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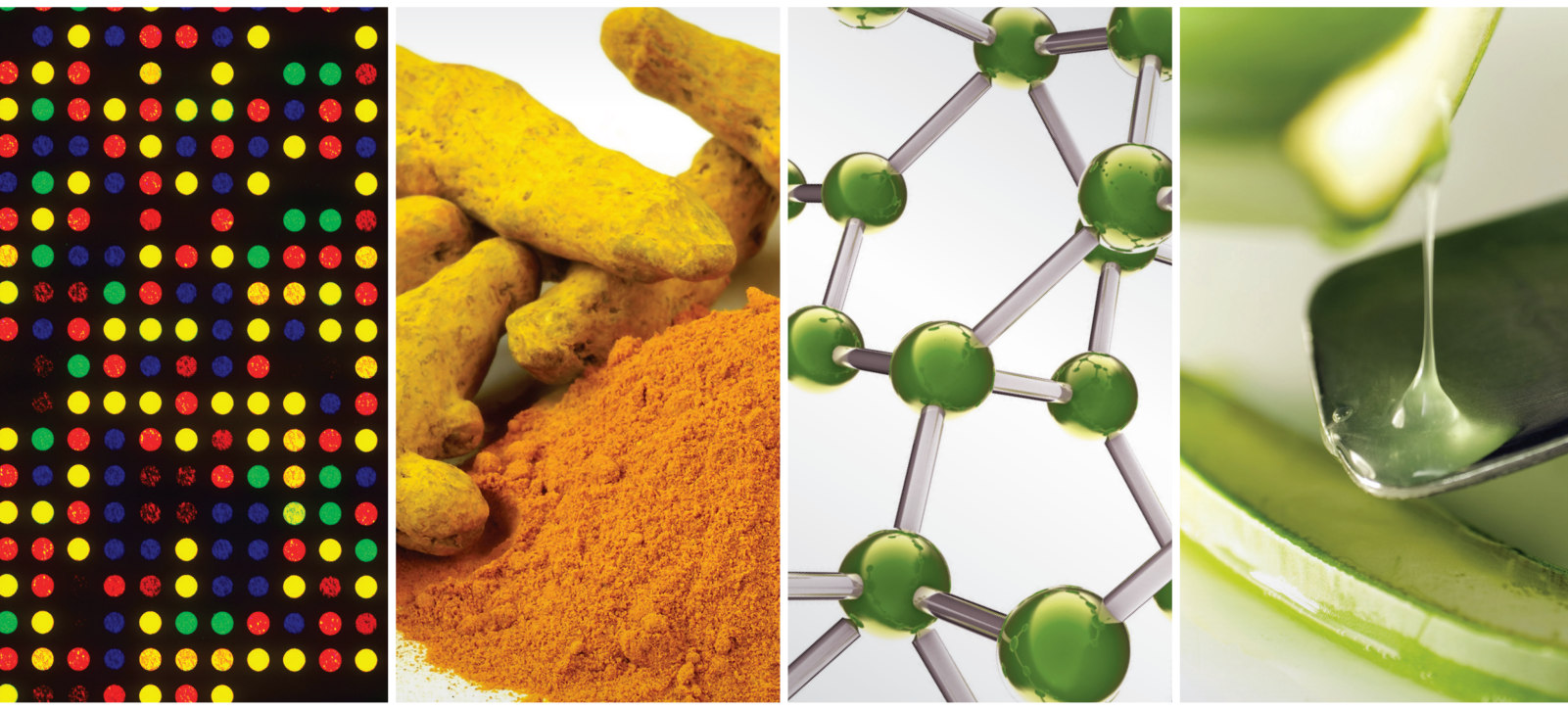
Hepatoprotective Potential of Herbal Medicine

Guest Editors: Ravirajsinh Jadeja, Ranjitsinh V. Devkar, and Srinivas Nammi



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Editorial Board

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Editorial

Hepatoprotective Potential of Herbal Medicine

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Occurrence of various types of hepatic ailments has been on the rise and has resulted in significant increase in morbidity and mortality worldwide. Viral hepatitis, alcoholic/nonalcoholic fatty liver disease, liver fibrosis, cirrhosis, hepatocellular carcinoma, and drug-induced liver injury are of major health concerns claiming millions of lives worldwide. Pharmaceutical drugs are often associated with liver injury and hence provide limited benefits in treating liver ailments. Use of herbal medicine to treat liver ailments has been mentioned in various ancient literature texts and a considerable number of medicinal plants have been evaluated using preclinical and clinical studies as hepatoprotective agents. Nevertheless, more rigorous studies are still required to screen and evaluate the use of herbal medicines in treating various liver diseases.

This special issue is a collection of five articles describing the use of herbal medicines against various liver diseases. An article by A. Sadeghipour et al. described lipid lowering effect of pomegranate (*Punica granatum* L.) peel extract in high fat diet fed rats. Authors also recommended that plant should be considered an excellent candidate for future studies on dyslipidemia. There are two research articles describing beneficial role of herbal medicines against alcoholic induced liver injury. P. Lodhi et al. showed that aqueous extract of *Camellia sinensis* (green tea) was able to ameliorate ethanol induced acute liver damage. An interesting article by L. An and F. Feng described network pharmacology-based antioxidant effect of Zhi-Zi-Da-Huang decoction, a classic traditional Chinese medicine (TCM) formula for alcoholic liver disease. It was concluded that network pharmacology is a useful tool for exploring the potential mechanism of action of TCM formula and new active ingredients. The only clinical study as a part of this special issue by M. V. Patel et al. showed benefits of a complex multiherbal regimen based on Ayurvedic medicine for the management of hepatic cirrhosis complicated with ascites. Silymarin is perhaps the most widely used compound for liver diseases. A study by J.-P. Wu et al. demonstrated that silymarin administration accelerates liver regeneration after partial hepatectomy.

Collectively this special issue provides recent updates on the use of herbal medicine in treating acute and chronic liver diseases. We hope that this special issue will be useful to scientific fraternity.

Acknowledgments

We would like to express our sincere thanks to the contributors of this special issue for their scientifically sound research articles. We extend our sincere thanks to the reviewers for critical assessment of each article, their constructive criticisms, and timely responses that made this special issue possible.

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Research Article

A Complex Multiherbal Regimen Based on Ayurveda Medicine for the Management of Hepatic Cirrhosis Complicated by Ascites: Nonrandomized, Uncontrolled, Single Group, Open-Label Observational Clinical Study

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Hepatic cirrhosis is one of the leading causes of death worldwide, especially if complicated by ascites. This chronic condition can be related to the classical disease entity *jalodara* in Traditional Indian Medicine (Ayurveda). The present paper aims to evaluate the general potential of Ayurvedic therapy for overall clinical outcomes in hepatic cirrhosis complicated by ascites (HCcA). In form of a nonrandomized, uncontrolled, single group, open-label observational clinical study, 56 patients fulfilling standardized diagnostic criteria for HCcA were observed during their treatment at the P. D. Patel Ayurveda Hospital, Nadiad, India. Based on Ayurvedic tradition, a standardized treatment protocol was developed and implemented, consisting of oral administration of single and compound herbal preparations combined with purificatory measures as well as dietary and lifestyle regimens. The outcomes were assessed by measuring liver functions through specific clinical features and laboratory parameters and by evaluating the Child-Pugh prognostic grade score. After 6 weeks of treatment and a follow-up period of 18 weeks, the outcomes showed statistically significant and clinically relevant improvements. Further larger and randomized trials on effectiveness, safety, and quality of the Ayurvedic approach in the treatment of HCcA are warranted to support these preliminary findings.

1. Introduction

Hepatic cirrhosis, especially if complicated by ascites, causes remarkable damage in human health and lives. Its management involves high costs for health care systems worldwide. Liver transplantation as one of the few treatment options bears risks and is largely unavailable or unaffordable for common patients in many countries, particularly in India. Therefore, alternative treatment measures are desirable.

Due to the high prevalence of chronic hepatic diseases in South Asia, Traditional Indian Medicine (Ayurveda) has generated extensive empirical knowledge in their treatment over several centuries.

In addition to observations of successfully treated cases in Ayurvedic clinics, preliminary scientific findings suggest that an exploration of traditional Ayurvedic literature and commonly implemented Ayurvedic treatment modalities might be worthwhile in this field.

Hepatic Cirrhosis—Epidemiological Data Worldwide. Hepatic cirrhosis represents the end stage of most chronic liver diseases, which can remain compensated for many years [1]. Decompensated cirrhosis can be characterized by the development of major complications like jaundice, variceal hemorrhage, ascites, or encephalopathy [2], of which ascites is the most common [3]. Approximately 50% of patients with

compensated cirrhosis develop ascites over a period of 10 years [4]. The occurrence of ascites is often a landmark in the natural progress of cirrhosis, as it requires hospitalization and many patients are referred for liver transplantation [3, 5–7]. In this sense, the disease carries the risk of life-threatening complications, partly due to a number of comorbidities. Established cirrhosis has a 10-year mortality of 34–66%, being largely dependent on the cause of cirrhosis; alcoholic cirrhosis has a worse prognosis than primary biliary cirrhosis and cirrhosis due to viral hepatitis [8]. Approximately 15% of patients with ascites die within the first 12 months after diagnosis and 44% within the first 60 months [9].

A recent survey reviewed 260 epidemiological studies on liver diseases in Europe. Alcohol consumption, viral hepatitis B and C, and metabolic syndromes related to obesity are the leading causes of cirrhosis and primary liver cancer here. Liver cirrhosis is responsible for around 170,000 deaths in Europe annually [10]. In the UK, liver disease is the 5th most common cause of death [11]. In the United States, chronic liver disease and cirrhosis ranked 12th among the leading causes of death in 2010 [12]. Figures are likely to be even higher in Asia and Africa, since childhood infection with HCV is more common there and the prevalence of cirrhosis in patients with chronic HCV increases with increasing duration of infection [13, 14].

