortalized normal liver epithelial cells, THLE-3, were maintained in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Carlsbad, CA) supplemented with 100 U/mL penicillin and 100 μg/mL streptomycin in the presence of 10% fetal bovine serum and incubated in a humidified atmosphere containing 5% CO_2 at 37°C.

2.3. Cell Proliferation Assay. Cell proliferation was measured using the CellTiter 96 Aqueous One Solution cell proliferation assay (Promega, Madison, WI, USA). Cells were seeded in 96-well plates at a concentration of 10^4 cells/well, allowed to adhere for 24 h, and subsequently exposed to different concentrated solutions as indicated below. Proliferation viability was measured according to the manufacturer's instructions after 48 h. The absorbance value at 490 nm was measured with an ELISA reader (BioTek, Vermont, USA). The stock solution of Huaier extract was diluted at final concentrations of 0, 2, 4, 8, and 16 mg/mL with complete 1640 medium. The viability ratio was calculated according to the following formula: The Viability Ratio = [(the absorbance of experimental group − the absorbance of blank group)/(the absorbance of untreated group − the absorbance of blank group)] × 100%.

2.4. Apoptosis Assay. Cell apoptosis was determined using the Annexin V-FITC apoptosis detection kit (Bio-science, Beijing, China). Briefly, 2×10^5 cells were seeded into a 6-well plate. After 48 h exposure to different concentrations of Huaier aqueous extract as above, all the adherent cells were collected with 0.25% trypsin without EDTA, including the floating cells in the medium. Annexin V-FITC and propidium iodide (PI) were used for staining according to the manufacturer's instructions. The double-stained cells were subsequently analyzed by a FACSCanto flow cytometer (Becton-Dickinson, Mountain View, CA, USA). At least 10,000 cells were counted each time.

2.5. Hoechst 33258 Staining. Following treatment with Huaier extract at various concentrations for up to 48 h in a 6-well plate, cells were washed twice with PBS and fixed in 1 mL of 4% paraformaldehyde for 10 min at 4°C. After washing twice with PBS, cells were stained with 500 μL Hoechst 33258 (Beyotime, Haimen, China) for 15 min at room temperature in the dark and then washed with PBS. Afterwards, the cells were mounted and examined under fluorescence microscopy (Olympus IX71, Tokyo, Japan). Apoptotic cells were identified by the condensation and fragmentation of their nucleus. The apoptotic ratio was obtained by the following calculation: The Apoptotic Ratio = apoptotic cell number/seeded cell number × 100%.

2.6. Cell Cycle Assay. Briefly, 2×10^5 cells were seeded into a 6-well plate and starved in serum-free medium on the second day. After 12 h starvation, the cells were treated with gradient concentrations of Huaier solution for 48 h. The cells were then trypsinized, washed with cold PBS, and fixed overnight with 70% cold ethanol at 4°C. The next day, the fixed cells were centrifuged at 1200 g for 5 min and washed once with PBS. After that, the cells were suspended in PI/RNase staining buffer for

30 min in the dark according to the manufacturer's instructions of Cell Cycle Detection Kit (KeyGEN, Nanjing, China). Then, the DNA contents of the cells were analyzed in a FACScan flow cytometer (Becton Dickenson, San Jose, CA, USA). At least 10,000 cells were collected for each measurement.

2.7. Western Blot Analysis. The cells treated with Huaier extract or SP600125 (a JNK1/2 inhibitor, Beyotime, Haimen, China) for 48 h were washed twice with ice-cold PBS and lysed in ice-cold protein lysis buffer supplemented with 1% (v/v) protease inhibitor cocktail and PMSF. The lysates were centrifuged at 12,000 rpm for 10 min at 4°C. The suspension protein concentrations were determined using a BCA Protein Assay kit (Beyotime, Haimen, China) and were then denatured by boiling. Total proteins (25 μg/lane) were resolved onto SDS-PAGE and transferred onto a PVDF membrane in a wet transfer system (Bio-Rad, USA) at 70 V at 4°C. For immunoblotting, the PVDF membrane was incubated with Tris-buffered saline plus Tween-20 (TBS-T) containing 5% nonfat milk for 1.5 h and then incubated with a specific primary antibody overnight at 4°C. Horseradish peroxidase- (HRP-) conjugated IgG was used as the secondary antibody and incubated for 2 h. Afterwards, reactive protein was detected using an enhanced chemiluminescence (ECl) commercial kit (Beyotime, Beijing, China). The results were recorded using the MicroChemi Bio-Imaging Systems (DNR Bio-Imaging Systems Ltd, Jerusalem, Israel) and Quantity One version 4.5.0 software (Bio-Rad, Hercules, CA, USA).

The primary antibodies used in this study were as follows: rabbit anti-pro-caspase 3 polyclonal antibody (Cat no. 19677-1-AP; Proteintech Group, Inc. Chicago, IL, USA), rabbit anti-cleaved-caspase 3 polyclonal antibody (Cat no. 25546-1-AP; Proteintech Group), rabbit anti-β-catenin polyclonal antibody (Cat no. 51067-2-AP; Proteintech Group), mouse anti-cyclin D1 monoclonal antibody (Cat no. 60186-1-Ig; Proteintech Group), mouse anti-GAPDH monoclonal antibody (Cat no. 60004-1-lg; Proteintech Group), rabbit anti-ERK polyclonal antibody (Cat no. 94; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), rabbit anti-p38MAPK polyclonal antibody (Cat no. 535; Santa Cruz Biotechnology), rabbit anti-JNK polyclonal antibody (Cat no. 571; Santa Cruz Biotechnology), mouse anti-pERK monoclonal antibody (Cat no. 7383; Santa Cruz Biotechnology), mouse anti-pp38MAPK monoclonal antibody (Cat no. 7973; Santa Cruz Biotechnology), mouse anti-pJNK monoclonal antibody (Cat no. 6254; Santa Cruz Biotechnology), and mouse anti-tubulin monoclonal antibody (Cat no. 0098; Cwbiotech, Beijing, China). The secondary antibodies included goat anti-rabbit IgG serum (1 : 40,000 dilution; Zhongshan Golden Bridge, Beijing, China) and goat anti-mouse IgG serum (1 : 40,000 dilution; Zhongshan Golden Bridge).

2.8. Statistical Analysis. All experiments were performed three times. Data were presented as means ± standard deviations (SD). The differences were analyzed using one-way ANOVA followed by the Student-Newman-Keuls test and all statistical analyses were performed using GraphPad Prism 5 software. Statistical differences are presented at probability levels of $P < 0.05$, $P < 0.01$, and $P < 0.001$.

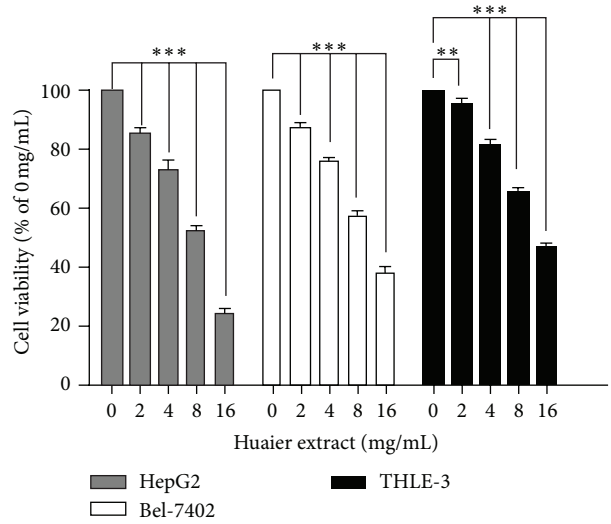

Figure 1: Effect of Huaier extract on the viability of HepG2, Bel-7402, and THLE-3 cells. HepG2, Bel-7402, and THLE-3 cells (10^4 cells/well) were treated with various concentrations (0, 2, 4, 8, and 16 mg/mL) of Huaier extract for 48 h. Cell viability was determined by using an MTS assay. The results represent the means ± SD of 3 independent experiments. $^{**}P < 0.01$ and $^{***}P < 0.001$, compared with that of the untreated control, respectively (0 mg/mL).

3. Results

3.1. Huaier Extract Inhibits Cell Proliferative Viability of HepG2 and Bel-7402 Cells. To evaluate the proliferative effect of Huaier extract on HepG2 and Bel-7402 cells, we measured cell proliferative viability using the MTS assay after the cells were dose-dependently treated with Huaier extract for 48 h. As shown in Figure 1, Huaier extract significantly suppressed cell viability of both HepG2 and Bel-7402 cells in a dose-dependent manner with IC_{50} value of 7.6 and 10.6 mg/mL, respectively, after 48 h. But the IC_{50} value in the case of THLE-3 was 13.8 mg/mL, which means that the Huaier extract is less toxic to the normal liver cells than to HCC cells.

3.2. Huaier Extract Induces Cell Apoptosis in HepG2 and Bel-7402 Cells. To demonstrate the apoptosis effect of Huaier extract, we used FCM analysis with Annexin V-FITC and PI double staining. After treatment with different doses of Huaier extract for 48 h, early apoptotic cells and late apoptotic cells were differentiated from viable or necrotic ones. In the control group, there were almost normal cells, rarely apoptotic cells, while in Huaier extract groups, the rates of apoptotic cells gradually increased along with increasing concentrations of Huaier extract. The rates of apoptosis in different Huaier extract (0, 2, 4, 8, and 16 mg/mL) groups were 5.50 ± 1.04%, 13.57 ± 0.58%, 29.40 ± 3.00%, 49.53 ± 8.50%, and 96.22 ± 3.06%, respectively, in HepG2 cells, and 1.5 ± 0.5%,

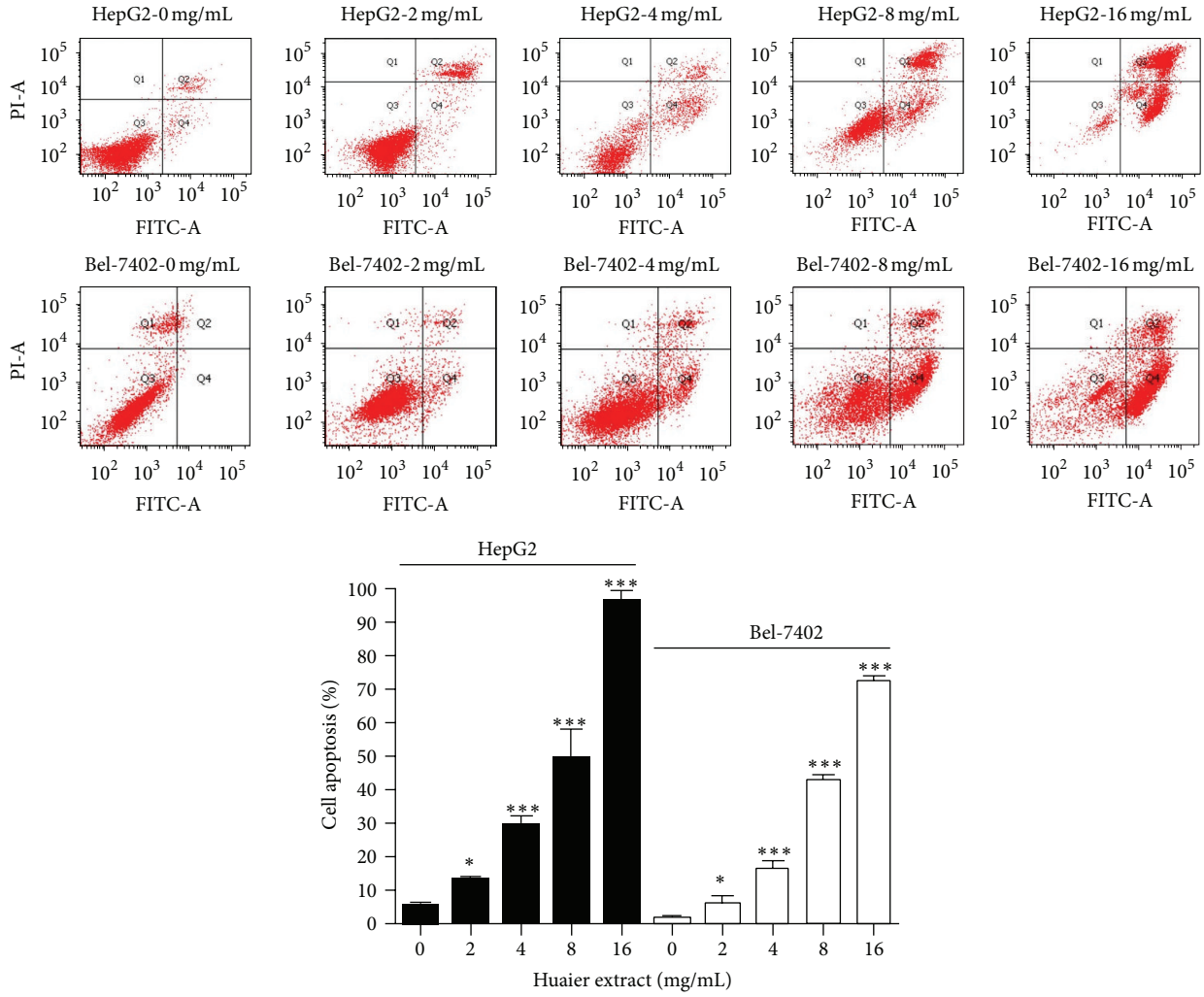

Figure 2: Effect of Huaier extract on the apoptosis of HepG2 and Bel-7402 cells. FCM analysis for apoptosis after treatment by Annexin V-FITC and PI staining on HCC cells with different doses of Huaier extract (0, 2, 4, 8, and 16 mg/mL) for 48 h. The ratios are expressed as the mean ratios ± SD in triplicate. $^{*}P < 0.05$ and $^{***}P < 0.001$, compared with that of the untreated control, respectively (0 mg/mL).

6.1 ± 2.1%, 16.6 ± 2%, 43 ± 1.5%, and 72.4 ± 1.6% respectively, in Bel-7402 cells (Figure 2).

3.3. Huaier Extract Induces Morphological Changes in HepG2 Cells. In addition, we verified the apoptotic effect of Huaier extract in HepG2 cells by morphological changes. After treatment with different doses of Huaier extract for 48 h, HepG2 cells were stained with Hoechst 33258. The normal cells in morphology are round and homogenous, while the morphological changes of cell apoptosis include cell shrinkage, nuclear condensation, and fragmentation. Fluorescence dye stains condense chromatin of apoptotic cells more brightly than chromatin of normal cells. The number of HepG2 cells adhering to the culture plates in Huaier extract treatment was greatly reduced compared to control group. The apoptotic morphological changes were observed in the Huaier extract-treated groups, whereas few apoptotic cells were found in the control group. The percentage of apoptotic cells in different Huaier extract (0, 2, 4, 8, and 16 mg/mL) groups was 4.27 ± 1.80%, 14.27 ± 1.20%, 27.60 ± 2.00%, 33.17 ± 1.90%, and 62.67 ± 2.40%, respectively, in HepG2 cells (Figure 3).

3.4. Huaier Extract Activates Caspase 3 and Induces the Expression of Cleaved Caspase 3 and Cleaved PARP in HepG2 and Bel-7402 Cells. To further confirm the apoptotic mechanisms of Huaier extract on HCC cells, we tested the expression of procaspase 3, cleaved caspase 3, and cleaved PARP with Western blot. Huaier extract activated the caspase 3, resulting in increased expression of cleaved caspase 3, cleaved PARP, and decreased expression of procaspase 3 (Figure 4).