In the course of the rising frequency of alcoholic and nonalcoholic fatty liver disease, a huge increase in the burden of liver diseases is expected over the next years [4]. In the UK, admissions to hospital and liver deaths are both rising at between 8 and 10% per year. Patients are presenting and dying from liver disease at an earlier age; the average age of death from liver disease is 59 years. Over the last 10 years there has been a 5-fold increase in the development of cirrhosis in 35- to 55-year-old patients [11]. Because of the increasing prevalence of chronic viral hepatitis and steatohepatitis and their high risk evolution toward end stage liver cirrhosis, preventive programs and early management of these conditions are considered as emerging health issues worldwide [14]. Treatments that may halt the progression of compensated to decompensated cirrhosis are currently being developed [15]. Liver transplantation, however, is the only option in patients with end stage disease. The cost of hepatic cirrhosis in terms of human suffering, hospital costs, and lost productivity is high [16, 17].

Hepatic Cirrhosis in India. Given the high incidence and prevalence of hepatitis B, hepatitis C, and fatty liver disease, hepatic cirrhosis is a common condition in India. Generally, Indian patients first seek help from conventional medicine, and in many cases liver transplantation is suggested to them. Actually, India would require up to 20,000 liver transplants per year. However, currently just 200–300 transplants per annum are possible within the framework of the Indian medical system. Liver transplantations are complex procedures; they require sophisticated infrastructures, expert medical teams, preservation of the transplant organs, expensive drugs, and prolonged stays in ICUs, all of which add significantly to the overall costs. A liver transplant requires about 50,000 USD, plus a lifelong commitment to immunosuppressants, costing about 2,500 USD per month, which is unmanageable for most Indian patients [18].

As to our observation, since the only curative yet unaffordable treatment option is liver transplantation in conventional medicine, in India, complementary and alternative medicine (CAM) treatments are often sought out instead, especially Ayurveda, the most established traditional whole medical system (WMS) in South Asia with a well developed infrastructure, recognized by the World Health Organization. (In the context of this paper, though useful for a reader not familiar with WMS Ayurveda, a detailed description of Ayurvedic medicine does not seem appropriate here. The interested reader may gather detailed information on the current infrastructure, institutional organization, education, practice, and research of Ayurveda from selected sources mentioned in Further Readings at the end of this paper.)

CAM and Ayurveda Related Drug Research in Liver Diseases including Hepatic Cirrhosis. Searching commonly accepted sources, only a paucity of scientifically evaluated CAM-options for the treatment of chronic liver diseases, especially of cirrhotic liver conditions, is retrievable.

S-Adenosylmethionine, polyenylphosphatidylcholine, betaine, and antioxidants like vitamins A, C, E, B6, and B12 as well as branched chain amino acids are examples of substances studied for their potential efficacy in cases of hepatic cirrhosis. Trials showed clinical improvements, but hardly any statistically significant improvements in liver function test [19–29].

For herbal drugs used in different fields of CAM limited data is available, which have shown beneficial effects on liver diseases in clinical trials [30–33]. Worldwide, *Silybum marianum* (L.) Gaertn. is currently the plant on which most research has been performed in the treatment of liver diseases [34–38]. From East Asia, the compound herbal drug TJ-9 is worth mentioning for chemoprevention in hepatocellular carcinoma, commonly prescribed as Xiao-Chai-Hu-Tang in China and as Sho-Saiko-To in Japan. It consists of an extract from the roots of *Scutellaria baicalensis* Georgi, *Glycyrrhiza glabra* L., *Bupleurum falcatum* L., and *Panax ginseng* C. A. Mey [39].

CAM research on chronic liver diseases also incorporates drugs commonly used in Ayurveda. Nobel laureate Baruch Blumberg, awarded for his hepatitis B virus surface-antigen discovery, had already in the 1980s initiated research on *Phyllanthus niruri* Sensu Hook. F. non Linn. (*P. niruri*), seeking measures to prevent and treat hepatitis B [40]. Reviews in recent years come to conclusions varying from being positive to being indifferent regarding *P. niruri*'s effect in this condition [41–43]. As an example among animal studies, carbon tetrachloride- (CCl4-) induced increase of serum glutamic-pyruvic transaminase (ALT) and elevation of MDA in liver of mice are significantly lowered by *P. niruri in vivo* and the coincubation of isolated rat hepatocytes with *P. niruri in vitro* significantly inhibits CCl4-induced decrease of mobility of membrane of liver cells and increase of intracellular free Ca~(2+)([Ca~(2+)]_(i)) concentrations of liver cells. These results suggest that the antilipid peroxidation effect and protection of membranes through *P. niruri* may

be related to its protective action against CCl4-induced liver injuries [44].

The positive action in chronic liver diseases of Liv 52, an Ayurvedic herbal compound preparation frequently used in India for chronic liver diseases, is well documented [45].

Piper longum Linn. fruit (*P. longum*), a traditional medicinal plant in Ayurveda, was examined for its hepatoprotective properties, concentrating on its main active ingredient piperine, which was reported to exert significant protection against acetaminophen-induced hepatotoxicity in mice [46].

Another medicinal plant in this context is *Picrorhiza kurroa* Royle ex Benth (*P. kurroa*). Cucurbitacin glycosides, isolated from the root of *P. kurroa*, exhibited liver protective and anti-inflammatory activities. Kutkin, a glycosidal bitter component of *P. kurroa* exhibited hepatoprotective activity in alcohol-induced hepatotoxicity in rats [47–51].

Boerhavia diffusa L. (*B. diffusa*) exhibits anti-inflammatory, hepatoprotective, and antioxidant effects and therefore can be considered as an option in cases of HCcA [52–55].

Tephrosia purpurea Pers. (*T. purpurea*) is widely used in the treatment of inflammation of the spleen and liver in the Ayurvedic tradition. Its powdered aerial parts prevent an elevation of GOT, GPT, and bilirubin levels [56, 57]. The cirrhotic and nodular changes induced by CCl4 were effectively prevented by *T. purpurea* showing that it might be acting by stabilizing cell membranes. These findings relate to an earlier study which reported that pretreatment of rats with Tefroli, a herbal product containing *T. purpurea* as one of the main ingredients, protected the rats against progress of hepatic fibrosis after chronic CCl4 intoxication [58].

Finally, *Eclipta alba* (L.) Hassk. (*E. alba*) holds a prominent position in the traditional use for hepatitis and spleen enlargements in Ayurveda and is considered to be a liver tonic [59]. The protective effect of *E. alba* on carbon tetrachloride-induced acute liver damage was also studied in 54 female guinea pigs in an experimental trial [60].

Hence, over the last years and decades, several research projects were dedicated to examining the effects of herbal drugs traditionally used in Ayurveda to support liver functions and treat common liver diseases. However, the effects of CAM herbal therapies on life-threatening chronic liver diseases have for the most part been evaluated on animal studies. Among the limited number of CAM clinical trials only a few deal with hepatic cirrhosis and so far no clinical study is known to the authors, which has directly evaluated Ayurvedic therapies in patients with HCcA.

Rationale for the Ayurvedic Treatment Protocol of the Present Study. On the basis of this preliminary evidence and as commonly practiced in the field of reverse pharmacology, treatment protocols incorporating empirical data from traditional treatment patterns of WMS Ayurveda can be considered as promising and ethically sound. In this sense, a standardized treatment protocol was developed from traditional Ayurvedic sources, supplemented by current research findings. It was implemented for several years at the P. D. Patel Ayurveda Hospital in Nadiad, India, before this observational study was conducted. Special attention was given to the proposition that medicinal plants exert more intense action, if embedded in complex treatment schemes. In Ayurveda, certain preparatory measures such as purificatory procedures (e.g., purgation) are considered to enhance the action of drugs. Also diet and lifestyle corrections are held to optimize the effect of any given treatment [61].

In current Ayurveda, HCcA is commonly related to the superordinate disease group of the so-called abdominal diseases (*udara roga*). Among eight types of "abdominal diseases" the most serious conditions are considered to be *jalodara* or *yakriddaludara*, which can be interpreted as different varieties of HCcA [62]. These nosological entities are described as having similar symptoms to another form of "abdominal disease" (*plihodara*) characterized by abdominal distention (due to splenomegaly), accompanied by weakness, anorexia, indigestion, constipation, excessive thirst, breathlessness, coughing, vomiting, cachexia, syncope or coma, and visible yellowish or indigo colored veins in the abdominal area [63].

The treatment protocol for the study was predominantly inspired by one of the most authoritative texts of Ayurveda (the *Caraka Samhita*). Initially, a procedure, which consists of a specific administration pattern of *P. longum* in increasing and tapering doses (*vardhamana-pippali-rasayana,* Table 2) [64], was performed on all patients. The microfine powder of dried *P. longum* fruit (family Piperaceae) was manufactured with the help of pulverizing machines by Sundar Ayurveda Pharmacy, that is, the pharmacy of the Pharmaceutical Department of J. S. Ayurved College, Nadiad, India, as all the following powders of dried plants. A large number of alkaloids and related compounds, the most abundant of which is piperine, together with methyl piperine, iperonaline, piperettine, asarinin, pellitorine, piperundecalidine, piperlongumine, and piperlonguminine are found in the fruit. According to classical Ayurveda, this procedure exerts a tissue-regenerating effect (*rasayana*), which can be interpreted as being hepatoprotective. The classical dosage patterns were found to be intolerable for patients of our hospital before the commencement of the study. Consequently, the study intervention was standardized to a lower dosage pattern.