3.5. Huaier Extract Induces Cells Arrest in S Phase of HepG2 and Bel-7402 Cells. To further investigate the effect of Huaier extract on the cell cycle, the cell cycle profiles of HepG2 and Bel-7402 cells were analyzed using flow cytometry. The cells were treated with Huaier extract at concentrations of 0, 2, 4, 8, and 16 mg/mL for 48 h and stained with PI. Huaier extract

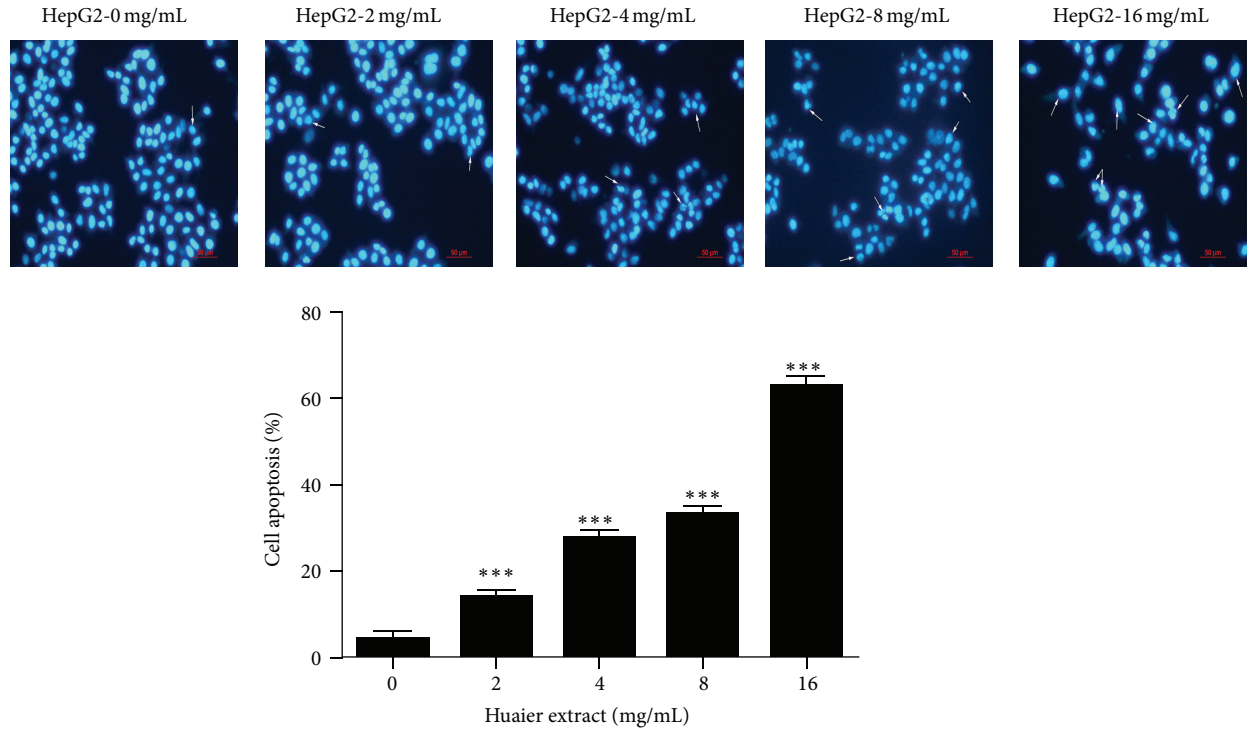

Figure 3: Effect of Huaier extract on the morphological changes of HepG2 cells. Hoechst 33258 staining was used to observe the apoptotic cells under a BX-60 fluorescence microscope (200x) after cells were treated with different doses of Huaier extract (0, 2, 4, 8, and 16 mg/mL) for 48 h. The arrow shows apoptotic cells. The values are expressed as the mean ratios ± SD from three independent experiments. $^{***}P < 0.001$, compared with that of the untreated control (0 mg/mL).

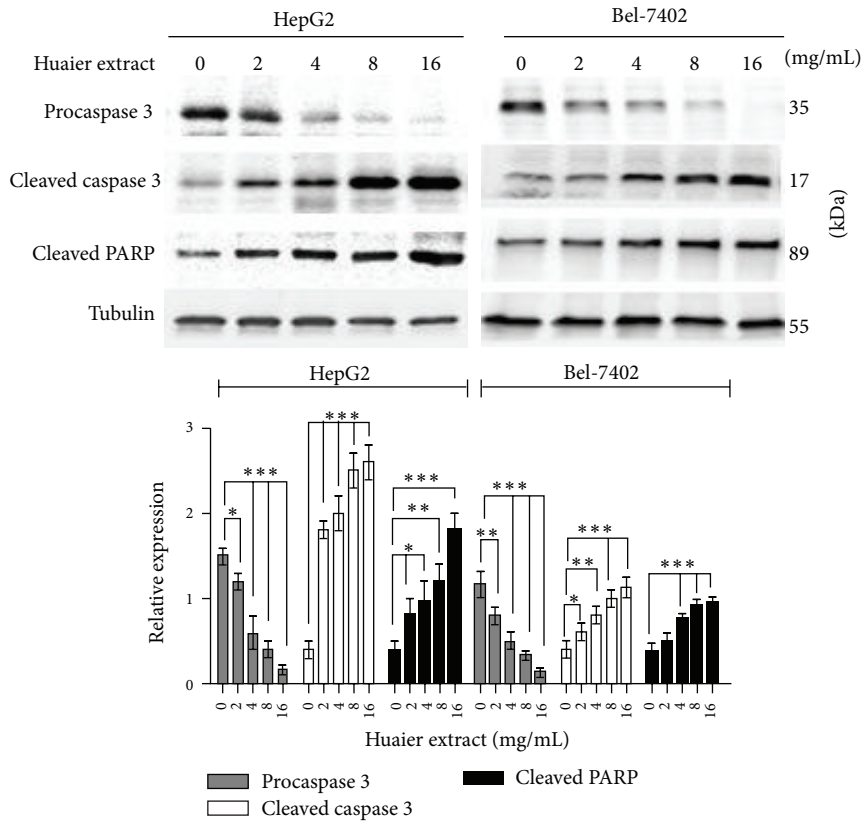

Figure 4: Effects of Huaier extract on expression of procaspase 3, cleaved caspase 3, and cleaved PARP in HCC cells. HCC cells were treated with various concentrations (0, 2, 4, 8, and 16 mg/mL) of Huaier extract for 48 h, and then cell lysates were subjected to Western blotting with procaspase 3, cleaved caspase 3, and cleaved PARP antibodies. The densitometric ratios were normalized to those of tubulin, and the results are expressed as the mean densitometric ratios ± SD in three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$, compared with that of the untreated control, respectively (0 mg/mL).

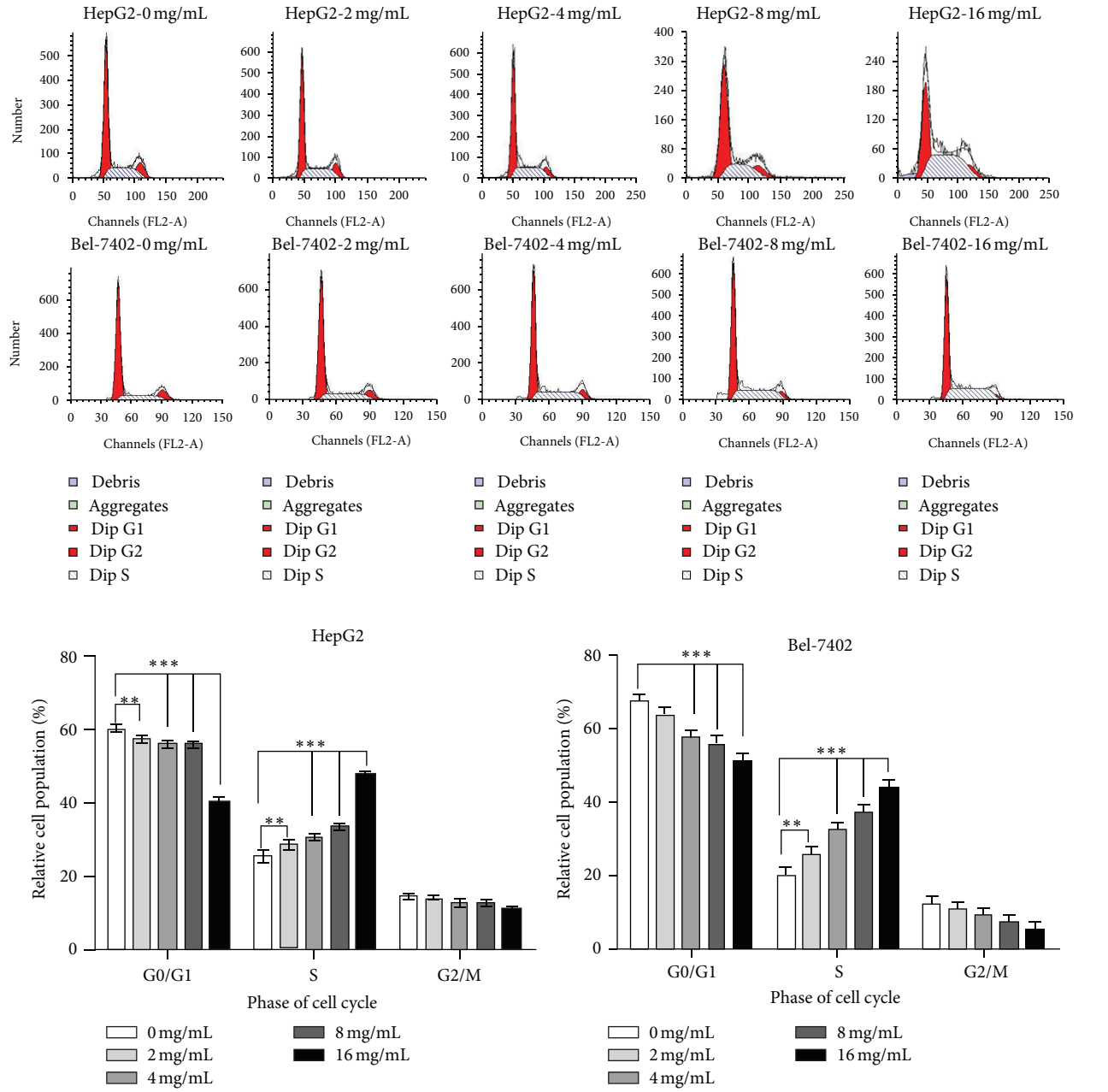

FIGURE 5: Effects of Huaier extract on the cell cycle of HCC cells by FCM. The cell cycle distributions in HepG2 and Bel-7402 cells were determined by PI staining and FCM analysis after being treated with different concentrations of Huaier extract (0, 2, 4, 8, and 16 mg/mL) for 48 h. The ratios are expressed as the mean ratios ± SD of three independent experiments. $^{**}P < 0.01$ and $^{***}P < 0.001$, compared with that of the untreated control, respectively (0 mg/mL).

treatment resulted in a significant increase in the percentage of cells in the S phase and a significant decrease in the percentage of cells in the G0/G1 phase. The percentage of cells accumulated in the S phase were 25.31 ± 1.53%, 28.43 ± 1.25%, 30.48 ± 0.76%, 33.55 ± 0.81%, and 47.57 ± 0.87%, respectively, in HepG2 cells, and 20.25 ± 2.06%, 25.87 ± 2.06%, 32.43 ± 2.02, 37.11 ± 2.05, and 43.61 ± 2.33, respectively, in Bel-7402 cells. The accumulation of G0/G1 phase cells was maximal in the control group and declined with increasing concentrations of Huaier extract. The decrease in the number of G0/G1 phase cells was 60.36 ± 0.71%, 57.43 ± 0.95%, 56.13 ± 0.96%, 55.76 ± 0.54%, and 40.38 ± 0.88%, respectively, in HepG2 cells, and 67.4 ± 2.07, 63.62 ± 2.30, 57.5 ± 1.84, 55.66 ± 2.41, and 51.23 ± 2.10, respectively, in Bel-7402 cells (Figure 5). These results indicate that Huaier extract suppresses HCC cells proliferation by inducing S phase cell cycle arrest.

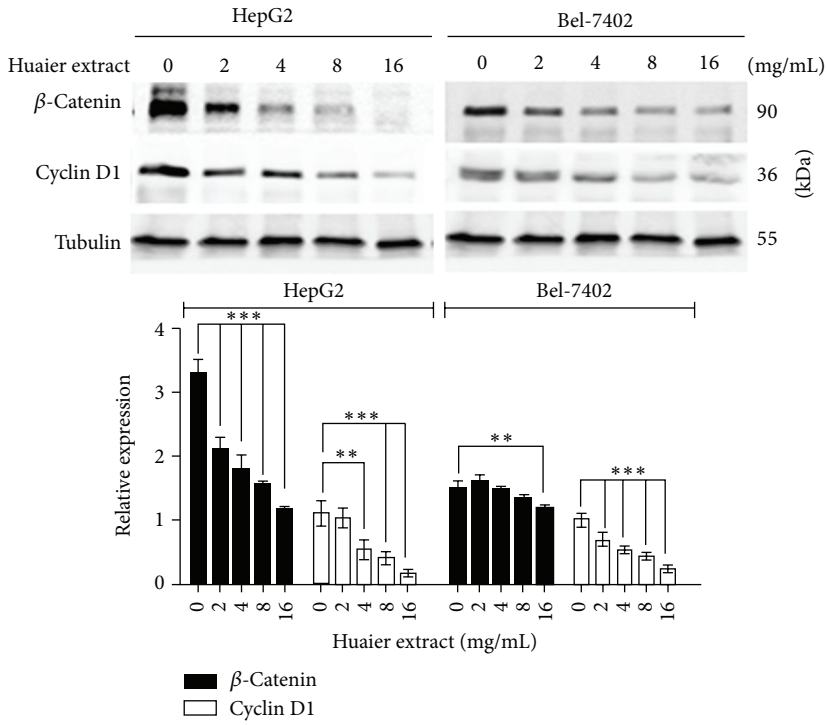

Figure 6: Effects of Huaier extract on expression of cycle related β-catenin and cyclin D1 in HCC cells. HCC cells were treated with various concentrations (0, 2, 4, 8, and 16 mg/mL) of Huaier extract for 48 h, and then cell lysates were subjected to Western blotting with β-catenin and cyclin D1 antibodies. The densitometric ratios were normalized to those of tubulin, and the results are expressed as the mean densitometric ratios ± SD in three independent experiments. $^{**}P < 0.01$ and $^{***}P < 0.001$, compared with that of the untreated control, respectively (0 mg/mL).

3.6. Huaier Extract Inhibits Cycle Related β-Catenin and Cyclin D1 Expression in HepG2 and Bel-7402 Cells. To elucidate the underlying mechanisms responsible for the proliferative inhibitory properties of Huaier extract on HepG2 and Bel-7402 cells, we detected changes of β-catenin and cyclin D1 expression by Western blotting and found that Huaier extract treatment significantly decreased the expression of β-catenin and cyclin D1 in a dose-dependent manner (Figure 6).

3.7. Huaier Extract Inhibits the Phosphorylation of MAPK in HCC Cells. MAPK has been shown to be involved in the induction of cycle related β-catenin and cyclin D1 in many types of cancer [24]. Given that treatment with Huaier extract inhibited expression of β-catenin and cyclin D1, we attempted to determine the MAPK related protein expression by Huaier extract treatment in HCC cells. By treating HepG2 and Bel-7402 cells with various concentrations of Huaier extract, it was determined that there was a significant dose-dependent decrease in the phosphorylation of ERK1/2, p38, and JNK1/2 in HCC cells (Figure 7). This finding indicates that Huaier extract may suppress the expression of β-catenin and cyclin D1 by inactivating the MAPK pathways.