The multidimensional approach of Ayurveda calls for certain "purification measures" in most diseases. Here, purgation induced by rhizomes and roots of *P. kurroa* was chosen in a dosage matched with the individual condition of each patient [65]. The powder of dried roots and rhizomes of *P. kurroa* (family Scrophulariaceae) mainly contains iridoid glycosides, cucurbitacins, unsaturated sterols/triterpenes, and polyphenols, especially kutkin, a bitter glycoside.

These "preparatory" treatments were embedded in a set of strict food and behavioral restrictions, based on classical Ayurvedic rationales [66]. Namely, a diet consisting only of boiled milk and maximum rest in an OPD setting were implemented.

Classically indicated drugs, like the powder of dried whole plants of *B. diffusa*, *T. purpurea*, and *E. alba*, were administered since the experimental studies cited above suggest a hepatoprotective action of these drugs and because (empirically) the combination of these drugs has been used for the treatment of HCcA over centuries in routine Ayurvedic

therapy. *B. diffusa* (family Nyctaginaceae) contains mainly alanine, arachidic acid, aspartic acid, behenic acid, boeravinones A through F, boerhaavic acid, and borhavine. In *T. purpurea* (family Fabaceae) the presence of flavones, flavanones (e.g., purpurin) and prenylated flavonoids, chalcones, and rotenoids was found. From *E. alba*, Ecliptasaponin C, a new triterpenoid glucoside, was isolated together with daucosterol and stigmasterol-3-O-glucoside.

To promote diuresis, the herbal compound formulation *Punarnavadi-kvatha* [67] based on the whole plant of *B. diffusa* and the herbomineral combination *Shveta-Parpati* [68] were chosen. The ingredients of *Punarnavadi-kvatha* are listed in Table 3. The method of preparation follows the description of ancient Ayurvedic texts for decoction (*kvatha*). 10 g of the course powder of mentioned dried plants are boiled in 160 mL water in an open vessel on mild heat until it is reduced to one-fourth of the original water quantity (40 mL). This preparation is boiled fresh twice daily and administered orally after cooling down. *Shveta-Parpati* contains ashes of 1 g ammonium chloride [NH_4Cl], 2 g potash alum [$KAl\ (SO_4)_2 12H_2O$], and 16 g potassium nitrate which are purified according to the classical method. All ingredients were triturated and afterwards deposited in a sealed earthen pot, which was kept in a furnace with fire for 8 hours. Then they were allowed to cool and powdered.

To this effect, the present nonrandomized, uncontrolled, single group, open-label observational clinical study scientifically explores effectiveness and safety of a treatment scheme, which generated clinically promising results in single cases of hepatic cirrhosis complicated by ascites in an Ayurvedic hospital.

2. Materials and Methods

2.1. Research Center. The P. D. Patel Ayurveda Hospital is a university teaching hospital of the J. S. Ayurved College, accredited by the Indian Government for under- and postgraduate Ayurveda education and run by a private charitable trust, the Mahagujarat Medical Society in Nadiad, India. All participants were screened and treated in both the in-patient department (IPD) and out-patient department (OPD) of the clinic.

The Institutional Ethics Committee of J. S. Ayurveda Mahavidyalaya, Nadiad, India, has given its permission to publish the data under the approval number 18/02-2014 (reference letter JSAM/14-15/26).

The laboratory consulted for all mentioned investigations is a part of the research center.

Adhering to standard operating procedures (SOP) and to classical descriptions all medicaments were purchased from Sundar Ayurveda Pharmacy, which is preparing all main Ayurvedic pharmaceuticals for the campus hospital. Collection of the used plant material complied with institutional, national, or international guidelines.

2.2. Patient Selection. The involved human subjects were all above 18 years of age. They were informed about the study details and gave consent to record and publish their data. Datasets were handled and displayed in a way which does not compromise anonymity or confidentiality or breach local data protection laws. Being an observational study, patients were chosen according to the inclusion and exclusion criteria.

Inclusion criteria were as follows: positive patient history and established diagnosis of HCcA according to international standards [15]; manifest clinical features, especially oedema, loss of appetite, general weakness, nausea and vomiting, increased measurement of abdominal girth (sign of ascites), and decreased urine output; manifest laboratory findings, especially hyperbilirubinemia, decreased serum albumin level, decreased albumin/globulin (A/G) ratio, raised serum alkaline phosphatase, raised liver transaminase enzymes, and confirmed abdominal ultrasound diagnosis of HCcA.

Exclusion criteria were as follows: hepatic cirrhosis due to cardiac causes, inherited metabolic causes, haemochromatosis and Wilson's disease; recent (≤3 month) life-threatening complications (like encephalopathy and excessive gastrointestinal bleeding), and other major comorbidities (like insulin-dependent diabetes mellitus, bleeding piles, manifest heart diseases, and renal failure). Female patients having pregnancy, postdelivery period, or lactation period and patients who were taking any psychiatric or other liver damaging medicines were also excluded.

Screening was performed between January 2007 and December 2010.

2.3. Study Protocol and Timeline. The study was planned to generate first exploratory data on the potential of Ayurvedic medicine for advanced liver diseases.

After screening, the main IPD observation period lasted for 6 weeks followed by an OPD follow-up of 18 weeks resulting in an overall 6-month observation period. During follow-up, patients were investigated for signs, symptoms, and laboratory findings (Table 1).

2.4. Therapy. The treatment conducted is standard care and best practice for the given disease at our hospital. All patients were treated according to the following treatment protocol standardized on the basis of classical Ayurvedic literature and traditional treatment patterns.

2.4.1. Phase 1. At the beginning of the IPD period, finely powdered dried fruit of *P. longum* was administered orally in an increasing and tapering dose pattern twice daily with milk before meals. This classical procedure was performed for a period of 13 days (Table 2).

2.4.2. Phase 2. On the morning of the 14th day, mild purgation was performed once, orally administering finely powdered dried rhizomes and roots of *P. kurroa* with warm water on an empty stomach in varying doses from 3 to 6 g according to the patients' individual sensitivity to purgatives (*koshtha*).

2.4.3. Phase 3. For the next 4 weeks a number of Ayurvedic drugs were given orally twice daily after meals (Table 3).

Table 1: Timeline of the study.

Phase	Event	Measure	Week
Prephase	Screening	(i) Inclusion and exclusion criteria	Week 0
Main observation period (IPD)	Baseline visit (V0)	(i) Clinical and laboratory investigations (ii) Admission to IPD	Week 1 (day 1)
Phase 1		(i) *Piper longum* Linn. administration (specific dose pattern) (ii) Strict diet and lifestyle measures	Weeks 1 & 2 (days 1–13)
Phase 2		(i) Purgation (ii) Strict diet and lifestyle measures	Week 2 (day 14)
Phase 3		(i) Drug administration (ii) Strict diet and lifestyle measures	Weeks 3–6
	First visit (V1)	(i) Clinical and laboratory investigations (ii) Discharge from IPD	Week 7
Follow-up (OPD) Phase 4		(i) Continued drug administration (ii) Diet and lifestyle measures	Weeks 7–24
	Second visit (V2)	(i) Clinical and laboratory investigations	Week 24

Table 2: Dose pattern of *Piper longum* Linn.

Day	1	2	3	4	5	6	7	8	9	10	11	12	13
Dose [g] twice daily					5	5	5	5	5				
				4						4			
			3								3		
		2										2	
	1												1

2.4.4. Diet and Lifestyle. In the IPD phase, a set of rigorous food (boiled pasteurized fat-free cow's milk) and behavioral restrictions (maximum rest in an IPD setting) was carried out. The participants were allowed to consume milk according to their digestive capacity varying from 2 to 3.5 litres per day. All other liquids and foods were prohibited.

2.4.5. Conventional Medication. Patients on conventional diuretics (e.g., furosemide and spironolactone) were advised to continue their medication initially. Their dosage was gradually reduced according to decreasing oedema as well as abdominal girth and increase in urine output and eventually stopped as soon as the patient responded to the Ayurvedic treatment satisfactorily.

2.4.6. Phase 4. During follow-up, patients were advised to continue all Ayurvedic medicaments at home except *P. longum*. In diet, they were instructed to take a special type of boiled beans (*Vigna radiata* (L.) Wilczek), rice, and boiled vegetables being easily digestible and nonslimy in consistency. Also nonsour fruits, like papaya, mango, sweet apple, and so forth, were permitted. All participants were strictly told to permanently abstain from all alcoholic beverages.

2.5. Assessment of Results. Treatment effects were measured by a predefined set of typical clinical features and laboratory parameters of HCcA. Additionally, the assessed outcomes included prognostic markers according to the Child-Pugh grade score.

2.5.1. Clinical Features (Scores). Following clinical features were selected as representative markers for the assessment of HCcA treatment during study visits (V0–V2): oedema, loss of appetite, general weakness, nausea and vomiting, abdominal girth, and daily urine output.

They were graded according to the score presented in Table 4. Changes in ascites were assessed by the measurement of abdominal girth and urine output in liters per day. All other investigations were also recorded before (V0) and after (V1) treatment and also during follow-up (V2).

Statistically, all outcomes were analyzed using a paired Student's test ("t" test). Explorative tests were not adjusted for multiple testing. A value of $p < 0.05$ was considered as statistically significant and $p < 0.001$ as statistically highly significant.

3. Results

3.1. Baseline (V0). 68 patients (48 males and 20 females) with HCcA were screened. Mean age was 46.45 years (<20 years of age: $n = 2$; 20–40 years of age: $n = 25$; >40 years of age: $n = 41$).

12 drop-outs happened due to personal reasons not related to the therapy, like social or family affairs (e.g.,

Table 3: Treatment protocol for Ayurvedic drugs.