3.8. Huaier Extract Inhibits the Expression of β-Catenin and Cyclin D1 and Induces S Phase Arrest via the JNK Signaling Pathway in HCC Cells. To further investigate whether JNK plays a role in reducing the expression of β-catenin and cyclin D1, HepG2 cells were pretreated with SP600125 (10 μM) for 1 h and then incubated with Huaier extract (8 mg/mL) for 48 h. Using Western blotting assay, it was found that treatment with either Huaier extract (8 mg/mL) or SP600125 (10 μM) reduced β-catenin protein levels by 19% and 28.4% and cyclin D1 protein levels by 41% and 51.7%, respectively, and that combination treatment reduced β-catenin and cyclin D1 protein levels by 54.7% and 60.7%. These data suggest that Huaier suppresses the expression of β-catenin and cyclin D1 through the downregulation of JNK signaling in HepG2 cells. Moreover, in a functional assay of cycle inhibitory properties, SP600125 also increased the percentage of HepG2 cells in the S phase, decreased the percentage of cells in the G0/G1 phase, and facilitated the Huaier extract induced S phase arrest in HepG2 cells (Figure 8). All these results indicate that Huaier extract suppresses HCC cells growth by inducing S phase cell cycle arrest through JNK signaling pathway.

4. Discussion

Huaier electuary ointment, of which the active ingredient is extracted from fungi of Huaier, has been used in clinic for the treatment of hepatocellular carcinoma with satisfactory results [26]. It has been showed that Huaier extract inhibits the proliferation and tumor angiogenesis and induces apoptosis of hepatocellular carcinoma cells [17]. Additionally, Huaier extract has also been indicated to suppress

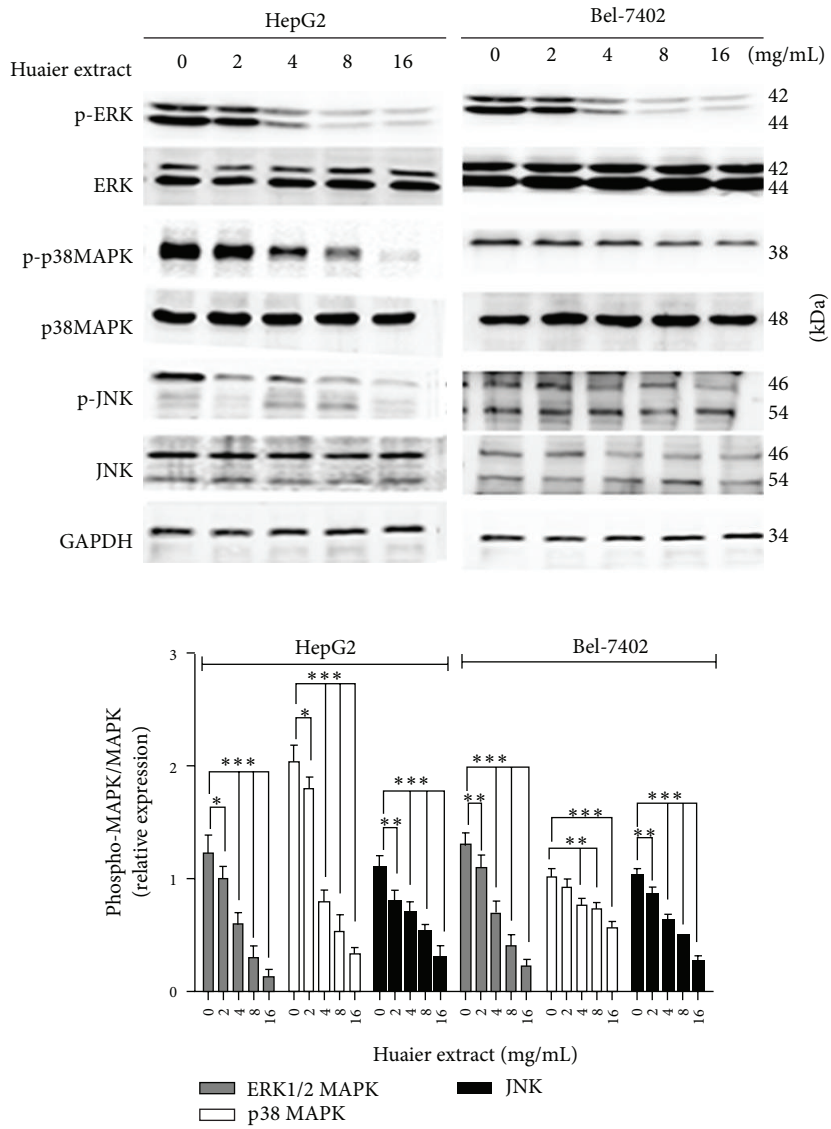

Figure 7: Effects of Huaier extract on expression of MAPK in HCC cells. HCC cells were treated with various concentrations (0, 2, 4, 8, and 16 mg/mL) of Huaier extract for 48 h, and then cell lysates were subjected to Western blotting using respective antibodies against MAPKs. Total ERK1/2, p38, JNK, and GAPDH were included as the loading controls, and the results are expressed as the mean densitometric ratios ± SD in three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$, compared with that of the untreated control, respectively (0 mg/mL).

the hepatocellular carcinoma biological activities of adhesion, migration, and invasion related to epithelial–mesenchymal transition (EMT) [16]. All these findings provide a certain rationale for therapeutic properties of Huaier extract in clinical applications of hepatocellular carcinoma. However, the potential molecular mechanisms are still elusive and require further validation.

In the current study, the results of MTS assay demonstrated that Huaier extract significantly attenuated the proliferation of HCC cells in a dose-dependent manner. To our knowledge, the inhibition of cell proliferation was involved in apoptosis and block of cell cycle progression. It has been reported that Huaier extract induced melanoma cells and breast cancer cells apoptosis via the increased expression of P53 and the modulation of Bcl-2/BAX protein expression [13, 18]. Moreover, Huaier extract also caused apoptosis in lung cancer cells via a miR-26b-5p-EZH2-mediated approach [11]. Ren et al. reported that Huaier extract induced HepG2 cells apoptosis by the detection of flow cytometry [17]. However, little is known on the exact apoptosis mechanism of Huaier extract on HCC cells. Of note, our data ascertained that Huaier extract induces the onset of apoptosis by activating caspase 3, increasing the expression of cleaved caspase 3 and cleaved PARP in HCC cells.

Eukaryotic cell proliferation is primarily regulated by the cell cycle, which consists of four phases: the G1 phase, the S phase, the G2 phase, and the M phase [27]. It is well established that the loss of key cell cycle checkpoints

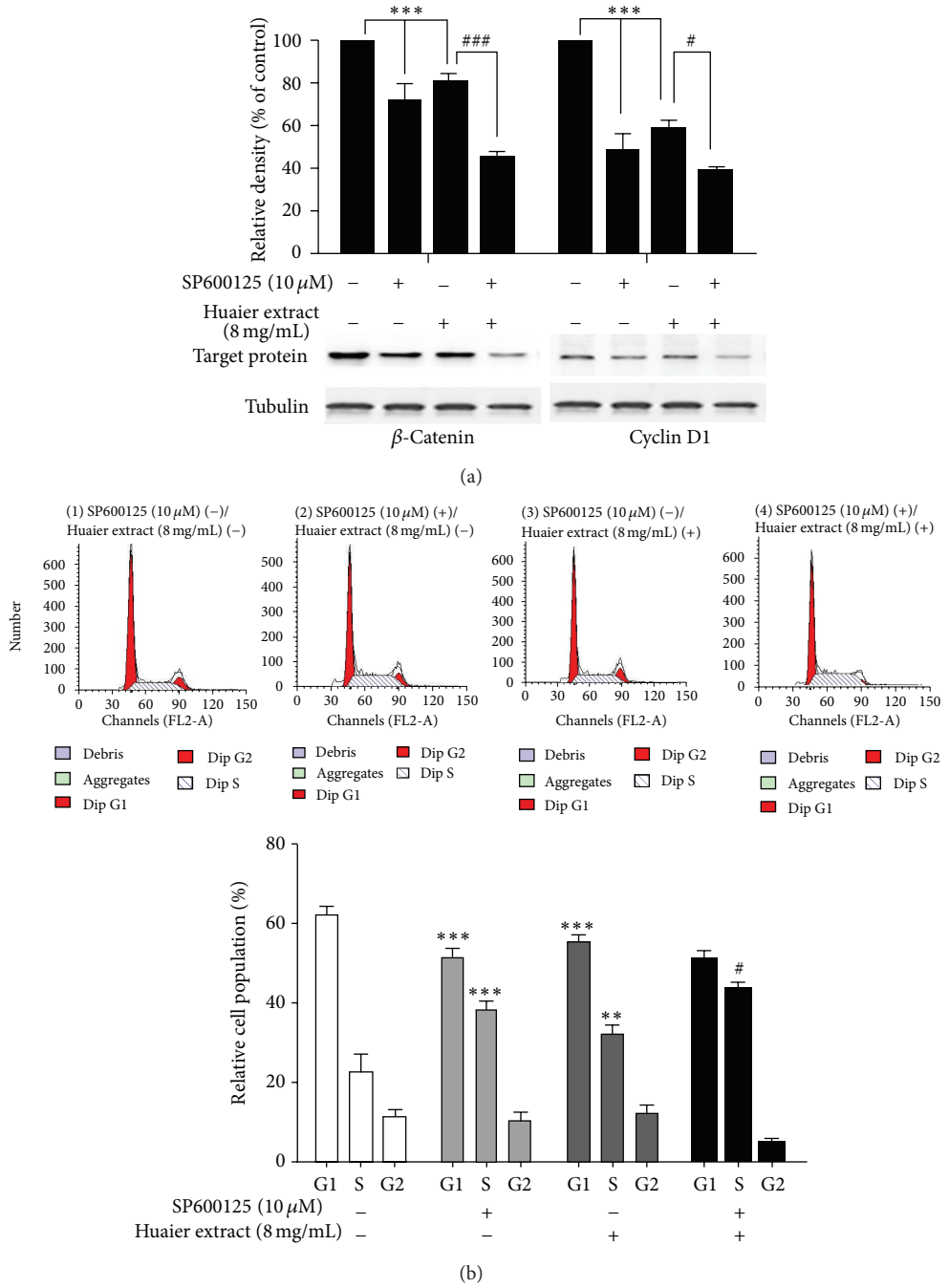

FIGURE 8: Effect of Huaier extract on the expression of β-catenin and cyclin D1, as well as cell cycle and inhibition after being treated with a JNK inhibitor (SP600125). (a) HepG2 cells were pretreated with SP600125 (10 μM) for 1 h and then incubated in the presence or absence of Huaier extract (8 mg/mL) for 48 h. β-catenin and cyclin D1 protein was determined by using Western blotting. (b) Cells were also assessed by cell cycle. Data are presented as the means ± SD of at least three independent experiments. $^{**}P < 0.01$, $^{***}P < 0.001$, untreated cells versus SP600125 or Huaier extract; $^{\#}P < 0.05$, $^{\#\#\#}P < 0.001$ Huaier extract versus SP600125 plus Huaier extract.

is a hallmark of cancer cells, which leads to abnormal proliferation and facilitates oncogenic transformation [28]. The G1/S transition is one of the two predominant checkpoints of the cell cycle and is responsible for the initiation and completion of DNA replication. The majority of studies have reported perturbation of the S/G2 phase transition with a decrease of cells in the G0/G1 phase of the cell cycle and an increase of cells in the S phase [28, 29]. In the present study, FACS analysis with PI staining revealed that the percentage proportion was increased in the S phase cells and reduced in the G0/G1 phase cells following Huaier extract treatment in a dose-dependent manner, indicating that the inhibitory effect

of Huaier extract on HCC cell proliferation is mediated by S phase cell cycle arrest.

In the current study, the expression of the important cycle regulatory protein, cyclin D1, and its upstream effector β-catenin were analyzed following the treatment of HepG2 and Bel-7402 cells with Huaier extract and the results were consistent with previous observations that S phase arrest is accompanied by the decreased expression of cyclin D1 and β-catenin [30, 31]. The modifications of these cell cycle-associated proteins induced by Huaier extract appear to block the cell progression through the S phase.

Activated MAPK pathways play a central role in HCC proliferation and cell cycle development [32, 33]. However, there are no reports and literatures of the relevant studies illustrating the relationship between Huaier extract and MAPK pathways on the proliferation of HCC. Therefore, we investigated the effect of Huaier extract on MAPK pathways in HCC cells and found that Huaier extract significantly downregulated the phosphorylation of ERK, p38MAPK, and JNK in HepG2 and Bel-7402 cells in a dose-dependent manner. What is more, we further investigated the role of JNK on cell cycle distribution and cycle related protein expression of cyclin D1 and β-catenin. Noticeably, treatment with SP600125 obviously decreased expression of cyclin D1 and β-catenin and also caused a significant increase in the percentage of cells in S phase and decrease in the percentage of cells in the G0/G1 phase, which was consistent with the treatment of Huaier extract on cells. All these results demonstrate that Huaier extract induces hepatocellular carcinoma cells arrest in S phase via JNK signaling pathway.

Overall, our findings verify that Huaier extract causes HCC cell apoptosis and induces hepatocellular carcinoma cells arrest in S phase via JNK signaling pathway, which advances our understanding on the molecular mechanisms of Huaier extract in hepatocarcinoma management. The clinical treatment for liver cancer is difficult due to its malignant biological characteristics such as invasion, metastasis, and malignant proliferation. In this study, we explored the antiproliferative mechanisms of Huaier extract on HCC cells, providing a novel prospect for liver cancer treatment. Huaier extract serves as a promising therapeutic drug for liver cancer, not only for a potent apoptosis inducing, antiangiogenic, and anti-invasive agent, but also for the block role in cell cycle progression.

Conflict of Interests

The authors declare no conflict of interests.

Acknowledgment

The present study was supported by the science and technology project of Shenyang (Grant no. F13-212-9-00).

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Hindawi Publishing Corporation
Evidence-Based Complementary and Alternative Medicine
Volume 2015, Article ID 125659, 11 pages
http://dx.doi.org/10.1155/2015/125659

Review Article

Curative Effects of Fuzheng Huayu on Liver Fibrosis and Cirrhosis: A Meta-Analysis

Shu Dong, Qi-Long Chen, and Shi-Bing Su

Research Center for Traditional Chinese Medicine Complexity System, Shanghai University of Traditional Chinese Medicine, Shanghai 201203, China

Correspondence should be addressed to Shi-Bing Su; shibingsu07@163.com

Received 4 September 2014; Revised 7 April 2015; Accepted 16 April 2015

Academic Editor: Thomas Ostermann

The Fuzheng Huayu (FZHY) formula is being used in antiliver fibrosis treatment in China. For systemic evaluation of the curative effects of FZHY on liver fibrosis and cirrhosis progress, a total of 1392 subjects (714 cases and 678 controls) were found to be eligible for meta-analysis in this study. Standard mean differences (SMDs) with 95% confidence interval (CI) were calculated for changes between FZHY groups and controls by employing fixed effects or random effects model. In the overall analysis, alanine transaminase (ALT) ($P = 0.003$, SMD = −0.87, 95% CI: −1.46 to −0.29), total bilirubin (TBil) ($P = 0.001$, SMD = −1.30, 95% CI: −2.10 to −0.50), hyaluronic acid (HA) ($P = 0.000$, SMD = −0.94, 95% CI: −1.30 to −0.58), laminin (LN) ($P = 0.000$, SMD = −0.80, 95% CI: −1.20 to −0.41), type III procollagen (PC-III) ($P = 0.000$, SMD = −1.27, 95% CI: −1.93 to −0.60), and type IV procollagen (IV-C) ($P = 0.000$, SMD = −0.78, 95% CI: −1.05 to −0.51) were decreased after FZHY treatment; however, albumin (ALB) was increased ($P = 0.037$, SMD = 1.10, 95% CI: 0.07 to 2.12) significantly. Furthermore, the Child-Pugh score was reduced significantly and the life quality was improved after FZHY treatment in cirrhosis patients. The results of this meta-analysis indicated that FZHY effectively improves the liver function, alleviates hepatic fibrosis, decreases Child-Pugh score, and relieves TCM symptoms caused by liver dysfunction, indicating that FZHY may contribute to the alleviation of liver fibrosis and cirrhosis.