Number	Botanical name	Ayurvedic name	Content ratio	Part used	Form	Dose
(1)	Compound decoction	*Punarnavadi-kvatha*		Various	Coarse powder/decoction	10 g/40 mL
(a)	*Boerhavia diffusa* L. L. Linn.	*Punarnava*	1 part	Whole plant	Coarse powder	
(b)	*Berberis aristata* DC.	*Daruharidra*	1 part	Whole plant	Coarse powder	
(c)	*Cyperus rotundus* L.	*Musta*	1 part	Root	Coarse powder	
(d)	*Curcuma longa* L.	*Haridra*	1 part	Rhizome	Coarse powder	
(e)	*Azadirachta indica* A. Juss.	*Nimba*	1 part	Bark	Coarse powder	
(f)	*Tinospora cordifolia* (Willd.) Hook. F. & Thoms.	*Guduci*	1 part	Stem	Coarse powder	
(g)	*Zingiber officinale* Rosc.	*Shunthi*	1 part	Rhizome	Coarse powder	
(h)	*Picrorhiza kurroa* Royle ex Benth.	*Katuki*	1 part	Root	Coarse powder	
(2)	Compound powder				Fine powder	5.5 g
(a)	*Tephrosia purpurea* Pers. Linn. Pers.	*Sharapunkha*		Root	Fine powder	2 g
(b)	*Phyllanthus niruri* Sensu Hook. F. non Linn.	*Bhumyamalaki*		Whole plant	Fine powder	3 g
(c)	Herbomineral combination	*Shveta-Parpati*			Ultrafine powder (ash)	0.5 g
(I)	Ammonium chloride [NH_4Cl]	*Navasara*	1 part		Ultrafine powder (ash)	
(II)	Potassium nitrate [KNO_3]	*Suryakshara*	16 parts		Ultrafine powder (ash)	
(III)	Potash alum [$KAl(SO_4)_2 12H_2O$]	*Sphatika*	2 parts		Ultrafine powder (ash)	
(3)	*Eclipta alba* (L.) Hassk.	*Bhrngaraja*		Whole plant	Fine powder	3 g

Table 4: Assessed clinical features' score (Grade 0–3).

Number	Symptom	Grade 0	Grade 1	Grade 2	Grade 3
(1)	Oedema	No oedema	Slight oedema on lower extremities	Severe oedema on lower extremities	Anasarca
(2)	Loss of appetite	Good appetite	Mild loss of appetite	Moderate loss of appetite	Complete loss of appetite
(3)	General weakness	No weakness	Mild weakness	Moderate weakness	Severe weakness
(4)	Nausea and vomiting	Absent	Occasional	Once or twice a week	Daily
(5)	Abdominal girth [cm]	Grading per [cm]-scale			
(6)	Urine output [L/day]	Grading per [L]-scale			

marriages or death of close family members, which are of major social relevance in South Asia). Overall, 56 patients' datasets were completely recorded.

Prolonged alcohol consumption and cryptogenic cirrhosis of liver were found as main causative factors in 26 patients. A positive patient history for different forms of hepatitis was present in 16 cases (Table 5). At the time of study entry (V0), all patients were treated with standard conventional medicine.

3.2. Treatment Effects

3.2.1. Clinical Features. Oedema was reduced by 83.9%. Appetite increased by 64.7%. General weakness declined

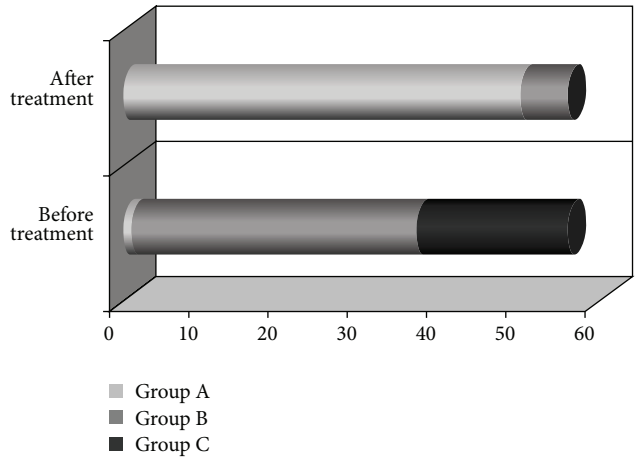

Figure 1: Effect on prognosis according to Child-Pugh grade score: overall change ($n = 56$).

Table 5: Baseline (V0) characteristics: underlying causes of HCcA ($n = 68$).

Basic cause (diagnosis)	Number of patients	Percentage [%]
Prolonged alcohol consumption	26	38
Alcohol and Hepatitis B	6	9
Hepatitis B	8	12
Hepatitis C	2	3
Cryptogenic cirrhosis	26	38

by 54.7%. Nausea and vomiting were relieved by 90.9%. Abdominal girth decreased by 19.7%. Urine output increased by 266.3%. All these clinical features were found to be statistically highly significant ($p < 0.001$) (Table 6).

3.2.2. Laboratory. Accordingly, all relevant laboratory values improved in a statistically significant fashion; albumin (40.7%) and bilirubin (66.5%) (Table 7) were mostly pronounced.

3.2.3. Child-Pugh Grade Score. At baseline (V0) 19 patients were graded in Group C (worst prognosis), 36 patients were graded in Group B (medium prognosis), and 1 was graded patient in Group A (best prognosis). After treatment (V1) 50 patients were found in Group A, 6 patients were found in Group B, and no patient was found in Group C (Figure 1).

56 patients completed the full 6-months treatment protocol. They adhered to the dietary and lifestyle advice and took their medicines regularly. During follow-up (V2) patients' general condition further improved and no setback of clinical features was observed. Liver function tests also further improved (see Table 7). With regard to the Child-Pugh grade score, 6 patients of Group B returned to Group A after completing the follow-up period; that is, all patients came to group A.

4. Discussion

In this study improvements in all clinical features and laboratory parameters were observed. The results were clinically relevant and statistically significant. Notably, albumin synthesis was initially impaired in all cases, but under this Ayurvedic treatment protocol albumin levels increased in all patients. Ascites could be reversed and controlled. None of the patients developed severe adverse effects or new complications and hence the treatment was found to be safe under the given conditions. Notably, in all participants conventional medication could be reduced or even withdrawn. Initially indicated invasive measures (e.g., liver transplantation) could be omitted, particularly through improvements of the Child-Pugh grade score in several patient cases. Patients were able to manage their routine everyday life affairs without becoming dependent on cost intensive conventional health care and maintenance measures within the observation period.

Hence, the treatment protocol can be considered as applicable and cost-effective and, probably, as a template for subsequent trials in this field. These results are promising and may be regarded as a novelty in clinical research on hepatic cirrhosis complicated by ascites, looking at an overall paucity of peer-reviewed publications on Ayurvedic treatment options in this field [61–66], and an absence of respective clinical study data available in internationally accessible medical databases.

From a conventional biomedical perspective, the probable mode of action of the study drugs may stem from their effects on specific metabolic liver functions and functions of associated organs, the details of which have still to be subject to future studies [40–60]. Taking the traditional Ayurvedic paradigms into account and Ayurveda's unique understanding of pathogenesis and treatment, more general terms should be used to explain their modes of action. Along the way, the challenges and problems arising in translational processes between systems' terminology remain an open field of study, in particular for qualitative research on WMS [67]. In summary, the study drugs' antioedemic, diuretic, immunomodulatory, rejuvenative, hepatosplenoprotective,

Table 6: Changes in clinical symptoms from baseline (V0) to week 7 (V1).

Number	Symptom score	Mean score		Improvement [%]	p value
		Baseline (V0)	Week 7 (V1)		
(1)	Oedema score	2.01 ± 0.68	0.33 ± 0.57	83.87 ± 0.62	<0.001
(2)	Loss of appetite score	2.62 ± 0.53	0.92 ± 0.52	64.70 ± 0.51	<0.001
(3)	General weakness score	2.67 ± 0.47	1.21 ± 0.5	54.67 ± 0.58	<0.001
(4)	Nausea and vomiting score	1.29 ± 0.59	0.12 ± 0.33	90.90 ± 0.4	<0.001
(5)	Abdominal girth [cm]	89.9 ± 10.6	72.1 ± 9.76	19.7 ± 7.17	<0.001
(6)	Urine output [liter/per day]	0.45 ± 0.29	1.63 ± 0.58	266.32 ± 0.5	<0.001

Table 7: Changes in clinical laboratory: baseline (V0), week 7 (V1), and follow-up (V2).

Investigation	Mean score	Improvement in percent [%]			p value
	Baseline (V0)	Week 7 (V1)	After 6 months (V2)	After Week 7 (V1)	Between V1 and V2
Serum bilirubin [mg/dL]	3.74 ± 4.59	1.25 ± 1.31	0.94 ± 0.63	66.5 ± 4.04	25.63 ± 0.70	<0.001
Serum albumin [g/dL]	2.71 ± 0.67	3.32 ± 0.45	3.43 ± 0.28	22.43 ± 0.54	3.27 ± 0.29	<0.001
Serum albumin/serum globulin [A/G]	0.73 ± 0.20	1.03 ± 0.20	1.17 ± 0.17	40.65 ± 0.19	13.33 ± 0.17	<0.001
Serum alkaline phosphatase [IU/L]	318.3 ± 102.2	210.8 ± 53.05	192.15 ± 29.42	33.8 ± 76.37	8.37 ± 30.60	<0.001
Serum globulin [g/dL]	3.77 ± 0.62	3.27 ± 0.43	2.96 ± 0.32	13.3 ± 0.51	9.33 ± 0.37	<0.001
Hemoglobin [g/dL]	8.78 ± 1.90	10.36 ± 1.52	11.33 ± 1.47	17.98 ± 0.93	9.36 ± 1.06	<0.001
Serum GPT [IU/L]	58.41 ± 44.38	40.62 ± 27.6	30.62 ± 9.07	30.5 ± 27.39	24.60 ± 21.62	<0.001
Serum GOT [IU/L]	84.52 ± 50.52	52.6 ± 31.21	44.27 ± 21.81	37.8 ± 34.92	14.67 ± 14.63	<0.001

and metabolism-promoting actions may be considered as being mainly responsible for the observed effects [68–75].