1. Introduction

Liver fibrosis is a consequence of chronic liver disease characterized by replacement of liver tissue by fibrosis, scar tissue, and regenerative nodules, leading to liver dysfunction [1]. Liver fibrosis can progress into liver cirrhosis that causes further hepatocellular dysfunction and increases intrahepatic resistance to blood flow, leading to hepatic insufficiency and portal hypertension, and seriously decreases patients' life quality [2]. Therefore, antiliver fibrosis is very important to prevent the occurrence of liver cirrhosis. However, clinical treatment for liver fibrosis, including early intervention or control of etiologies and hepatic inflammation and regulation of hepatic extracellular matrix (ECM) metabolism and stellate cell (HSC) activation [3, 4], still lacks effective medications.

It has been reported that Chinese medicine significantly contributed to the treatment of liver fibrosis [5–7]. Fuzheng Huayu (FZHY) formula, traditional Chinese medicine (TCM) 319, is a SFDA-approved antifibrotic medicine in China [8]. It consists of 6 Chinese herbal medicines [9] and has functions to promote blood flow, dissolve blood stasis, tonify spirit, and nourish liver [10]. Current studies have showed that FZHY has the effect of antifibrosis in patients with chronic hepatitis B [11, 12] and experimental rats [13] and reduced hyaluronidase and improved the life quality in liver cirrhosis patients [14].

Moreover, FZHY can effectively suppress the pathway related with autocrine activation in HSCs and/or fibrotic liver tissue through inhibiting the vascular endothelial growth factor (VEGF) expression level [13], downregulated α-smooth muscle actin (α-SMA) expression [15], and regulated the action of transforming growth factor-β1 (TGF-β1) signaling transduction and Smads activation [16, 17]. FZHY has probably induced the apoptosis of stellate cells by activating p38 mitogen-activated protein kinase (MAPK) and inhibiting stress-activated protein kinases and Jun N-terminal kinase (SAPK/JNK) [18]. In addition, FZHY has also enhanced

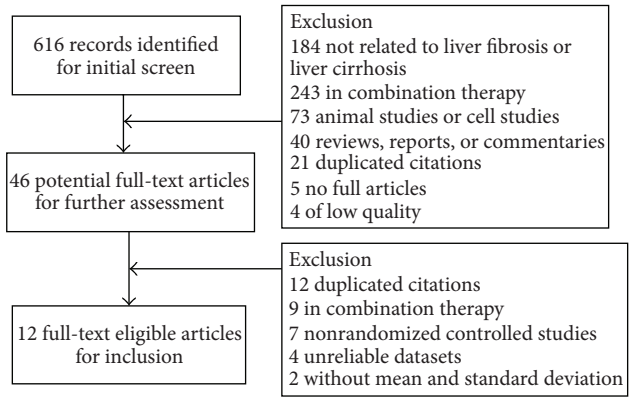


Figure 1: Flow diagram of studies identified with criteria for inclusion and exclusion.

the activation of hepatic NK cells and the production of interferon-gamma (IFN-γ) effectively against liver fibrosis [19].

Furthermore, it has been reported that the antioxidative stress effect of FZHY worked through downregulating the expressions of cytochrome P450 2E1 [20] and tumor necrosis factor receptor type I (TNFR1) [21]. In other diseases, curative effects of FZHY also were being investigated against renal interstitial fibrosis, which is related to the reversal of tubular epithelial-to-mesenchymal transition induced by TGF-β1 [10].

Recently, the efficacy of FZHY against liver fibrosis is being evaluated in multicenter, randomized controlled clinical trials. In particular, a phase II clinical trial of FZHY has been successfully completed in USA, which further indicated the effect of FZYH against liver fibrosis [22]. To systematically assess the curative effect of FZHY on liver fibrosis and cirrhosis underlying the clinical trials, a meta-analysis was conducted on all eligible published studies in the current study.

2. Methods

2.1. Identification and Eligibility of Relevant Studies. A systematic search was performed using PubMed, Springer, EBSCO Medline, Web of Science, SciVerse ScienceDirect, China National Knowledge Infrastructure (CNKI), and Wan-Fang and VIP database (last search: June 20, 2014) with the following MeSH terms and keywords: Fuzheng Huayu, FZHY, TCM 913, liver fibrosis, liver cirrhosis, and effect. The searches were limited to clinical trials. All eligible studies were examined carefully twice, and their bibliographies were double checked. Twelve full-text eligible articles were selected from 616 candidate studies (Figure 1).

2.2. Inclusion Criteria. The following criteria were used for the study selection: (1) evaluating the effects of FZHY as a treatment for liver fibrosis and liver cirrhosis; (2) FZHY alone or based on basic therapy for treatment, other TCM formulas, medication, placebo, or basic therapy for control; (3) full-text articles; (4) random case-control clinical trials; (5) reliable sufficient data estimating mean and standard deviation.

2.3. Exclusion Criteria. Studies were excluded as follows: (1) the study did not meet the criteria above; (2) it used FZHY in combination therapy for treatment; (3) it involved animal studies or *in vitro* studies; (4) it did not use the intervention or the outcomes were unreliable; (5) it did not report the primary research such as review articles, letter to the editor, and commentaries or represented duplicate publications of other studies.

2.4. Quality Assessment. Methodological quality of RCTs was assessed using five-point Jadad scale [23], including (1) description of randomization; (2) adequate and appropriate randomization method; (3) description of single- or double-blindness; (4) assessors blinded to treatment conditions; (5) description of withdrawals and dropouts. In addition, allocation concealment and dropouts were also taken into the assessment. All trials were reviewed by at least 2 reviewers and any disagreement was resolved through the involvement of a third reviewer in consensus conferences.

The methodological quality of the RCTs included in our study was assessed by the method of Jadad. The scores range from 1 to 5, 1 or 2 being considered as low quality trials and 3 to 5 as high quality.

2.5. Data Extraction. Information was carefully extracted from all eligible publications independently by 2 investigators according to the inclusion criteria listed above. For conflicting evaluation, the agreements were reached following consensus and discussion. In each study, the characteristics were collected, such as first author's name, year of publication, design of experiment, general information of participants, history of liver disease, total numbers of trials and controls, interventions for each group, and outcomes of each indicator in trials and controls. We did not define any other indicators in our meta-analysis.

2.6. Statistical Methods. The statistical tests were performed with STATA version 11.0 (Stata Corporation, College Station, TX) to quantify and compare the efficacy outcomes of the FZHY group versus controls. Continuous data were reported as mean ± standard deviation (SD). Heterogeneity of studies was checked by the random effects model. P value which is equal to or more than the nominal level of 0.05 for the Q statistic indicated a lack of heterogeneity across studies, allowing for the use of the fixed effects model, otherwise, perform the random effects model [24]. According to the P value of heterogeneity of the results between two groups, we chose the random effects model or fixed effects model to perform data analysis. Continuous outcomes were presented as weighted standard mean differences (SMDs) with 95% confidence intervals (CI). The strength of effect of FZHY on liver fibrosis and cirrhosis was measured by the P value of the test of SMD with forest plot.

There are mainly two common tests for publication bias test, Egger's test and Begg's test. In this meta-analysis, the publication bias was diagnosed by Begg's test, which was based on rank correlation method and the value of $\Pr > |z|$ was calculated to judge if there is publication bias in

meta-analysis. The value of Pr > |z| less than 0.05 was considered representative of statistically significant publication bias; ≥0.05 was considered representative of no statistically significant publication bias [25].

3. Results

3.1. Characteristics of Included Studies. According to search strategy defined above, 616 articles were retrieved in this meta-analysis. Based on the inclusion and exclusion criteria, 12 articles [11, 14, 26–35] with 1392 subjects were finally selected (714 cases in treatment group and 678 cases in control group). Male patients with liver cirrhosis were dominated in the subjects. The main characteristics, intervention, and outcome measures of the individual studies were shown in Table 1.

3.2. Quality Assessment. The design features clarified as randomization, parallel control, single- or double-blinding, allocation concealment, and withdrawals/dropouts were shown in Table 2. The curative effect assessment performed by the clinicians was not adopted.

In the studies, 12 articles were conducted randomized with a parallel control and 4 articles were with blinding method. The withdrawals/dropouts were also conducted. The measurements of serum indices of liver function and liver fibrosis were independently conducted by laboratory staff in these studies. According to the scores of Jadad scale, 8 studies were of low quality with the score of 2, while other 4 studies were of high quality (Table 2).

3.3. Total Effectiveness Rate. The total efficacy rate was assessed in 3 trails [26, 27, 31] among 12 articles. The results showed that the total efficacy rate in FZHY treatment group was no less than 90%, which was stringently higher with controls.

3.4. Serum Indices of Liver Function. According to the P value of heterogeneity test, a fixed effects model was used for the data analysis before treatment ($P > 0.05$), while a random effects model was used for the data analysis after treatment ($P \leq 0.05$). The results showed that the values of ALT, AST, and TBil decreased significantly, but the ALB value was increased in FZHY group. Before treatment, the SMDs with 95% CI of ALT, AST, TBil, and ALB were 0.06 (−0.06, 0.19), 0.03 (−0.01, 0.16), −0.07 (−0.22, 0.0.07), and −0.25 (−0.38, −0.11) in patients; however, after treatment, the relative values were changed to −0.87 (−1.46, −0.29), −0.22 (−0.48, 0.03), −1.30 (−2.10, −0.50), and 1.10 (0.07, 2.12), respectively. The forest plots of ALT, AST, TBil, and ALB after treatment were shown in Figure 2 and Supplementary Material (see Supplementary Material available online at http://dx.doi.org/10.1155/2015/125659). The P value of SMD was shown in Table 3.

3.5. Serum Indices of Liver Fibrosis. Heterogeneity analyses implied that the data of HA, LN, PC-III, and VI-C before treatment fits a fixed effects model ($P > 0.05$), while the data of HA, LN, PC-III, and VI-C after treatment should use a random effects model ($P \leq 0.05$). Meta-analysis results showed that the SMDs of HA, LN, PC-III, and VI-C with 95% CI were 0.08 (−0.03, 0.18), 0.01 (−0.10, 0.12), 0.02 (−0.10, 0.13), and 0.11 (−0.00, 0.22) before treatment; similarly, the relative values were −0.94 (−1.30, −0.58), −0.80 (−1.20, −0.41), −1.27 (−1.93, −0.60), and −0.78 (−1.05, −0.51) after treatment. It suggested that the levels of serum HA, LN, PC-III, and VI-C in the treatment groups were lower than those in control groups (Figure 3 and Supplementary Material). The P value of SMD was shown in Table 3.

3.6. Other Indices. The histologic results showed that liver inflammation grade [11] and fibrosis stage [35] decreased markedly after treatment, whereas no obvious improvement was seen in controls. According to the results of B ultrasound examination [30, 31] and FibroScan [35], FZHY had a better effect on improving the diameter of portal vein and thickness of spleen. In cirrhosis patients [14], FZHY could decrease the Child-Pugh score significantly ($P < 0.01$) compared with the control groups. The effect of FZHY on TCM symptoms [14], life quality [34], and social ability [30] has also been evaluated which showed that FZHY could improve the signs and symptoms of patients compared with the control groups. For the reason that the number of trails evaluating these parameters above was limited, we described these results instead of performing meta-analysis for these data.

3.7. Adverse Effect. In this study, 8 trails were observed as adverse events in FZHY treatment. It has been reported that there were one case of mild nausea [33] and 3 cases of mild discomfort in stomach [28]. In another clinical trial, 3 patients in the treatment group experienced mild abdominal distention and 5 in the placebo group developed mild nausea after 2 years of treatment [34]. And the other 4 trails reported no adverse events.

3.8. Heterogeneity and Bias Analysis. The heterogeneity of studies was calculated by Cochran's Q and I^2 test, where the $P \leq 0.05$ or $I^2 \geq 40\%$ was considered to be significant. The heterogeneity was not significant for these studies ($P > 0.05$) before FZHY treatment; however, the statistical values were stringently heterogeneous in data after treatment ($P < 0.05$) (Table 3).

Furthermore, the publication bias was performed by Begg's test. The forest plots of ALT, AST, TBil, ALB, HA, LN, PC-III, and VI-C were identified, respectively (Figures 2 and 3), and did not reveal any asymmetry for studies. In particular, except for PC-III, the Pr > |z| value of other plots was more than 0.05; it indicated that the studies have no publication bias (Table 3).

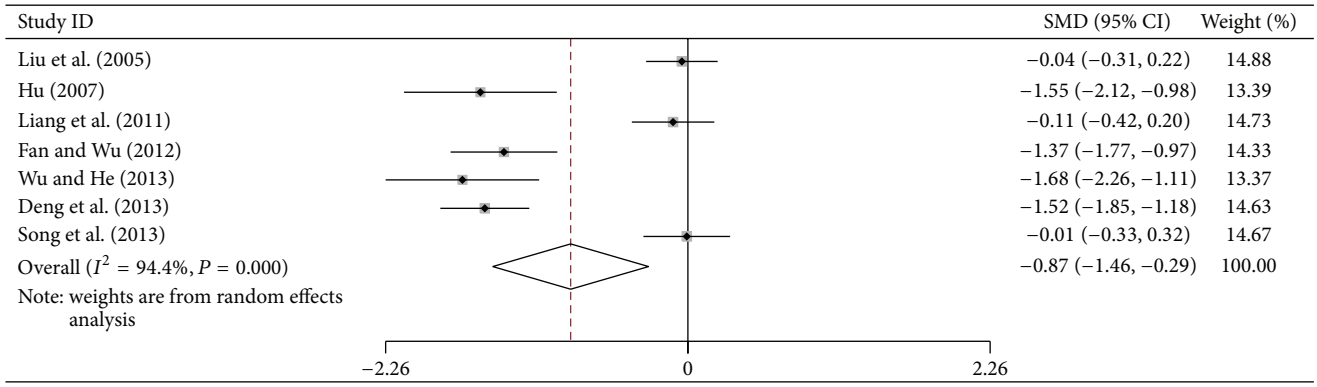
4. Discussion

Liver fibrosis is a common pathologic process for almost all chronic liver diseases which will result in liver cirrhosis or even hepatocellular carcinoma [36]. The primary therapy of

Table 1: Main characteristics, intervention, and outcome measures of the individual studies in the meta-analysis.

Studies and published years [No]	Diseases	Cases (T/C)	Intervention regimens		Time (wk)	Outcomes assessment
			Treatment group	Control group		
Liu et al. 2005 [11]	Hepatitis B-caused fibrosis	110/106	0.93 g per FZHY capsule, five capsules orally, tid	0.93 g per Huoluo Shugan capsule, five capsules orally, tid	24	Histologic examination, liver function, liver fibrosis, B ultrasound examination, safety assessment parameter
Zhao et al. 2006 [26]	Hepatitis B-caused fibrosis	30/30	FZHY recipe orally, twice	Conventional protection liver therapeutics	12	Clinical syndrome, liver fibrosis
Hu 2007 [27]	Cirrhosis	32/30	5 FZHY capsules orally, tid	Conventional protection liver therapeutics	12	Liver function, liver fibrosis
Qiu 2010 [28]	Hepatitis B-caused fibrosis	60/50	0.3 g per tablet in FZHY, 1.5 g tid orally	Conventional protection liver therapeutics	24	Liver fibrosis
Wang 2011 [29]	Hepatitis B-caused fibrosis	60/55	0.3 g per tablet in FZHY, 1.5 g tid orally	Conventional protection liver therapeutics	12	Liver fibrosis
Liang et al. 2011 [30]	Posthepatitic cirrhosis	90/90	4 FZHY tablets orally, tid	Conventional protection liver therapeutics, placebo	24	Social ability score, liver function, liver fibrosis, coagulation, B ultrasound examination, safety assessment
Fan and Wu 2012 [31]	Hepatitis B-caused fibrosis	60/60	0.3 g per tablet in FZHY, 1.5 g orally, tid	Conventional protection liver therapeutics	24	Liver function, liver fibrosis, B ultrasound examination
Zhu 2012 [32]	Posthepatitic fibrosis	28/28	0.3 g per tablet in FZHY, 1.5 g orally, tid	10 g silymarin orally, tid	24	Clinical syndrome, liver fibrosis
Wu and He 2013 [33]	Schistosomiasis hepatic fibrosis	33/31	3 FZHY capsules orally, tid	Conventional protection liver therapeutics	24	Clinical syndrome, liver function, liver fibrosis
Deng et al. 2013 [34]	Posthepatitic cirrhosis	90/90	4 FZHY tablets orally, tid	Four placebo tablets orally, tid	24	Liver function, liver fibrosis, coagulation, hemodynamics, degree of esophagogastric varices, score of symptoms and life quality, adverse events, two-year survival
Song et al. 2013 [14]	Hepatitis B-caused cirrhosis	110/106	0.4 g per tablet in FZHY, 1.6 g orally, tid	0.4 g per tablet in placebo, 1.6 g orally, tid	12	ALT, AST, HA, TCM syndrome score, Child-Pugh
Wang et al. 2014 [35]	Fibrosis	30/30	3 FZHY tablets orally, tid	6 g Anluo Huaxian pill orally, twice	48	Histologic examination, FibroScan, liver fibrosis, B ultrasound examination

No: reference number; T: treatment group; C: control group; wk: weeks.



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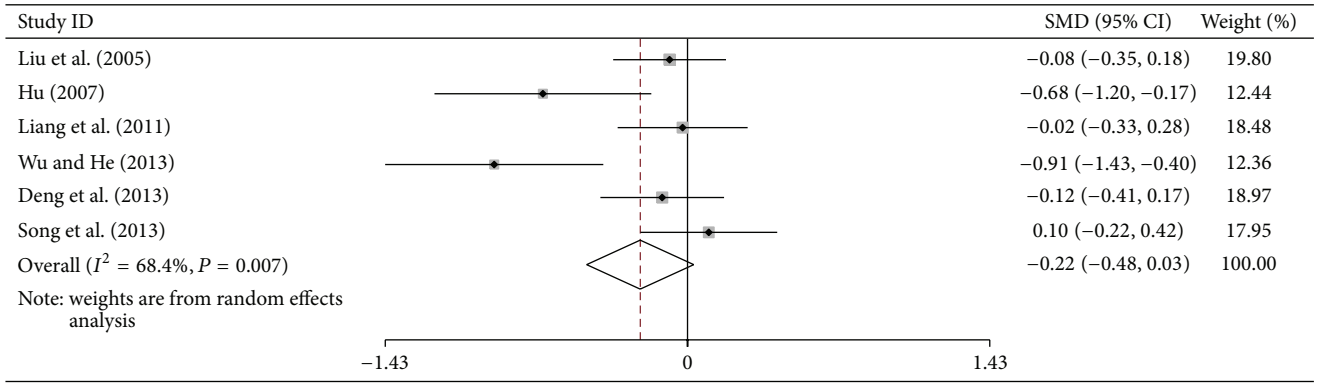

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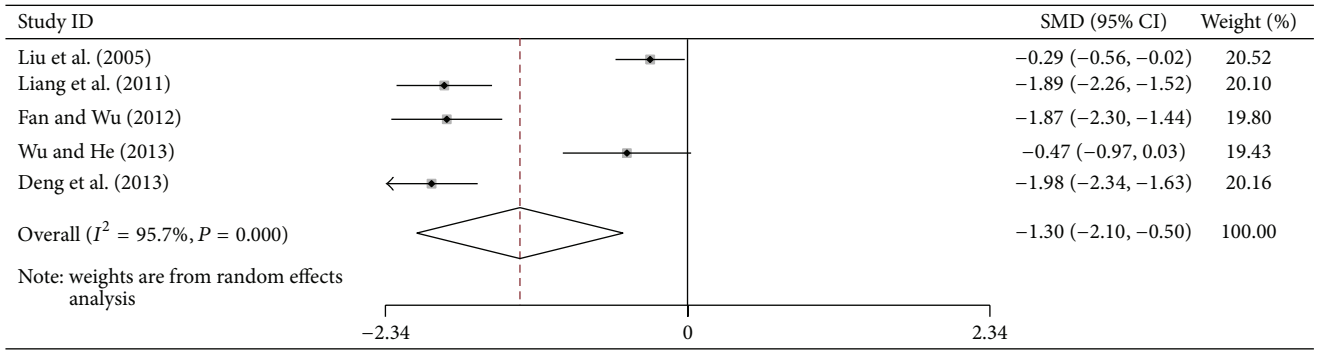

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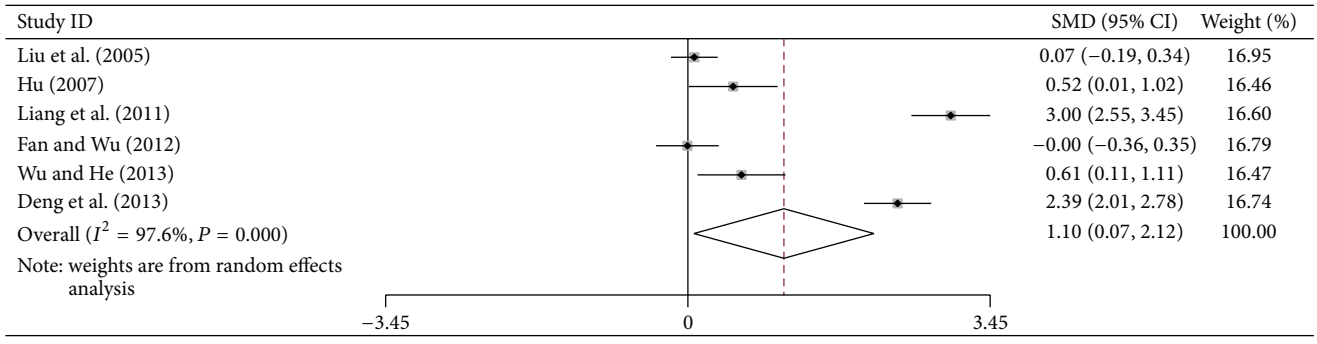

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Figure 2: Forest plots of ALT, AST, TBil, and ALB after treatment: (a) ALT, (b) AST, (c) TBil, and (d) ALB.

Table 2: The quality evaluation of included trials using Jadad assessment scale.

Studies	Quality					Jadad score
	Randomization	Parallel control	Blindness	Allocation concealment	Withdrawal assessment	
Liu et al. [11]	y	y	y	n	y	4
Zhao et al. [26]	y	y	n	n	n	2
Hu [27]	y	y	n	n	n	2
Qiu [28]	y	y	n	n	n	2
Wang [29]	y	y	n	n	n	2
Liang et al. [30]	y	y	y	n	y	4
Fan and Wu [31]	y	y	n	n	n	2
Zhu [32]	y	y	n	n	n	2
Wu and He [33]	y	y	n	n	n	2
Deng et al. [34]	y	y	y	n	y	4
Song et al. [14]	y	y	y	n	y	4
Hao et al. [35]	y	y	n	n	n	2

y: yes, score = 1; n: no, score = 0.

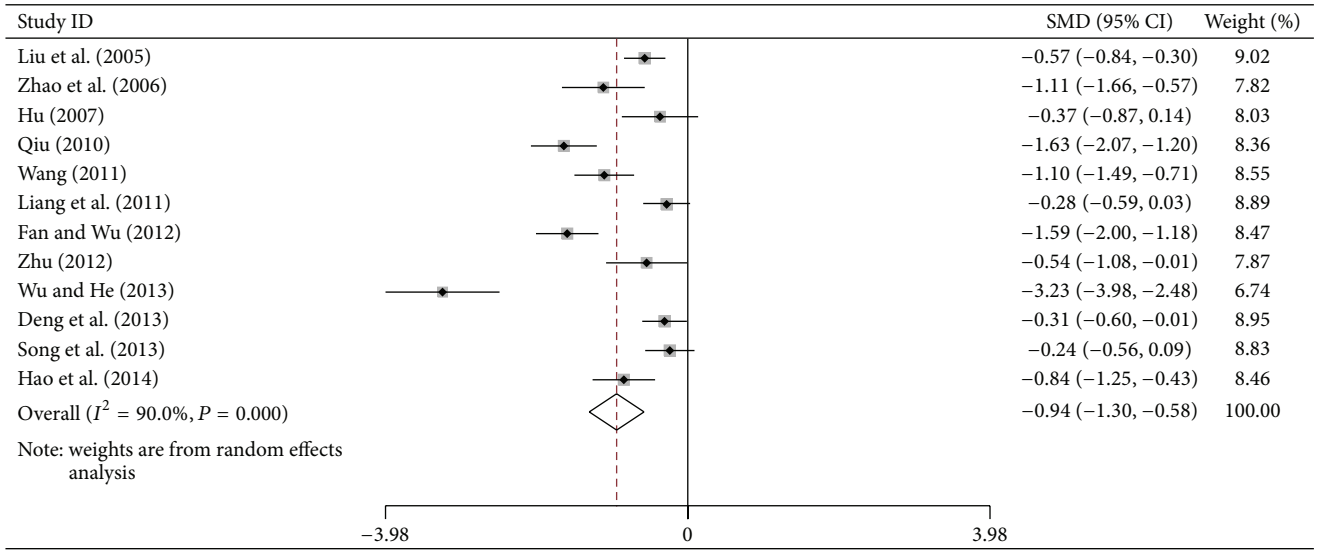
Table 3: SMD, heterogeneity, and publication bias for liver function and fibrosis before and after FZHY treatments.

Parameters	Treatments	SMD (P value)	Heterogeneity			Pr > |z| (P value)
			Chi-squared	I-squared	P value	
ALT	Before	0.321	7.61	0.212	0.268	0.230
	After	0.003	107.21	0.944	0.000	0.230
AST	Before	0.714	1.78	0.000	0.879	0.452
	After	0.084	15.83	0.684	0.007	0.452
TBil	Before	0.335	5.31	0.247	0.257	0.806
	After	0.001	93.31	0.957	0.000	0.806
ALB	Before	0.001	5.86	0.147	0.320	1.000
	After	0.037	207.03	0.976	0.000	0.452
HA	Before	0.150	1.71	0.000	0.999	0.837
	After	0.000	110.45	0.900	0.000	0.115
LN	Before	0.907	1.63	0.000	0.998	0.640
	After	0.000	109.31	0.909	0.000	0.161
PC-III	Before	0.716	9.25	0.000	0.509	0.043
	After	0.000	275.96	0.964	0.000	0.020
VI-C	Before	0.053	5.00	0.000	0.891	0.533
	After	0.000	53.25	0.812	0.000	0.161

antifibrosis in cirrhosis patients is etiological [37] and symptomatic treatment to reduce fibrosis, which has a beneficial impact on portal hypertension and other complications [38]. Currently the main Western medications include interferon and vitamin E [39] which were not approved to be used in clinic officially [40]. Moreover, some Chinese herbal medicines were used against liver fibrosis and cirrhosis such as Danshen and Huangqi injection [41], Qianggan capsule, and Biejia Ruangan tablets [42, 43], but these Chinese herbal medicines have not proved a good and definite effect. Therefore, finding more safe and effective drugs to prevent and reverse liver fibrosis is an urgent task for patients with liver disease.

FZHY formula, as an effective TCM, has been investigated in both animal experiments [44, 45] and clinical trials [46] for several years, which has revealed holistic effects such as improving liver function and serum fibrotic parameters and cirrhosis, decreasing portal pressure, and regulating immune function and amino acids balance [47]. FZHY has also alleviated renal fibrosis [48], against experimental pulmonary fibrosis [49]. Although many multicenter trials were undertaken, considering the limited participants, the coincident trial conditions, and assessment measures, a systemic review is in need to assess the effect of FZHY.

Meta-analysis is systemic and beneficial to assess the accuracy of each trail, which gives a more objective evaluation and explains the heterogeneity between different trails [50]. It has been used in many trails of different diseases [51–53] and used to assess the effect of many kinds of Chinese herbal medicine such as Danshen injection and Huangqi injection against liver cirrhosis [41]. While regarding FZHY, a widely used TCM recipe to treat liver fibrosis and cirrhosis



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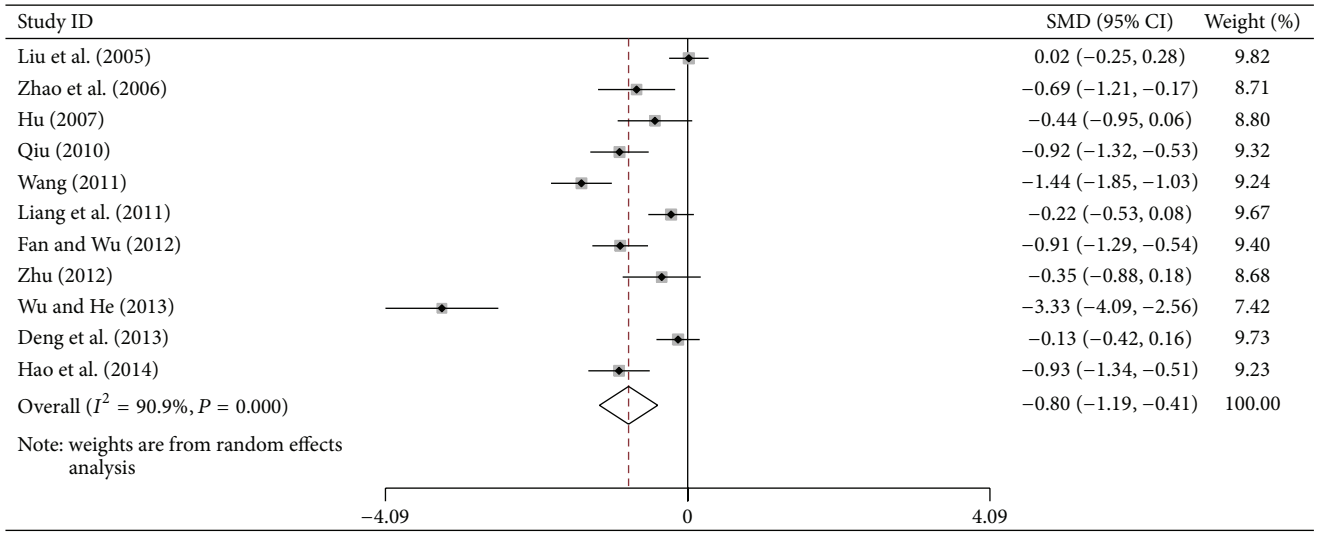