4.1. Limitations and Future Directions. The study is a non-randomized, uncontrolled, single group, open-label observational clinical study. This design therefore bears major limitations. Various nonspecific effects, the setting, and the time course may have contributed to the beneficial course of the disease. Thus they may have influenced the results. Furthermore, changes in diet and the restricted alcohol consumption may have influenced the results. If the study therapies would have been studied with more patients and under controlled, randomized conditions, the effectiveness of the therapy could have been presented with more power, certainty, and accuracy. Another limitation is that patients with serious complications like severe gastrointestinal bleeding and hepatic encephalopathy were not included in this study. Though important for the Child-Pugh Score, patients with hepatic encephalopathy were a priori excluded during the screening process. Moreover, the inclusion of further assessment criteria like lipid profile data, PBMC transcripts profiles or miRNA evaluations, pre- and posttreatment USG, and liver biopsies would be helpful to give clues on histological changes in future studies. From a whole systems perspective it might also be considered a limitation that mind-body aspects of Ayurvedic medicine were not included in the treatment protocol [76].

Notably, the transferability of these results to Western clinical contexts, particularly looking at Ayurvedic treatment availability, economic aspects, and safety issues, remains another important field of discussion [77, 78]. For example, many of the therapies and drugs used in this study are not commonly available in Western countries, especially not in standardized and rigorously tested dosage forms, and, moreover, are usually much more expensive than in their countries of origin. Thus, the conclusions of this study remain to some extent regional to South Asia where Ayurveda is a recognized as a full-fledged and widely practiced system of medicine [79].

5. Conclusions

On the basis of these findings, the Ayurvedic treatment protocol could be a potentially safe and effective complement in the case of HCcA therapy. If replicated in larger trials, Ayurvedic medicine could represent a promising tool to postpone the need of liver transplantations, increase the QoL of patients with HCcA, and reduce overall treatment costs. However, randomized and controlled studies with larger numbers of patients are indicated to further evaluate the results of this Ayurvedic treatment protocol. Cross-cultural and interdisciplinary aspects should also be taken into account in future studies on WMS Ayurveda.

Further Readings

WHO

http://www.who.int/dg/speeches/2008/20081107/en/

http://www.who.int/intellectualproperty/studies/B.Patwardhan2.pdf
http://www.who.int/medicines/areas/traditional/BenchmarksforTraininginAyurveda.pdf
http://whqlibdoc.who.int/hq/2000/WHO_EDM_TRM_2000.1.pdf

Department of AYUSH, Ministry of Health and Family Welfare (AYUSH, CCIM)

http://www.indianmedicine.nic.in/
http://ccimindia.org/http://ccimindia.org/
http://www.nia.nic.in/

Universities

http://www.ayurveduniversity.com/
http://www.bhu.ac.in/ims/ayurveda/ayur_highlights.htm

Research

http://www.ccras.nic.in/
http://ayushportal.nic.in/

Research Database

http://www.dharaonline.org/Forms/Home.aspx

Journals

http://nopr.niscair.res.in/
http://www.ijaronline.com/
http://www.ancientscienceoflife.org/
http://www.ayujournal.org/
http://www.jaim.in/
http://bjournals.ub.rug.nl/index.php/ejim/

Phytotherapy

http://www.nmpb.nic.in/
http://nopr.niscair.res.in/
http://www.medicinalplants.in/

Abbreviations

A/G: Albumin/globulin ratio
ALT: Serum glutamic-Pyruvic Transaminase
B. diffusa: *Boerhavia diffusa* L.
CAM: Complementary and alternative medicine
CCI4: Carbon tetrachloride
E. alba: *Eclipta alba* (L.) Hassk.
HCcA: Hepatic cirrhosis complicated by ascites
HCV: Hepatitis C virus
ICU: Intensive care unit
IPD: In-patient department
OPD: Out-patient department
P. longum: *Piper longum*
P. kurroa: *Picrorhiza kurroa*
P. niruri: *Phyllanthus niruri*Sensu Hook. F. non Linn.
QoL: Quality of life
SOP: Standard operating procedures
T. purpurea: Tephrosia purpurea Pers.
V0: Baseline visit
V1: First visit (week 7)
V2: Second visit (follow up, after 6 months)
WHO: World Health Organization
WMS: Whole medical system.

Disclosure

The institutes, where this study was performed, belong to a charitable organization engaged in health and education services in the region, including the laboratory and the pharmacy. The medicines implemented in this study are commonly used traditional Ayurvedic formulations from classical textbooks. They are not branded products of any company and were supplied by the pharmacy of our campus. The study was not funded by any other organization.

Conflict of Interests

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

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References

[1] G. Garcia-Tsao and J. K. Lim, "Management and treatment of patients with cirrhosis and portal hypertension: recommendations from the department of veterans affairs, Hepatitis C Resource Center Program and the National Hepatitis C Program," *The American Journal of Gastroenterology*, vol. 104, no. 7, pp. 1802–1829, 2009.

[2] G. D'Amico, G. Garcia-Tsao, and L. Pagliaro, "Natural history and prognostic indicators of survival in cirrhosis: a systematic review of 118 studies," *Journal of Hepatology*, vol. 44, no. 1, pp. 217–231, 2006.

[3] P. Ginés, E. Quintero, V. Arroyo et al., "Compensated cirrhosis: natural history and prognostic factors," *Hepatology*, vol. 7, no. 1, pp. 122–128, 1987.

[4] N. C. Fisher, J. Hanson, A. Phillips, J. N. Rao, and E. T. Swarbrick, "Mortality from liver disease in the West Midlands, 1993–2000: observational study," *British Medical Journal*, vol. 325, no. 7359, pp. 312–313, 2002.

[5] G. D'amico, A. Morabito, L. Pagliaro, and E. Marubini, "Survival and prognostic indicators in compensated and decompensated cirrhosis," *Digestive Diseases & Sciences*, vol. 31, no. 5, pp. 468–475, 1986.

[6] J. Llach, P. Gines, V. Arroyo et al., "Prognostic value of arterial pressure, endogenous vasoactive systems, and renal function in cirrhotic patients admitted to the hospital for the treatment of ascites," *Gastroenterology*, vol. 94, no. 2, pp. 482–487, 1988.

[7] I. M. Lucena, R. J. Andrade, G. Tognoni, R. Hidalgo, and F. de la Cuesta, "Multicenter hospital study on prescribing patterns for prophylaxis and treatment of complications of cirrhosis," *European Journal of Clinical Pharmacology*, vol. 58, no. 6, pp. 435–440, 2002.

[8] H. T. Sørensen, A. M. Thulstrup, L. Mellemkjar et al., "Long-term survival and cause-specific mortality in patients with cirrhosis of the liver: a nationwide cohort study in Denmark," *Journal of Clinical Epidemiology*, vol. 56, no. 1, pp. 88–93, 2003.

[9] R. Planas, S. Montoliu, B. Ballesté et al., "Natural history of patients hospitalized for management of cirrhotic ascites," *Clinical Gastroenterology and Hepatology*, vol. 4, no. 11, pp. 1385.e1–1394.e1, 2006.

[10] M. Blachier, H. Leleu, M. Peck-Radosavljevic, D.-C. Valla, and F. Roudot-Thoraval, "The burden of liver disease in Europe: a review of available epidemiological data," *Journal of Hepatology*, vol. 58, no. 3, pp. 593–608, 2013.

[11] The British Society of Gastroenterology: Commissioning Report, 2014, http://www.bsg.org.uk/clinical/commissioning-report/management-of-patients-with-chronic-liver-diseases.html.

[12] S. L. Murphy, J. Xu, and K. D. Kochanek, "Deaths: final data for 2010," *National Vital Statistics Reports*, vol. 61, no. 4, 2013.

[13] R. D'Souza, M. J. Glynn, I. Ushiro-Lumb et al., "Prevalence of hepatitis C-related cirrhosis in elderly asian patients infected in childhood," *Clinical Gastroenterology and Hepatology*, vol. 3, no. 9, pp. 910–917, 2005.

[14] F. Ullah, S. Khan, A. K. Afridi, and S. U. Rahman, "Frequency of different causes of cirrhosis liver in local population," *Gomal Journal of Medical Sciences*, vol. 10, pp. 178–181, 2012.

[15] I. Grattagliano, E. Ubaldi, L. Bonfrate, and P. Portincasa, "Management of liver cirrhosis between primary care and specialists," *World Journal of Gastroenterology*, vol. 17, no. 18, pp. 2273–2282, 2011.

[16] D. Schuppan and N. H. Afdhal, "Liver cirrhosis," *The Lancet*, vol. 371, no. 9615, pp. 838–851, 2008.

[17] A. M. Minino, M. P. Heron, S. L. Murphy, and K. D. Kochanek, "Deaths: final data for 2004," *National Vital Statistics Reports*, vol. 55, no. 15, pp. 1–120, 2007, http://www.cdc.gov/nchs/data/nvsr/nvsr55/nvsr55_19.pdf.

[18] D. Suryanarayan, "India needs 20,000 cadaver livers a year: Mohamed Rela," http://hepatitiscresearchandnewsupdates.blogspot.de/2010/06/india-needs-20000-cadaver-livers-year.html.

[19] C. S. Abittan and C. S. Lieber, "Alcoholic liver disease," *Current Treatment Options in Gastroenterology*, vol. 2, no. 1, pp. 72–80, 1999.