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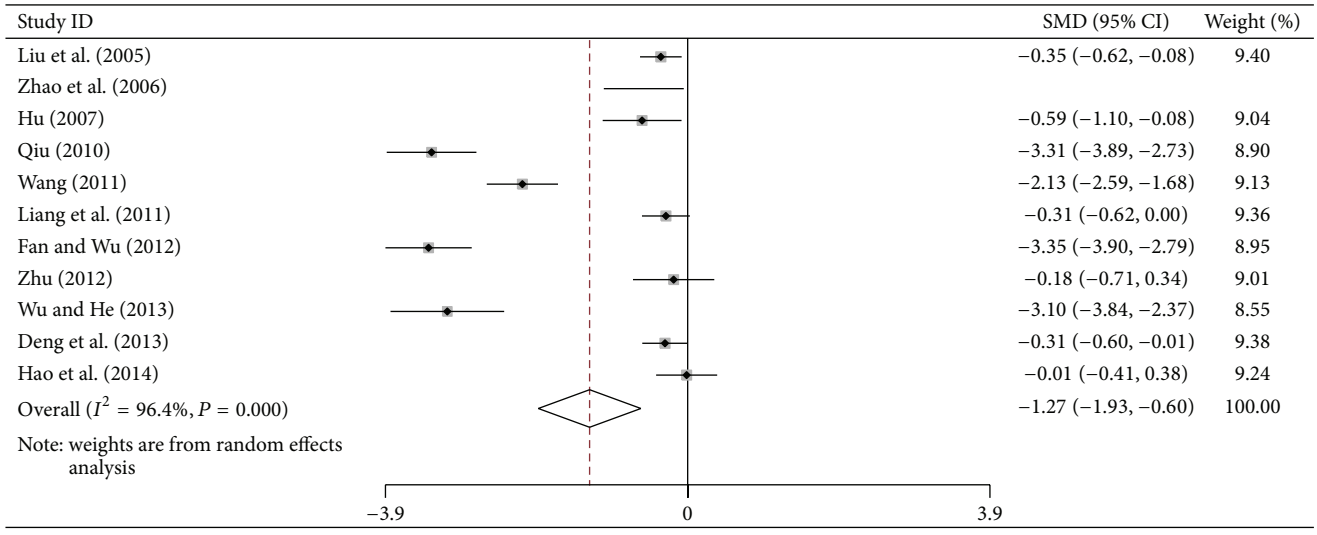

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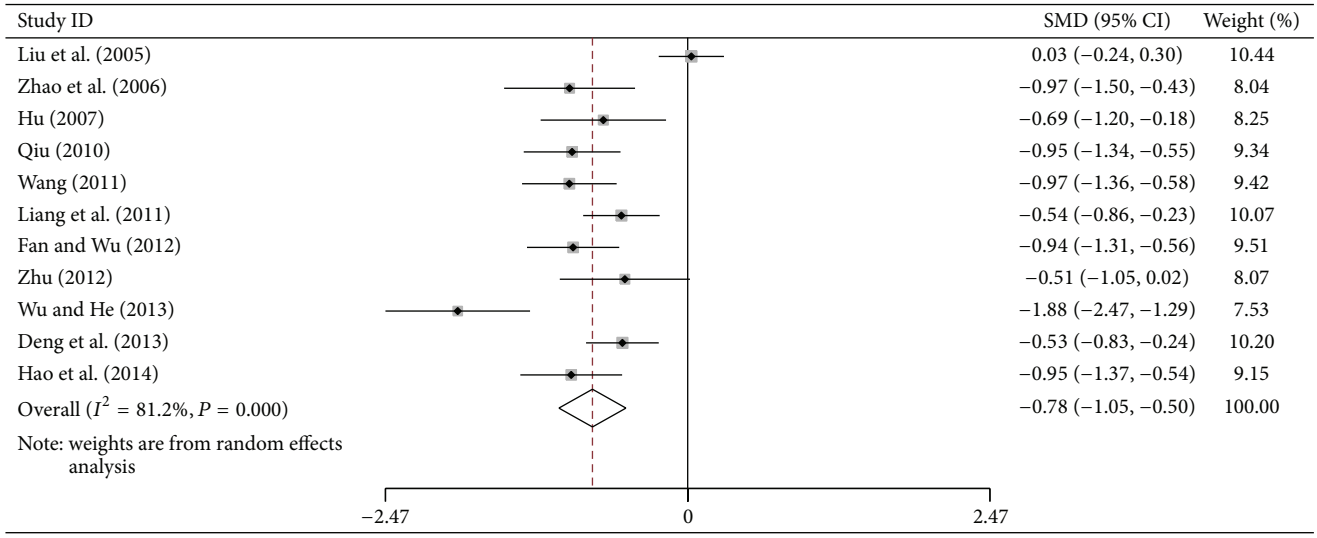

(d)

Figure 3: Forest plots of HA, LN, PC-III, and VI-C after treatment: (a) HA, (b) LN, (c) PC-III, and (d) VI-C.

in China, there was no systemic analysis to assess its effects of RCTs recently. In this study, we assessed the curative effects of FZHY on liver fibrosis and cirrhosis. This meta-analysis showed that the curative effect in FZHY-treated groups was better than that of controls. Generally, the serum ALT, AST, TBil, and ALB are the main markers for evaluating liver function; furthermore, the serum HA, LN, PC-III, and IV-C are important markers for evaluating liver fibrosis [54, 55]. In meta-analysis, the forest plots showed that there was significantly statistical difference in SMD test of ALT (P = 0.003), TBil (P = 0.001), ALB (P = 0.037), HA (P = 0.000), LN (P = 0.000), PC-III (P = 0.000), and IV-C (P = 0.000) after FZHY treatment, while there was no statistical difference for AST (P = 0.084). In 3 trails of liver cirrhosis, the Child-Pugh scores also decreased significantly after treatment ($P < 0.01$) [14]. Histologic results showed that FZHY could decrease liver inflammation grades and fibrosis stage [35], which markedly with the reverse rate 52% in FZHY group in liver biopsy [11].

In addition, clinical research indicated that FZHY also had a better effect on improving the diameter of portal vein and thickness of spleen [30, 31, 35] and effectively reduces the risk of variceal bleeding, improves survival rates in liver cirrhosis patients with varices, especially in the treatment of the capsule and propranolol combination [56], and alleviates ascites [57]. Moreover, FZHY could definitely improve mental disturbance and social activity deficit in patients with chronic hepatitis B-caused cirrhosis [58]. The results of this meta-analysis were also confirmed with self-control clinical trials [59].

TCM syndrome (ZHENG and TCM pattern) determined by clinical symptom and sign can be quantified by TCM syndrome score and used to evaluate the clinical efficacy of Chinese herbal medicine [60]. The effects of FZHY on TCM symptoms [14], life quality [34], and social ability [30] had been evaluated. The results showed that FZHY improves TCM syndrome score and psychology score compared with the control groups. And the accurately TCM pattern differentiation could guide the appropriate TCM treatment with FZHY in patients with hepatitis B-caused cirrhosis [14]. Interestingly, FZHY had also been predicted to have the effect of antihyperlipidemia and antihyperglycemia, through a high-throughput data analysis of hepatitis B-caused cirrhosis treatment [61].

In this meta-analysis, there was no statistically significant heterogeneity in all the comparisons after treatment, and with the outcome of Begg's test, we concluded that there was no statistically significant bias in the overall studies.

Thus, we revealed that FZHY may improve liver function and alleviate liver fibrosis which was consistent with most of the related studies as summarized in meta-analysis. According to the results of Jadad scores, sensitivity analysis, and publication bias test, we believe the outcomes of this meta-analysis were reliable. However, the limitations of the meta-analysis should be acknowledged. Firstly, the methodological design of individual studies was not coincident which included inclusion criteria, source of controls, patients' status, and the drugs used in control group. Secondly, the diversity of treatment dose and the small sample number and the lack of long term follow-ups degraded the validity of the evidence in the clinical trials. Thirdly, liver fibrosis and cirrhosis may be caused by a variety of factors, such as alcohol, medicine, and virus. In this study, the trails included were hepatitis B-caused fibrosis mostly, so this study was limited to reflect the effect of FZHY on liver fibrosis caused by variety of factors. In addition, although some indices of B ultrasound, FibroScan, and the safety evaluation in the trials were reported, they were insufficient to perform meta-analysis; therefore, more high quality random control trails are required. Despite the limitation, our meta-analysis significantly increased the statistical power of the analysis based on substantial number of cases and controls from 12 different studies. The results of

meta-analysis did not draw different conclusions, indicating that our results were robust.

5. Conclusions

The meta-analysis results suggested that FZHY can effectively improve liver function and life quality by relieved symptoms, alleviating hepatic fibrosis and decreasing Child-Pugh score, with less adverse effect. Therefore, FZHY might be a safe and effective Chinese medicine against liver fibrosis and cirrhosis. Considering that this systematic review had the limitations in the coincident methodological design of each trail and the small number of the included trails, rigorously designed multicenter, double-blind, randomized, and large-scale controlled trials are further required.

Conflict of Interests

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

Acknowledgments

This work was supported by Key Program of National Natural Science Foundation of China (81330084), Shanghai Municipal Science and Technology Commission Project (12401900401), E-Institutes of Shanghai Municipal Education Commission (E03008), Scheming Project of Shanghai Municipal Education Commission (2012JW25), and The Natural Science Foundation of Shanghai Science and Technology Commission (14ZR1438800).

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Hindawi Publishing Corporation
Evidence-Based Complementary and Alternative Medicine
Volume 2015, Article ID 238495, 9 pages
http://dx.doi.org/10.1155/2015/238495

Research Article

Dynamical Regulation Analysis Identifies Molecular Mechanisms of Fuzheng-Huayu Formula against Hepatitis B-Caused Liver Cirrhosis

Qi-Long Chen,[1] Yi-Yu Lu,[1] Jing-Hua Peng,[2] Shu Dong,[1] Bin Wei,[1] Ya-Nan Song,[1] Qian-Mei Zhou,[1] Hui Zhang,[1] Ping Liu,[3] and Shi-Bing Su[1]

[1]*Research Center for TCM Complexity System, Shanghai University of TCM, Shanghai 201203, China*
[2]*Institute of Liver Diseases, Shuguang Hospital Affiliated to Shanghai University of TCM, Shanghai 201203, China*
[3]*Shanghai University of TCM, Shanghai 201203, China*

Correspondence should be addressed to Ping Liu; liupingg1@126.com and Shi-Bing Su; shibingsu07@163.com

Received 4 August 2014; Revised 20 September 2014; Accepted 21 September 2014

Academic Editor: Qing He

Fuzheng-Huayu (FZHY) tablet was formulated based on Chinese medicine theory in treating liver fibrosis. A clinical trial has indicated that FZHY can against hepatitis B-caused liver cirrhosis (HBC), but the underlying mechanism of FZHY efficacy is unclear. Here, we report that miRNA expression levels are remarkably changed when FZHY formula was used in HBC patient's treatment as a paradigm of trials. Then, we functionally characterize the significant impact of potential kernel miRNAs by miRNA-target network analysis. Enrichment analysis show that the FZHY formula dramatically effecting the molecular regulated module in HBC. Thus, we infer that FZHY plays a critical function in HBC treatment process and directly regulated many important pathways, including but not limited to cell cycle, p53 signaling pathway, and TGF-β signaling pathway, suggesting a new strategy for investigating the molecular mechanism of FZHY treatment.

1. Introduction

Liver cirrhosis (LC) is a consequence of chronic liver disease characterized by liver fibrosis, scar tissue, and regenerative nodules, leading to the destruction of hepatic microstructure and liver dysfunction. Usually, cirrhosis is caused by hepatitis viruses, alcoholism, nonalcoholic steatohepatitis (NASH), and autoimmune liver disease as well as fatty liver disease. Noticeably, infection of hepatitis B virus (HBV) in human liver that induces the development of liver cirrhosis is increasing, annually, the HBV caused cirrhosis more than 1.5 million people in the world [1]. The five-year survival rate of patients with severe HBV caused cirrhosis only is about 50% [2] and rigorously and clinically lacks the effective drugs for the therapy of hepatitis B-caused cirrhosis in the past decades.

Fuzheng-Huayu (FZHY) tablet, a Chinese herbal formula, containing herbs such as *Radix Salvia miltiorrhiza*, *Cordyceps*, *Semen Persicae*, was formulated based on Chinese medicine theory in treating liver fibrosis and was approved. Pharmacological studies and clinical trials [3] have demonstrated that FZHY has a significant effect against liver fibrosis, in particular the effects observed from clinical trials in treating liver fibrosis caused by chronic hepatitis B. Furthermore, the actions on inhibition of hepatic stellate cell activation [4] and regulation of TGF-beta 1 signaling transduction pathway [5] also were effected for FZHY against liver fibrosis. In previous clinical trial, we have found the therapeutic efficacy of FZHY on hepatitis B-caused cirrhosis (HBC), but the underlying mechanism of FZHY efficacy is to a large extent still elusive.

MicroRNA (miRNA) is a class of small, endogenous, noncoding RNA molecules [6, 7], which suppress the translation of target mRNAs or induce mRNAs degradation [8–10]. Depending on the grade of concordance between miRNA sequence and target mRNA, the negative regulatory effect for target mRNAs more like as a rheostats to make fine-scale adjustments to protein output [11]. Generally, miRNAs have higher stability in circulation systems, tissue, and organ

[12], and they are often detected in blood under pathological conditions caused by cell turnover, cell destruction, and pathological injury. Furthermore, miRNAs are relevance as regulators of gene expression, therefore affecting crucial processes in diseases development, especially, and offer great potential as biomarkers for diseases detection due to their remarkable stability in blood [12, 13].

In previous study, the multicenter, double blind, equal-randomized, and placebo-controlled trials were used to evaluate the curative effects of FZHY treatment in hepatitis B-caused cirrhosis (HBC). The results demonstrated that FZHY can clearly improve the TCM symptoms and quality of life of HBC patients. In this work, we hypothesized that miRNAs profiling in serum has potential as ideal biomarkers, which are associated with FZHY efficiency on HBC and thus focused on the miRNAs profiling analysis, the differences, and similarities in the FZHY and Placebo treatment in HBC. The aim was to investigate the molecular mechanism of FZHY efficacy on HBC through a regulation of miRNAs.

2. Materials and Methods

2.1. Clinical Specimens. The clinical serums of 6 HBC patients before trial and after trial (6 months) were collected, including 3 patients of FZHY intervention group and 3 patients of Placebo intervention. The selected criteria of samples including HA (hyaluronic acid), ALT (alanine aminotransferase), AST (aspartate aminotransferase), and Child-Pugh score in FZHY group were distinctly decreased compared to Placebo group. In addition, 7 serums of normal controls were randomly obtained from Shanghai Longhua Hospital. The diagnostic criteria of western medicine for HBC followed the guidelines that are defined by the Chinese Society of Hepatology and Chinese Society of Infectious Diseases in 2005 [14].

This research project was conducted according to the guidelines of the Declaration of Helsinki and the principles of Good Clinical Practice (China) and approved with the local ethics committee of Shanghai University of TCM. Furthermore, informed consent was received from all patients of this study. The clinical data of patients with CHB-caused cirrhosis were shown in Table 1.

2.2. Serum Sample Collection and RNA Isolation. All serum samples were from the peripheral venous blood of HBC subjects and healthy donors, which were immediately frozen in liquid nitrogen and then stored at −80°C. The RNAs in serum were extracted using a miRVana PARIS kit (Ambion, Austin, TX) according to the manufacturer's protocol and based on the RNase-free DNase I (Promega, Madison, WI) to eliminate DNA contamination. The concentration of RNAs isolated from serum ranged from 1.5 to 12 ng/μL.

2.3. miRNA Microarray and Data Analysis. The miRNA profiles of 12 HBC subjects (6 before trial samples and 6 after trial samples) and 7 controls were generated using Agilent Human miRNA microarray V3 (Agilent Technologies Inc., USA). Hybridization signals were detected with the Agilent Microarray Scanner; the data were extracted using Feature Extraction V10.7 (Agilent Technologies, CA). All raw data were transformed to log2 and normalized each expression by zero mean and unit sample variance.

Using random variance model t-test of R package, the differential expression (DE) miRNAs were calculated among FZHY group, Placebo group, and Control group, where the fold-change >1.5 and $P < 0.001$ were considered to be significant. Heat map and hierarchical cluster analysis of expression data were performed using Cluster 3.0 and Tree-View programs. Class prediction of samples was performed using a statistical algorithm of the support vector machine (SVM) incorporating differential expression (DE) miRNAs at a univariate parametric significance level of $P = 0.01$. The prediction rate was estimated via 10-fold and 10-time cross-validation and the bootstrap method for small sample data.

2.4. Identification of miRNA-Target Genes and miRNA-Target Network Constructing. The validated miRNA-target genes were predicted using three databases involving TarBase (v6.0) [15], miRecords (2013) [16], and miRTarBase (2013) [17], which hosted the largest collection of manually curate experimentally data. Furthermore, the programs of miRanda, miRDB, miRWalk, and RNAhybrid were used to predict the unverified miRNA-target genes, where $P < 0.001$ was considered to be significant. All predictions were merged and acted as final data for building miRNA-target network, of which the profiles were constructed using Cytoscape software (version 3.1). In the network, nodes represent miRNAs or target genes, and the edges represent the connection strength.

2.5. Enrichment Analysis of Target Genes. Of the inferred miRNA-target genes, those showing a significant ($P < 0.05$) expression difference among the samples before and after FZHY or Placebo treatment were analyzed for pathways involving these genes using DAVID online analysis [18, 19], and significance analysis was determined when P values were corrected for false discovery rate (FDR). Gene sets containing less than 5 genes overlapping were removed from the DAVID analysis. In our analysis, GO terms and pathways with an FDR-adjusted P value of less than 0.05 were retained.

3. Results

3.1. Differential Expressed miRNAs Regulated by FZHY Treatment in HBC. We calculated the miRNAs profiles among FZHY group, Placebo group, and Controls before trials. Before trials, there are 8 DE miRNAs between FZHY group and Controls and 9 DE miRNAs between Placebo group and Controls. However, after trials, 158 DE miRNAs were calculated between FZHY group and Controls (FZHY/Control), and 147 DE miRNAs were selected between Placebo group and Controls (Placebo/Control). Furthermore, 111 DE miRNAs were detected between before and after trials in FZHY group (Before/After FZHY), and 68 miRNAs were obtained in Placebo group (Before/After Placebo). The consecutive heat maps and hierarchical cluster showed the classification of miRNAs expression profiles in HBC compared to normal subjects (Figure 1).

Analysis of the DE miRNAs profiles showed that 138 miRNAs were overlapped between FZHY and Placebo

Table 1: Clinical data of patients with CHB-caused cirrhosis (HBC).

Patient ID	Age	Gender	Clinical types	Intervention types	HA before/after	ALT before/after	AST before/after	Child-Pugh score before/after
S27	56	M	HBC	FZHY	406/148.2	151/23	162/37	5/5
S28	57	M	HBC	FZHY	355.5/108.5	92/19	113/28	8/5
S29	59	F	HBC	FZHY	182.7/70.6	27/20	28/31	11/7
S30	73	M	HBC	Placebo	238.7/363.5	28/114	34/97	5/6
S31	47	F	HBC	Placebo	116/180.2	21/35	44/54	7/8
S32	51	M	HBC	Placebo	57.4/164.1	53/63	51/68	5/9

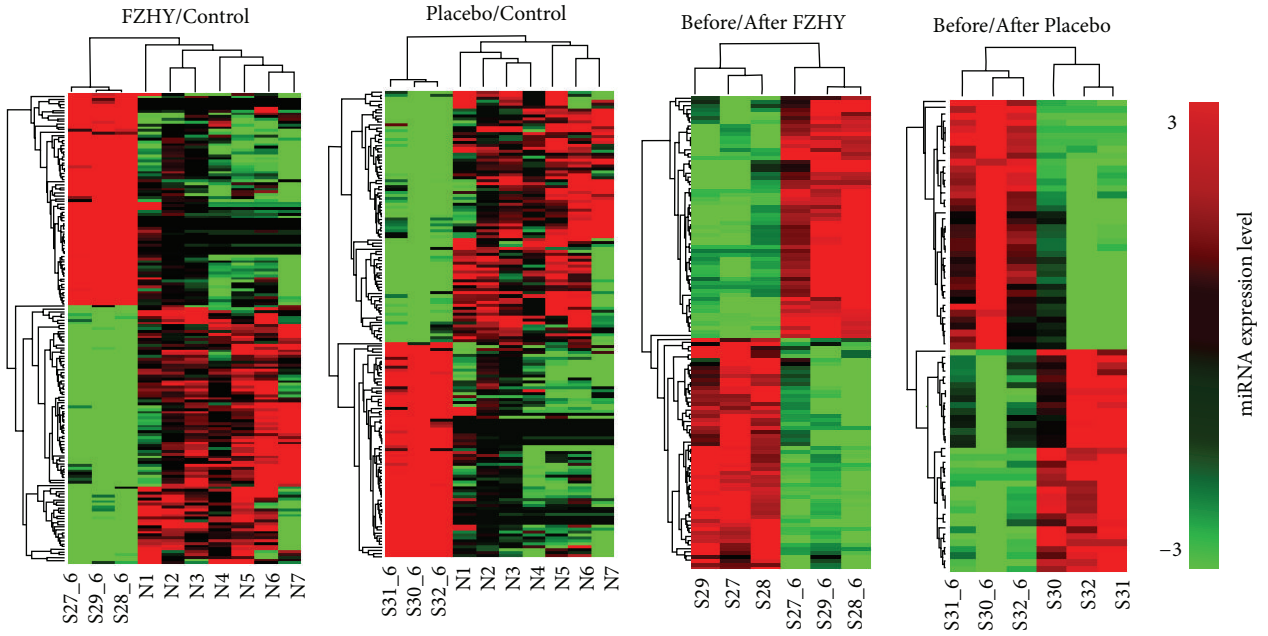

Figure 1: Heat map of differential expressed miRNAs among the FZHY/Control, Placebo/Control, Before/After FZHY treatment, and Before/After Placebo treatment was shown based on different colors, respectively. Red color represents miRNA upexpression and green color represents downexpression. Relationship among the samples was divided by binary tree classification and showed at the upper portion. Hierarchical cluster of miRNAs was displayed at nearside.

groups after trials. Interestingly, 43 overlapped miRNAs were selected between the Before/After FZHY group and Placebo group. This result indicates that the overlapped miRNAs might play important regulated functions for FZHY treated process in HBC patients. To evaluate the variety of the overlapped miRNAs, we calculated the ratio value of each miRNA between FZHY and Placebo groups based on expression levels and obtained 58 miRNAs (ratio > 1). Similarly, 20 miRNAs (ratio > 1) were selected from Before/After FZHY and Placebo groups.

3.2. Overview of miRNA-Target Networks. The consecutive miRNA-target networks of each stage were constructed using the DE miRNAs and predicted target genes (Figure 2). To reveal the details of network, the global networks were divided into overlapped network and independent network in FZHY (FZHY/Control) and Placebo (Placebo/Control) groups (Figure 2(a)). Similarly, the Before/After FZHY and Before/After Placebo networks were also divided into the overlapped network and independent network, respectively (Figure 2(b)). By analysis of the nodes of network, we found that the independent networks only consist of 20 miRNAs (12.7%) and 9 miRNAs (6.1%) in FZHY (FZHY/Control) and Placebo (Placebo/Control) groups, respectively. However, the independent network consists of 68 miRNAs (61.3%) in Before/After FZHY group and 25 miRNAs (36.8%) in Before/After Placebo group. This phenomenon suggested that the diversity of miRNA expression levels has been changed between FZHY and Placebo groups.

Generally, miRNA inhibits translation or induces mRNA degradation by binding to the 3′-UTRs of target mRNAs [8]; here, we mainly focus on upexpression miRNAs for each stage. Following the network, we found that the topological profile of each network is more likely similar to "Medusa" model [20], which consists of regulatory core by hub nodes and is represented most prominently in the network. It indicates that the hub nodes of network are determinants of the realized gene expression profiles, but the periphery nodes that should be regulated are not regulated.

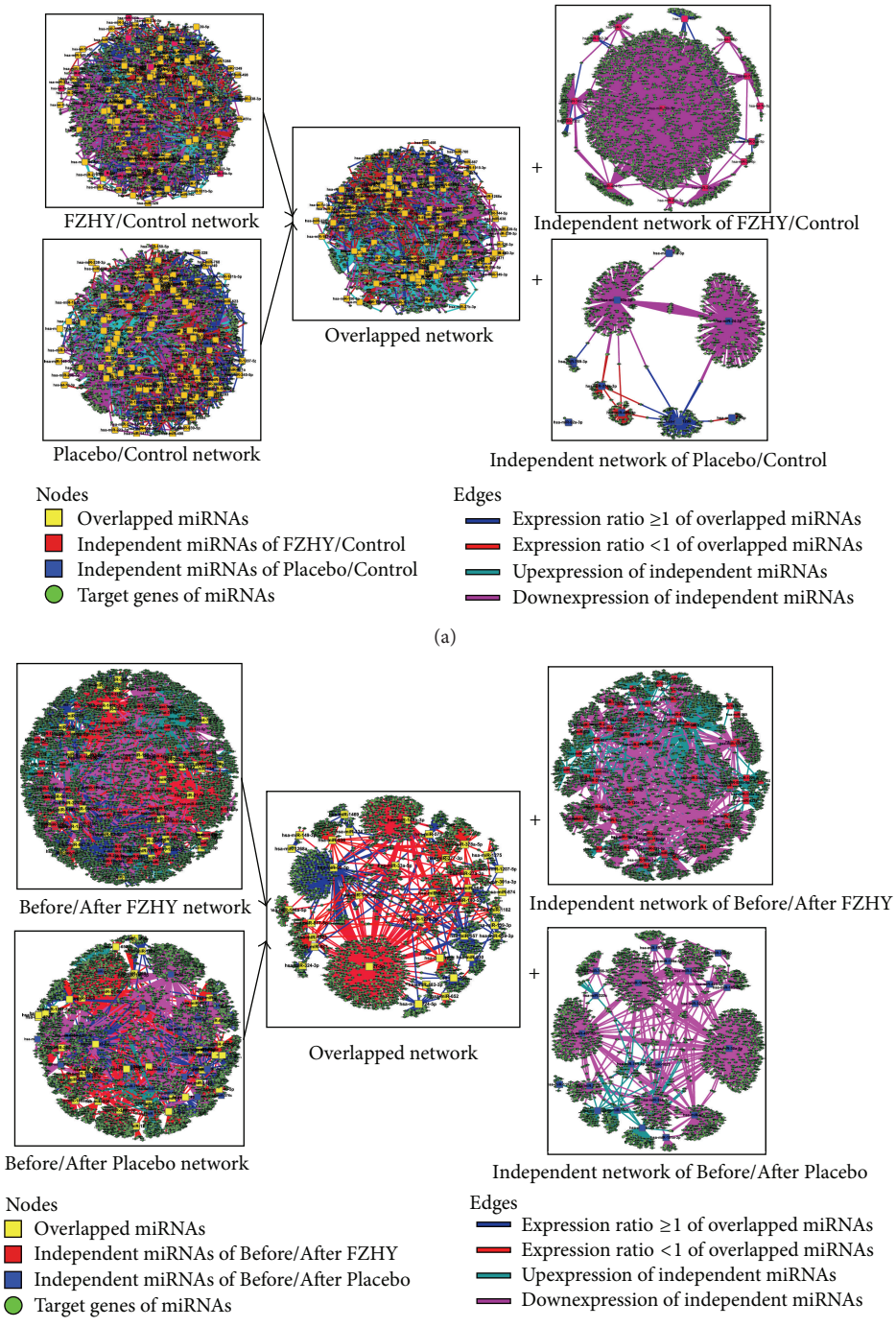

Figure 2: The global profiles of miRNA-target networks in FZHY and Placebo trials were showed. (a) The global profiles of FZHY/Control and Placebo/Control miRNA-target networks were constructed, respectively, and included the overlapped network and independent networks of FZHY/Control and Placebo/Control. (b) The global profiles of Before/After FZHY and Before/After Placebo miRNA-target networks were constructed, respectively, and included the overlapped network and independent networks of Before/After FZHY and Before/After Placebo.

3.3. Potential Kernel miRNAs Selection. To estimate the hub nodes form networks, we have calculated the betweenness centrality (BC), closeness centrality (CC), and degree (d) of each node. Based on the value distribution, the thresholds of BC and CC were defined as 0.01 in the network, respectively. The candidate kernel nodes were selected by the conditions of BC $\geq$ 0.01, CC $\geq$ 0.01, and $d \geq 2$ average (d). Thus, we detected 23 miRNAs in FZHY/Control, 19 miRNAs in Placebo/Control, 15 miRNAs in Before/After FZHY, and 8 miRNAs in Before/After Placebo groups. Interestingly, there are 19 overlapped miRNAs between FZHY/Control and Placebo/Control groups and 4 overlapped miRNAs between

Before/After FZHY and Before/After Placebo groups. We calculated the overlapped miRNA expressed level ratio between FZHY/Control and Placebo/Control groups, as well as between Before/After FZHY and Before/After Placebo groups. To classify the overlapped miRNAs, the miRNAs expression ratio value were calculated, >1 represents this miRNA level was increased, whereas, ≤1 means it was decreased.

Then, these miRNAs were divided into two groups. Group A was associated with FZHY efficacy, which consists of the overlapped miRNAs (ratio < 1) and independent upexpression miRNAs of FZHY/Control and Before/After FZHY groups. Group B was associated with Placebo trial, including the overlapped miRNAs (ratio ≤ 1) and independent upexpression miRNAs of Placebo/Control and Before/After Placebo groups. Finally, 30 miRNAs were selected as potential kernel miRNA stringently associated with FZHY efficacy and 16 miRNAs highly correlated with Placebo trial (Table 2).

3.4. Enrichment Analysis for Potential Kernel miRNA Targets. To understand the potential kernel miRNAs holistically, we conducted functional enrichment analysis for the target genes of them using DAVID analysis [18, 19]. GO term (5% top terms) analysis reveals that Group A miRNA targets are mainly associated with intracellular, nucleus, protein binding, membrane-bounded organelle, protein modification process, posttranslational protein modification, cellular macromolecule metabolic process, cell, cellular process, regulation of cellular metabolic process, and negative regulation of cellular process (Figure 3(a)). Similarly, Group B miRNA targets are mainly associated with intracellular part, nucleotide binding, protein binding, purine nucleoside binding, ribonucleotide binding, intracellular membrane-bounded organelle, intracellular membrane-bounded organelle, macromolecule localization, cytoplasm, and cellular process (Figure 3(b)). Interestingly, there are 15 overlapped GO terms ($P < 0.001$) between Group A and Group B (Figure 3(e)).

Importantly, KEGG pathway analysis shows an impressive functional association of Group A miRNA targets with various cancer-related pathways, such as pathways in cancer ($P = 2.09 \times 10^{-5}$), colorectal cancer ($P = 1.42 \times 10^{-3}$), p53 signaling pathway ($P = 4.55 \times 10^{-3}$), small cell lung cancer ($P = 1.58 \times 10^{-2}$), endocytosis ($P = 3.37 \times 10^{-2}$), TGF-beta signaling pathway ($P = 2.32 \times 10^{-2}$), chronic myeloid leukemia ($P = 2.58 \times 10^{-2}$) as well as cell cycle ($P = 2.31 \times 10^{-2}$), and prostate cancer ($P = 2.11 \times 10^{-2}$) (Figure 3(c)). In Group B, except 6 overlapped pathways, the miRNA targets are also associated with gap junction ($P = 3.18 \times 10^{-3}$), melanoma ($P = 3.41 \times 10^{-3}$), hypertrophic cardiomyopathy ($P = 9.17 \times 10^{-3}$), adipocytokine signaling pathway ($P = 1.05 \times 10^{-2}$), mTOR signaling pathway ($P = 1.43 \times 10^{-2}$), prostate cancer ($P = 3.73 \times 10^{-2}$), and glioma ($P = 3.04 \times 10^{-2}$) (Figure 3(d)). Furthermore, BIOCARTA pathway only appeared in Group A ($P < 0.05$) and was mainly associated with ALK in cardiac myocytes ($P = 7.57 \times 10^{-4}$), TGF-β signaling pathway ($P = 2.78 \times 10^{-3}$), PKC-catalyzed phosphorylation ($P = 6.39 \times 10^{-3}$), thrombin signaling ($P = 1.76 \times 10^{-2}$), regulation of BAD phosphorylation ($P = 2.15 \times 10^{-2}$), and signaling pathway from G-protein families ($P = 3.65 \times 10^{-2}$) (Figure 3(f)).