[20] J. M. Mato, J. Cámara, J. F. de Paz et al., "S-adenosylmethionine in alcoholic liver cirrhosis: a randomized, placebo-controlled, double-blind, multicenter clinical trial," *Journal of Hepatology*, vol. 30, no. 6, pp. 1081–1089, 1999.

[21] S. Chitturi and G. C. Farrell, "Herbal hepatotoxicity: an expanding but poorly defined problem," *Journal of Gastroenterology and Hepatology*, vol. 15, no. 10, pp. 1093–1099, 2000.

[22] P. Angulo and K. D. Lindor, "Treatment of nonalcoholic fatty liver: present and emerging therapies," *Seminars in Liver Disease*, vol. 21, no. 1, pp. 81–88, 2001.

[23] C.-T. Liu, P.-T. Chuang, C.-Y. Wu, Y.-M. Weng, W. Chen, and C.-Y. Tseng, "Antioxidative and *in vitro* hepatoprotective activity of *Bupleurum kaoi* leaf infusion," *Phytotherapy Research*, vol. 20, no. 11, pp. 1003–1008, 2006.

[24] A. Rambaldi and C. Gluud, "S-adenosyl-L-methionine for alcoholic liver diseases," *The Cochrane Database of Systematic Reviews*, no. 2, Article ID CD002235, 2006.

[25] M. Cave, I. Deaciuc, C. Mendez, Z. Song, S. Joshi-Barve, and C. McClain, "Nonalcoholic fatty liver disease: predisposing factors and the role of nutrition," *The Journal of Nutritional Biochemistry*, vol. 18, no. 3, pp. 184–195, 2007.

[26] F. Lirussi, L. Azzalini, S. Orando, R. Orlando, and F. Angelico, "Antioxidant supplements for non-alcoholic fatty liver disease and/or steatohepatitis," *Cochrane Database of Systematic Reviews*, no. 1, Article ID CD004996, 2007.

[27] K. J. Moriarty, H. Platt, S. Crompton et al., "Collaborative care for alcohol-related liver disease," *Clinical Medicine*, vol. 7, no. 2, pp. 125–128, 2007.

[28] Y. Nakaya, K. Okita, K. Suzuki et al., "BCAA-enriched snack improves nutritional state of cirrhosis," *Nutrition*, vol. 23, no. 2, pp. 113–120, 2007.

[29] Y. Urata, K. Okita, K. Korenaga, K. Uchida, T. Yamasaki, and I. Sakaida, "The effect of supplementation with branched-chain amino acids in patients with liver cirrhosis," *Hepatology Research*, vol. 37, no. 7, pp. 510–516, 2007.

[30] S. Luper, "A review of plants used in the treatment of liver disease: part 1," *Alternative Medicine Review*, vol. 3, no. 6, pp. 410–421, 1998.

[31] L. Langmead and D. S. Rampton, "Review article: herbal treatment in gastrointestinal and liver disease—benefits and dangers," *Alimentary Pharmacology & Therapeutics*, vol. 15, no. 9, pp. 1239–1252, 2001.

[32] A. Sharma, M. S. Sharma, A. Mishra, S. Sharma, B. Kumar, and A. Bhandari, "Review on Thar plants used in liver diseases," *International Journal on Research Methodologies in Physics and Chemistry*, vol. 1, no. 2, pp. 224–236, 2011.

[33] A. Del Prete, A. Scalera, M. D. Iadevaia et al., "Herbal products: benefits, limits, and applications in chronic liver disease," *Evidence-Based Complementary and Alternative Medicine*, vol. 2012, Article ID 837939, 19 pages, 2012.

[34] H. A. Salmi and S. Sarna, "Effect of silymarin on chemical, functional, and morphological alterations of the liver. A double-blind controlled study," *Scandinavian Journal of Gastroenterology*, vol. 17, no. 4, pp. 517–521, 1982.

[35] K. Hruby, G. Csomos, M. Fuhrmann, and H. Thaler, "Chemotherapy of *Amanita phalloides* poisoning with intravenous silibinin," *Human Toxicology*, vol. 2, no. 2, pp. 183–195, 1983.

[36] J. C. Trinchet, T. Coste, V. G. Lévy et al., "Treatment of alcoholic hepatitis with silymarin. A double-blind comparative study in 116 patients," *Gastroentérologie Clinique et Biologique*, vol. 13, no. 2, pp. 120–124, 1989.

[37] P. Ferenci, B. Dragosics, H. Dittrich et al., "Randomized controlled trial of silymarin treatment in patients with cirrhosis of the liver," *Journal of Hepatology*, vol. 9, no. 1, pp. 105–113, 1989.

[38] A. Parés, R. Planas, M. Torres et al., "Effects of silymarin in alcoholic patients with cirrhosis of the liver: results of a controlled, double-blind, randomized and multicenter trial," *Journal of Hepatology*, vol. 28, no. 4, pp. 615–621, 1998.

[39] H. Oka, S. Yamamoto, T. Kuroki et al., "Prospective study of chemoprevention of hepatocellular carcinoma with sho-saiko-to (TJ-9)," *Cancer*, vol. 76, no. 5, pp. 743–749, 1995.

[40] P. S. Venkateswaran, I. Millman, and B. S. Blumberg, "Effects of an extract from *Phyllanthus niruri* on hepatitis B and woodchuck hepatitis viruses: in vitro and in vivo studies," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 84, no. 1, pp. 274–278, 1987.

[41] J. Liu, H. Lin, and H. McIntosh, "Genus Phyllanthus for chronic hepatitis B virus infection: a systematic review," *Journal of Viral Hepatitis*, vol. 8, no. 5, pp. 358–366, 2001.

[42] Y. Xia, H. Luo, J. P. Liu, and C. Gluud, "Phyllanthus species for chronic hepatitis B virus infection," *Cochrane Database of Systematic Reviews*, vol. 4, Article ID CD008960, 2011.

[43] Y. Xia, H. Luo, J. P. Liu, and C. Gluud, "Phyllanthus species versus antiviral drugs for chronic hepatitis B virus infection," *Cochrane Database of Systematic Reviews*, no. 4, Article ID CD009004, 2013.

[44] S. Zhou, C. Xu, N. Zhou et al., "Mechanism of protective action of *Phyllanthus urinaria* L. against injuries of liver cells," *China Journal of Chinese Materia Medica*, vol. 22, no. 2, pp. 109–111, 1997.

[45] W. W. Fleig, M. Y. Morgan, and M. A. Hölzer, "The ayurvedic drug LIV 52 in patients with alcoholic cirrhosis. Results of a prospective, randomized, double-blind, placebo-controlled clinical trial," *Journal of Hepatology*, vol. 26, supplement 1, p. 127, 1997.

[46] E. P. Sabina, A. D. H. Souriyan, D. Jackline, and M. K. Rasool, "Piperine, an active ingredient of black pepper attenuates acetaminophen-induced hepatotoxicity in mice," *Asian Pacific Journal of Tropical Medicine*, vol. 3, no. 12, pp. 971–976, 2010.

[47] B. L. Pandey and P. K. Das, "Immunopharmacological studies on Picrorhiza kurroa royle-ex-benth part IV: cellular mechanisms of anti-inflammatory action," *Indian Journal of Physiology and Pharmacology*, vol. 33, no. 1, pp. 28–30, 1989.

[48] G. L. Floersheim, A. Bieri, R. Koenig, and A. Pletscher, "Protection against *Amanita phalloides* by the iridoid glycoside mixture of *Picrorhiza kurroa* (kutkin)," *Agents and Actions*, vol. 29, no. 3-4, pp. 386–387, 1990.

[49] B. Shukla, P. K. S. Visen, G. K. Patnaik, and B. N. Dhawan, "Choleretic effect of picroliv, the hepatoprotective principle of *Picrorhiza kurroa*," *Planta Medica*, vol. 57, no. 1, pp. 29–33, 1991.

[50] R. Rastogi, S. Saksena, N. K. Garg, N. K. Kapoor, D. P. Agarwal, and B. N. Dhawan, "Picroliv protects against alcohol-induced chronic hepatotoxicity in rats," *Planta Medica*, vol. 62, no. 3, pp. 283–285, 1996.

[51] A. B. Vaidya, D. S. Antarakar, J. C. Doshi et al., "*Picrorhiza kurroa* Royle-ex-Benth as a hepatoprotective agent—experimental and clinical studies," *Journal of Postgraduate Medicine*, vol. 42, pp. 105–108, 1996.

[52] R. H. Singh and K. N. Udupa, "Studies on the Indian indigenous drug Punarnava (*Boerhaavia diffusa* L.) Part IV. Preliminary controlled clinical trial in nephrotic syndrome," *Journal of Research in Indian Medicine*, vol. 7, no. 3, pp. 28–33, 1972.

[53] K. A. Manu and G. Kuttan, "Punarnavine induces apoptosis in B16F-10 melanoma cells by inhibiting NF-κB signaling," *Asian Pacific Journal of Cancer Prevention*, vol. 10, no. 6, pp. 1031–1038, 2009.

[54] K. A. Manu and G. Kuttan, "Immunomodulatory activities of Punarnavine, an alkaloid from *Boerhaavia diffusa*," *Immunopharmacology and Immunotoxicology*, vol. 31, no. 3, pp. 377–387, 2009.

[55] M. T. Olaleye, A. C. Akinmoladun, A. A. Ogunboye, and A. A. Akindahunsi, "Antioxidant activity and hepatoprotective property of leaf extracts of *Boerhaavia diffusa* Linn against acetaminophen-induced liver damage in rats," *Food and Chemical Toxicology*, vol. 48, no. 8-9, pp. 2200–2205, 2010.

[56] M. Ramamurthy and M. Srinivasan, "Hepatoprotective effect of *Tephrosia purpurea* in experimental animals," *Indian Journal of Pharmacology*, vol. 25, no. 1, pp. 34–36, 1993.

[57] A. Khatri, A. Garg, and S. S. Agrawal, "Evaluation of hepatoprotective activity of aerial parts of *Tephrosia purpurea* L. and stem bark of *Tecomella undulata*," *Journal of Ethnopharmacology*, vol. 122, no. 1, pp. 1–5, 2009.

[58] C. K. Chauhan, S. A. Nanivadekar, and F. R. Billimoria, "Effect of a herbal hepatoprotective product on drug metabolism in patients of cirrhosis and hepatic enzyme function in experimental liver damage," *Indian Journal of Pharmacology*, vol. 24, no. 2, pp. 107–110, 1992.

[59] K. Prabu, N. Kanchana, and A. M. Sadiq, "Hepatoprotective effect of *Eclipta alba* on paracetamol induced liver toxicity in rats," *Journal of Microbiology and Biotechnology Research*, vol. 1, no. 3, pp. 75–79, 2011.

[60] K. Ma-Ma, N. Nyunt, and K. M. Tin, "The protective effect of Eclipta alba on carbon tetrachloride-induced acute liver damage," *Toxicology and Applied Pharmacology*, vol. 45, no. 3, pp. 723–728, 1978.

[61] R. Rohilla, T. Garg, A. K. Goyal, and G. Rath, "Herbal and polymeric approaches for liver-targeting drug delivery: novel strategies and their significance," *Drug Delivery*, pp. 1–17, 2014.

[62] S. Palbag, B. K. Dey, and N. K. Singh, "Ethnopharmacology, phytochemistry and pharmacology of *Tephrosia purpurea*," *Chinese Journal of Natural Medicines*, vol. 12, no. 1, pp. 1–7, 2014.

[63] D. Manvar, M. Mishra, S. Kumar, and V. N. Pandey, "Identification and evaluation of anti Hepatitis C virus phytochemicals from *Eclipta alba*," *Journal of Ethnopharmacology*, vol. 144, no. 3, pp. 545–554, 2012.

[64] A. C. Donepudi, L. M. Aleksunes, M. V. Driscoll, N. P. Seeram, and A. L. Slitt, "The traditional ayurvedic medicine, *Eugenia jambolana* (Jamun fruit), decreases liver inflammation, injury and fibrosis during cholestasis," *Liver International*, vol. 32, no. 4, pp. 560–573, 2012.

[65] R. K. Dhiman and Y. K. Chawla, "Herbal medicines for liver diseases," *Digestive Diseases and Sciences*, vol. 50, no. 10, pp. 1807–1812, 2005.

[66] J. C. Patel, "Cirrhosis of liver with ascites treatment based on principles of ayurved," *Indian Journal of Medical Sciences*, vol. 55, no. 9, pp. 481–482, 2001.

[67] C. M. Witt, A. Michalsen, S. Roll et al., "Comparative effectiveness of a complex Ayurvedic treatment and conventional standard care in osteoarthritis of the knee—study protocol for a randomized controlled trial," *Trials*, vol. 14, no. 1, article 149, 2013.

[68] R. K. Sharma and B. Dash, in *Agnivesha's Caraka Samhita*, vol. 3, chapter 1, no. 32–40, pp. 46–47, Chowkhamba Sanskrit Series Office, Varanasi, India, 4th edition, 2000.

[69] R. K. Sharma and B. Dash, in *Agnivesha's Caraka Samhita*, vol. 3, chapter 13, no. 47, p. 535, Chowkhamba Sanskrit Series Office, Varanasi, India, 4th edition, 2000.

[70] R. K. Sharma and B. Dash, *Agnivesha's Caraka Samhita*, Text with English Translation, Volume 3, Chowkhamba Sanskrit Series Office, Varanasi, India, 4th edition, chapter 13, no. 38, p. 531, 2000.

[71] R. K. Sharma and B. Dash, *Agnivesha's Caraka Samhita*, Text with English Translation, Volume 3, Chowkhamba Sanskrit

Series Office, Varanasi, India, 4th edition, chapter 13, no. 78, pp. 543-544, 2000, vol. 1, no. 3.36–40, pp. 47-48, 2000.

[72] R. K. Sharma and B. Dash, *Agnivesha's Caraka Samhita*, Text with English Translation, Volume 3, Chowkhamba Sanskrit Series Office, Varanasi, India, 4th edition, chapter 13, no. 77, pp. 543-544, 2000.

[73] R. K. Sharma and B. Dash, *Agnivesha's Caraka Samhita*, Text with English Translation, Volume 3, Chowkhamba Sanskrit Series Office, Varanasi, India, 4th edition, chapter 13, no. 107–194, pp. 551–571, 2000.

[74] G. Sen, *Bhaishajya ratnavali*, Chaukhambha Sanskrit Sansthan, Udara Roga Adhikara, 11th edition, pp. 43-44, 1993, edited by: B. S. Mishra.

[75] Y. T. Achary, *Siddha Yoga Sangraha*, Shree Baidyanath Ayurved, Nagpur, India, 11th edition; vol. 18, no. 2, p. 98, 2000.

[76] C. Kessler, M. Wischnewsky, A. Michalsen, C. Eisenmann, and J. Melzer, "Ayurveda: between religion, spirituality, and medicine," *Evidence-Based Complementary and Alternative Medicine*, vol. 2013, Article ID 952432, 11 pages, 2013.

[77] R. B. Saper, S. N. Kales, J. Paquin et al., "Heavy metal content of ayurvedic herbal medicine products," *Journal of the American Medical Association*, vol. 292, no. 23, pp. 2868–2873, 2004.

[78] R. B. Saper, R. S. Phillips, A. Sehgal et al., "Lead, mercury, and arsenic in US- and Indian-manufactured Ayurvedic medicines sold via the internet," *The Journal of the American Medical Association*, vol. 300, no. 8, pp. 915–923, 2008.

[79] WHO, http://apps.who.int/medicinedocs/documents/s17552en/s17552en.pdf.

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Research Article

Silymarin Accelerates Liver Regeneration after Partial Hepatectomy

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Partial hepatectomy (PHx) is a liver regeneration physiological response induced to maintain homeostasis. Liver regeneration evolved presumably to protect wild animals from catastrophic liver loss caused by toxins or tissue injury. Silymarin (Sm) ability to stimulate liver regeneration has been an object of curiosity for many years. Silymarin has been investigated for use as an antioxidant and anticarcinogen. However, its use as a supportive treatment for liver damage is elusive. In this study, we fed silymarin (Sm, 25 mg/kg) to male Sprague-Dawley rats for 7 weeks. Surgical 2/3 PHx was then conducted on the rats at 6 hrs, 24 hrs, and 72 hrs. Western blot and RT-PCR were conducted to detect the cell cycle activities and silymarin effects on hepatic regeneration. The results showed that silymarin enhanced liver regeneration by accelerating the cell cycle in PHx liver. Silymarin led to increased G1 phase (cyclin D1/pRb), S phase (cyclin E/E2F), G2 phase (cyclin B), and M phase (cyclin A) protein and mRNA at 6 hrs, 24 hrs, and 72 hrs PHx. HGF, TGFα, and TGFβ1 growth factor expressions were also enhanced. We suggest that silymarin plays a crucial role in accelerated liver regeneration after PHx.

1. Introduction

Liver tissue presents excellent recovery ability from surgical or toxic injury. Hepatocyte regeneration is the ability to regenerate through a compensatory growth process and then return to a nonproliferative state. We know that most liver cancer patients must receive partial liver surgical resection to protect their remaining liver function. After surgery, the hepatocytes regenerate by increasing their cell numbers. The native hepatocyte function cannot maintain the integrated whole liver function. Many hepatic liver regeneration growth drugs have been tested in partially hepatectomized (PHx) animals. Silymarin is a flavonoid complex extracted from Milk Thistle plant seeds (*Silybum marianum*). Milk Thistle seeds have been used as a medicine for centuries to treat liver diseases and they are still used today. At the turn of

the 20th century, medical herbalists were using silymarin for its hepatoprotective and regeneration properties. Clinical studies have shown that silymarin (Sm) can halt and even reverse early stage liver disease in recovering alcoholics. It has also been shown to be successful in treating hepatitides B and C [1–3]. Silymarin has anti-inflammatory properties resulting from inhibiting cytokines production. Silymarin in turn increases liver regeneration and the formation of new hepatocytes. However, whether silymarin can help DNA replication and mitosis in regenerative cells is still unknown. We do know that it is absorbed in the intestine, concentrates in the bile, and probably has an enterohepatic circulation that continues to recycle through the liver [4–6]. Partial hepatectomy (PHx) triggers hepatocyte proliferation-mediated liver repair and is used widely to study the mechanisms governing liver regeneration. When injured, the liver "knows" how to accurately regulate its mass by growing and when to stop. Excessive liver mass is regulated by apoptosis. The quiescent hepatocyte regulates the disorder phenomena in the liver to maintain mass and function to protect animals in the wild from the catastrophic effects of food toxins [7–9]. There are interesting differences in the timing of cell cycle progression between partial hepatectomy and silymarin treatment after PHx. Despite being very well described, the reasons for these differences remain unclear. It is important to determine the hepatocyte replication role in liver regeneration. Based on the knowledge of liver regulation and regeneration, several growth factors are required to balance between cell cycle stimulator and inhibitor genes expressed after PHx. This may explain the liver regeneration growth process regulation [10]. We suggest that silymarin may act as a cell progression agent to induce the cell cycle progress to rebuild the lost hepatic tissue. Silymarin may enhance three available growth factors (HGF, TGFα, and TGFβ1), acting as a cell cycle progression agent that triggers liver regeneration to protect the liver as a hepatoprotective agent for liver disease. HGF is a mitogenic agent for many different cell types with major effects on liver hepatic growth [11–13]. Matrix breakdown may cause a rapid release of HGF into the plasma, which occurs shortly after PHx [14]. TGFβ1 is known to be involved in the activation of a proteolytic cascade with increased proteolysis of some hepatic biomatrix components [15]. HGF and TGFα may have paracrine effects on regenerating hepatocytes [16, 17]. HGF, TGFα, and TGFβ1 may stimulate DNA synthesis in hepatocytes, changing the cell from proliferation or death during regeneration [18, 19]. Almost immediately, we expect major changes in the complete mitogens expression for hepatocytes and in the expression of a relatively large number of genes, after silymarin treatment [20, 21].