Subsequently, we constructed the target-pathway network using miRNA targets and related KEGG pathways (Figures 4(a) and 4(b)). The topological profiles of network were calculated using ClusterONE algorithm [21], which was defined as P value < 0.001, node size ≥ 5, and network density ≥ 0.05, and then obtained the kernel nodes related cluster from network. As showed in Figures 4(c) and 4(d), the cluster of Group A contained 6 pathways and 9 kernel genes, and the cluster of Group B contained 7 pathways and 8 kernel genes. Conspicuously, we notice that 5 genes (CDK6, E2F3, CCND1, SMAD4 and CDKN1B) are associated with cell cycle, colorectal cancer, pancreatic cancer, chronic myeloid leukemia, and small cell lung cancer in the cluster of Group A (Figure 4(c)). Interestingly, there are 3 genes (IGF1, CDK6, and CCNE1) which also acted in similar roles in the cluster of Group B, which was correlated with p53 signaling pathway, as well as glioma, prostate cancer, small cell lung cancer, pathways in cancer, and melanoma (Figure 4(d)). It suggested that the cell cycle might play an important role in FZHY treatment process, while p53 signaling pathway is a major component in Placebo trial.

4. Discussion

As a class of gene regulators, miRNA has an important combinatorial hallmark in gene regulation process; in particular, the exceptional stability of circulating miRNAs in serum is the basis of their value in clinical use [13]. In this work, we generated miRNAs expression maps of FZHY group and Placebo group by miRNA microarray and reported that the miRNAs levels were prominently changed in both of them. As shown in results, after 6-month treatment, the DE miRNAs are strongly increasing in FZHY (158 miRNAs) and Placebo groups (147 miRNAs). Although FZHY and Placebo groups have similar DE miRNAs, the ratio value of overlapped miRNAs suggested that there are great differences between FZHY and Placebo treatment and also indicated that these miRNAs are nonnegligible factors in the process of FZHY treatment. Subsequently, the consecutive miRNA-target networks were constructed, and the topological profiles are more likely similar to "Medusa" model; it suggests that the kernel nodes of network are determinants in the realized gene expression levels [22, 23].

Actually, the summation of gene expression and network connectivity can quantitative evaluated the module conservation in complex diseases [24], which provides a new avenue in understanding of molecular mechanism and distinguishing functional processes in disease progression [24, 25]. Here, we obtained 30 kernel miRNAs from network, which are highly correlated with the curative effects of FZHY in HBC treatment process (Table 2). FZHY is a Chinese herbal formula and contained many complex compounds, and we speculate that these kernel miRNAs might format a molecular group and holistically play important regulated functions in HBC treatment process. To understand the potential functions of these kernel miRNAs, we conducted enrichment analysis

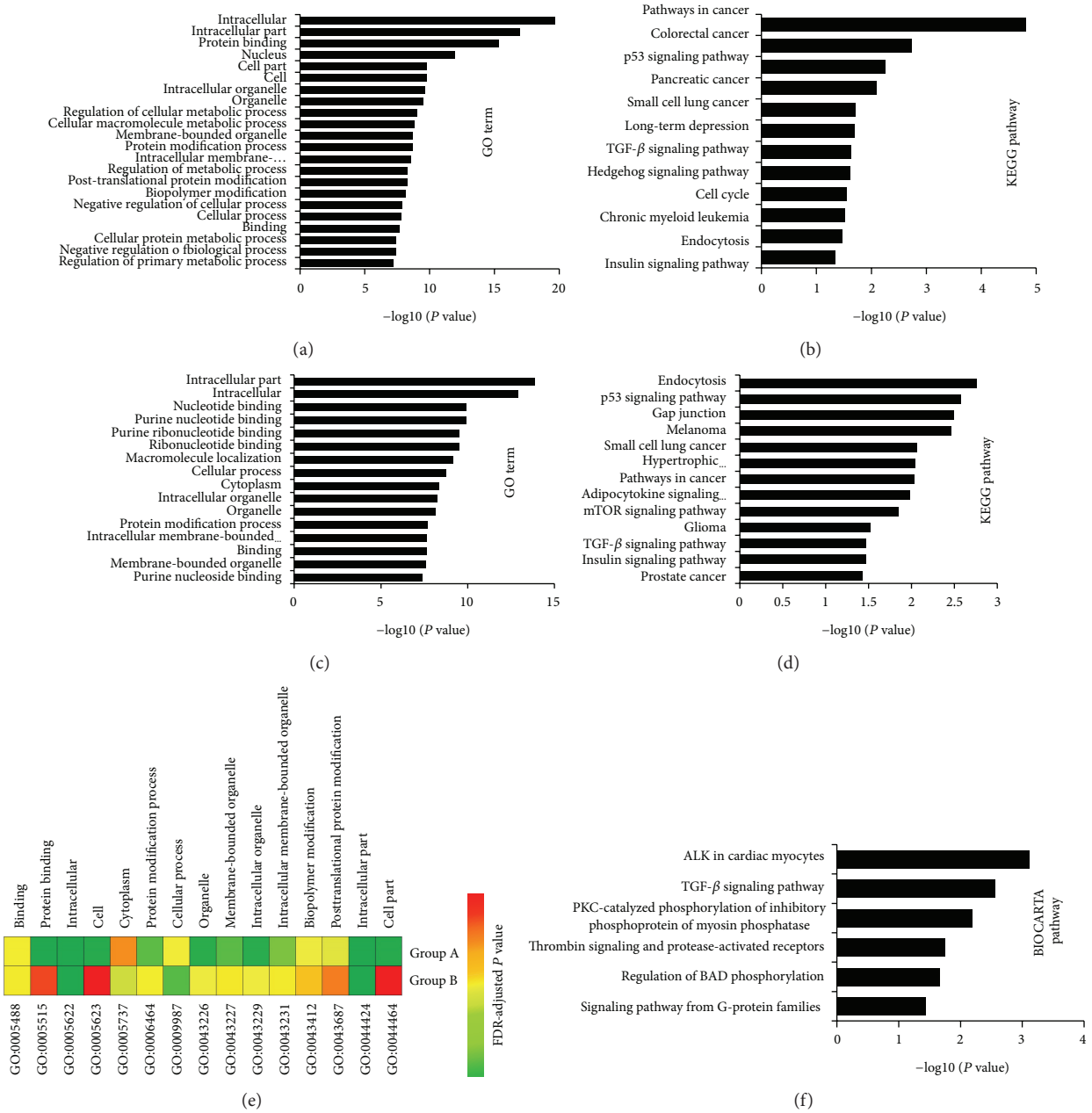

Figure 3: GO term and pathway by target genes of kernel miRNAs. Significant analysis was determined when P values were corrected for false discovery rate (FDR). Gene sets containing less than 5 genes overlapping were removed from the DAVID analysis. (a) Group A miRNA targets related GO term, (b) Group A miRNA targets related KEGG pathway, (c) Group B miRNA targets related GO term, (d) Group B miRNA targets related KEGG pathway, and (e) the distribution of overlapped GO terms between Group A and Group B (FDR-adjusted P value < 0.001) were showed. Colors were scaled according to the proportion of overlaps. (f) The BIOCARTA pathway of Group A miRNA targets, while Group B miRNA targets have not correlated with any pathway when $P < 0.05$.

for the target genes of them. As shown in Figures 3(a) and 3(c), cellular macromolecule metabolic process, cellular process, and regulation of cellular metabolic process as well as negative regulation of cellular process are highly correlated with FZHY group; however, the Placebo group mainly associated with purine nucleoside binding, ribonucleotide binding, intracellular membrane-bounded organelle, intracellular membrane-bounded organelle, and macromolecule localization. Furthermore, the discrepant P values (PDR-adjusted) suggest that they might play different roles in the FZHY and Placebo trials process, although they have many overlapped GO terms (Figure 3(e)) and pathways. The results suggested that there were different molecular regulated modules between FZHY and Placebo treatment process in HBC.

To determine the biological consequence of pathway-mediated landscape in FZHY and Placebo trials, we constructed the target-pathway networks using kernel miRNA targets and KEGG pathways. The cluster results of network showed that cell cycle is an important pathway in FZHY treatment process, and p53 signaling pathway is a major

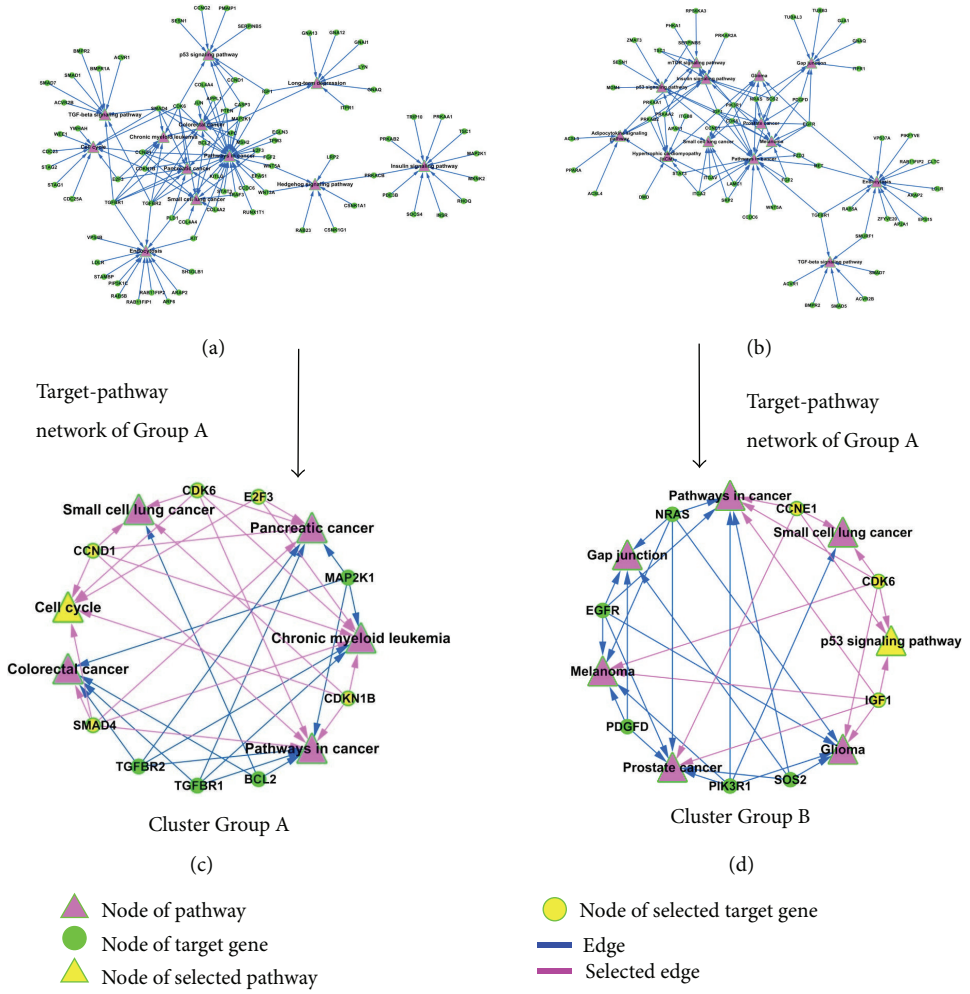

Figure 4: The global profile of target-pathway network and related cluster was showed. (a) Group A miRNA targets related target-pathway network, (b) Group B miRNA targets related target-pathway network, (c) the cluster of Group A miRNAs related target-pathway network, and (d) the cluster of Group B miRNAs related target-pathway network.

Table 2: The potential kernel miRNAs selected by FZHY and Placebo treatments.

Group A (FZHY related miRNAs)				Group B (Placebo related miRNAs)	
has-miR-1207-5p	has-miR-765	hsa-miR-19b-3p	hsa-miR-320d	hsa-miR-101-3p	hsa-miR-301b
has-miR-1268a	hsa-miR-101-3p	hsa-miR-21-5p	hsa-miR-340-5p	hsa-miR-1299	hsa-miR-335-5p
has-miR-134	hsa-miR-103a-3p	hsa-miR-221-3p	hsa-miR-374a-5p	hsa-miR-130a-3p	hsa-miR-33a-5p
has-miR-135a-3p	hsa-miR-128	hsa-miR-25-3p	hsa-miR-374b-5p	hsa-miR-142-3p	hsa-miR-340-5p
has-miR-1471	hsa-miR-142-5p	hsa-miR-26b-5p	hsa-miR-424-5p	hsa-miR-148b-3p	hsa-miR-363-3p
has-miR-26a-5p	hsa-miR-15a-5p	hsa-miR-27b-3p	hsa-miR-93-5p	hsa-miR-16-5p	hsa-miR-381-3p
has-miR-483-5p	hsa-miR-186-5p	hsa-miR-29c-3p		hsa-miR-19b-3p	hsa-miR-410
has-miR-663a	hsa-miR-19a-3p	hsa-miR-30e-3p		hsa-miR-21-5p	hsa-miR-497-5p

component in Placebo treatment process. The imbalance of G1/S and G2/M phases of cell cycle is associated with dysfunction in hepatocarcinoma [26], while p53 signaling pathway is highly correlated with the pathogenesis of numerous cancer types [27]. It implicates that the FZHY can significantly increase the cancer-related miRNAs levels, then mediates the related target genes, and transforms the original progressions of cell cycle and p53 signaling pathway in HBC patients.

On the other hand, we noticed that the TGF-β signaling pathway commonly appeared in KEGG and BIOCARTA pathway of FZHY treatment group. TGF-β (transforming growth factor-beta) is an important regulatory tumor suppressor factor in epithelial cells [28]. TGF-β1 expression level was correlated with tumor progression, metastasis, angiogenesis, and poor prognostic outcome in various types of human cancer [29–32]. TGF-β also is a central regulator in

chronic liver disease, which contributes to all stages of disease progression from initial liver injury through inflammation and fibrosis to cirrhosis and hepatocellular carcinoma [33]. We speculate that TGF-β signaling pathway might act as an important marker to discriminate the curative effects of FZHY and Placebo treatment in HBC patients and may contribute to fighting against liver cirrhosis.

5. Conclusion

In conclusion, FZHY formula can remarkably change miRNAs expression levels of HBC patients and mediate the related molecular regulated module in HBC treatment process. Here, we infer that FZHY plays a critical function in HBC treatment process and directly regulated many important pathways, including but not limited to cell cycle, p53 signaling pathway, and TGF-β signaling pathway. It provides us with a new clue to investigate the molecular mechanisms of FZHY treated HBC process.

Conflict of Interests

The authors declare that they have no financial and personal relationships with other people or organizations that can inappropriately influence their work, and there is no potential conflict of interests including employment, consultancies, stock ownership, honoraria, paid expert testimony, patent applications and registrations, and grants or other funding.

Acknowledgments

This work was supported by Program of National Natural Science Foundation of China (81473443), Key Program of National Natural Science Foundation of China (81330084), Natural Science Foundation of Shanghai Science and Technology Commission (14ZR1438800), Scheming Project of Shanghai Municipal Education Commission (2012JW25), Leading Project of Integrated Traditional and Western Medicine of Shanghai University of Traditional Chinese Medicine (2013), and Key Science Foundation of Anhui Province (KJ2012A260).

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