2. Materials and Methods

2.1. Animals and Treatments. Sprague-Dawley rats were obtained from the Animal House of the National Science Council in Taiwan. The rats were housed in an environmentally controlled room, at 22 ± 5°C temperature, with relative humidity of about 60%. The animals were provided with standard food pellets and tap water. All rats were acclimatized for 1 week prior to the beginning of all experiments.

2.2. Preparation of Hot-Water Extract Hepatoprotective Herbal Drug, Silymarin, from Milk Thistle. The hot-water extract was prepared by boiling Milk Thistle (*Silybum marianum L.*) seed meal with distilled water for 1 hr in 100°C water. The use of hot water as an extraction solvent for Milk Thistle at temperatures above 100°C was explored. The compounds yield necessary to reach their maxima was reduced from 200 to 55(%), when the extraction temperature was increased from 100 to 140°C. The extract was filtered, freeze-dried, and kept at 4°C. The silymarin (Sm) extraction yield was 6.8 mg/g (22-23). The dried extract was dissolved in distilled water before use. Silymarin (Sm, 25 mg/kg) oral gavages at the end of the 7 weeks experiment occurred.

2.3. Experimental Partial Hepatectomy (PHx) and Sham Groups. Three randomly selected animals were used for each time point. After injecting ketamine subcutaneously at a dose of 30 mg/kg, liver resections consisting of 70%~85% (2/3) of the liver mass were performed in the partial hepatectomy group. Animals were anesthetized for the partial hepatectomy (PHx) using isoflurane inhalation. After 70% of hepatectomy procedure completion, the animals were administered buprenex (0.3 mg/kg in 3 mL NaCl) and then placed under a lamp to prevent hypotermy. Upon awakening, the animals were then placed into cages. The animals in the PHx and corresponding group were sacrificed at 6 hrs, 24 hrs, and 72 hrs after the operation. The animal group in which no surgery was performed was used as the control liver group and referred to as the time "0" group in the quantitated graphs. All animals were sacrificed by cervical dislocation. The remnant liver lobes were excised and washed in PBS and then immediately frozen in liquid nitrogen.

2.4. Western Blot Analysis. Liver extracts (20 μg) were run on by 12% SDS-PAGE gel for 90 min, at 100 V, and then transferred to polyvinylidene difluoride (PVDF) (Hybond-C Extra Supported, 0.45 μm; Amersham, Piscataway, NJ, USA) membranes. Membranes were blocked in 5% nonfat milk (diluted in Tris-buffered saline and 0.1% Tween 20) for 30 min and probed with the appropriate primary antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) against HGF, TGFα, TGFβ1, cyclin D1, pRb, cyclin E, E2F, cyclin A, and cyclin A at 4°C overnight and then incubated with HRP-conjugated secondary antibody (Promega, Madison, WI, USA). After extensive washing, the targeted proteins were detected using an enhanced chemiluminescence system (ECL).

2.5. Reverse Transcriptase PCR (RT-PCR). 0.5 μg of the total RNA was derived from liver plus primers using RT-PCR. The first-strand synthesis kit was applied according to the manufacturer's instructions; the PCR primers included (1) TGFβ1: forward primer of TGFβ1 (5′AGGAGACCATTC-CCCTGACT3′); reverse primer of TGFβ1 (5′TTCTTCCTC-CACTTCCCCTT3′), (2) cyclin B: forward primer of cyclin B (5′ACCTACAGTGAAGATGCACACC3′), reverse primer of cyclin B (5′CCTGTAGTTCTTGTTTCCTGCAC3′), (3)

Table 1: Liver regeneration after PHx.

	6 hrs	24 hrs	72 hrs	168 hrs
Animal number (N)	6	6	8	10
Body weight (g)	236.7 ± 23.59	259.2 ± 10.68	234.4 ± 19.16	216.7 ± 11.25
p value versus 6 hrs		$p = 0.059$	$p = 0.844$	$p = 0.0314$
Postoperative liver weight (g)	3.7 ± 0.67	5.3 ± 0.36	8.7 ± 1.71	10.8 ± 0.62
p value versus 6 hrs		$p < 0.001$	$p < 0.0001$	$p < 0.0001$
Partial liver weight (g)	6.2 ± 0.94	6.9 ± 0.45	7.7 ± 0.63	5.8 ± 0.60
p value versus 6 hrs		$p = 0.086$	$p = 0.004$	$p = 0.309$
Remnant liver weight (g)	4.7 ± 0.99	4.7 ± 0.46	2.8 ± 0.64	3.9 ± 0.47
p value versus 6 hrs		$p = 0.934$	$p = 0.001$	$p = 0.058$
Liver regeneration (%)	–19.2	10.62	76.32	119.55

cyclin A: forward primer of cyclin A (5′-GCAGAGTTC-TGATGGAGAGA-3′), reverse primer of cyclin A (5′-ACA-GTCTTGCAGGTGACATC-3′), (4) HGF: forward primer of HGF (5′-CTGCTGCAGGAGACCATGTA-3′), reverse primer of HGF (5′-CTCCGTGTGGGACAGGTAGT-3′), and (5) GAPDH: forward primer of GAPDH (5′-GGGTGT-GAACCACGAGAAAT-3′), reverse primer of GAPDH (5′-CCACAGTCTTCTGAGTGGCA-3′). The RT-PCR results were analyzed based on the assessment of product sizes upon ethidium bromide-agarose gel electrophoresis. For each gene, we determined the cycle number of PCR reactions in which the PCR reaction was not saturated. The following PCR conditions were used: the initial denaturation step was conducted at 95°C, with the annealing temperature and extension at 72°C. The final extension was conducted at 72°C for 10 minutes. These conditions were applied to all reactions and the PCR products were electrophoresed on 1.2% agarose gel.

2.6. Immunofluorescence Analysis. Liver tissue sections were washed with 4% paraformaldehyde for 10 min and permeated using 0.01% Triton X-100, followed by blocking with 5% BSA in PBS for 30 min at room temperature. Sections were incubated with antibody Ki-67 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at room temperature for 1 hr. Following a wash in PBS, sections were incubated for 1 hr at room temperature in FITC-conjugated rabbit anti-mouse secondary antibody diluted 1 : 500 in PBS. The images were captured using a Leica DMI400B inverted Fluorescence microscope linked to camera.

2.7. Immunohistochemistry Assay. Liver tissues were fixed in 10% buffered formalin. Fixed samples were embedded in paraffin, sectioned, and stained with hematoxylin and eosin (H&E) for histological examination. To identify cytoplasmic lipid vacuoles, 5 μm thick sections were prepared from frozen tissues and incubated with BrdU. DNA synthesis was determined by counting BrdU-positive nuclei. The labeling was expressed as the total cells counted. Immunohistochemistry was performed using mouse anti-BrdU (Santa Cruz Diagnostics, Santa Cruz, CA) as antibody, using DAB stained to detect the targeted antibody expression.

2.8. Statistical Analysis. All data are expressed as the means ± S.E. For western blot and RT-PCR analysis, quantitation was carried out by scanning and analyzing the intensity of the hybridization signals using the FUJIFILM Imagine program. Statistical data analysis was performed using SigmaStat software. Student's t-test comparison was made when PHx and PHx + Sm rats are being compared against the sham group. Comparison between PHx and PHx + Sm was also made using Student's t-test. A p value of less than 0.05 and 0.01 was considered statistically significant. A two-way ANOVA was used when 24 hrs sham and 72 hrs sham rats were compared against 6 hrs sham rats and when 24 hrs PHx and 72 hrs PHx rats were compared against 6 hrs PHx rats, and comparison between 24 hrs PHx and 72 hrs PHx was made. A p value of less than 0.05 and 0.01 was considered to be statistically significant.

3. Results

During regeneration after 70% hepatectomy, the liver was divided one or two times and began to regenerate and then return to quiescence. We know that liver regeneration is a physiological response induced for maintaining homeostasis. According to Table 1, the result showed us the postoperative liver weight (g) which increased from 3.7 ± 0.67 (6 hrs PHx) to 5.3 ± 0.36 (24 hrs PHx) ($p < 0.001$ versus 6 hrs PHx), to 8.7 ± 1.71 (72 hrs PHx) ($p < 0.0001$ versus 6 hrs PHx), and to 10.8 ± 0.62 (168 hrs PHx) ($p < 0.0001$ versus 6 hrs PHx). Partial liver weight (g) at 72 hrs PHx has significantly increased but decreased at 168 hrs. In turn, the remnant liver weight (g) also decreased at 72 hrs PHx, from 4.7 ± 0.99 (at 6 hrs PHx) to 2.8 ± 0.64 (at 72 hrs PHx, $p < 0.001$ versus at 6 hrs PHx), but with no significant difference at 168 hrs. Thus, the liver regeneration (%) increased from 10.62% at 24 hrs to 76.32% at 72 hrs and from the highest to 119.55% at 168 hrs PHx. Given the above evidence, liver regeneration is time dependent. Partial hepatectomy (PHx) is a complex physiological response that takes place after the loss of hepatocytes caused by viral injury or secondary liver resection. During liver regeneration, a series of resections take place to maintain homeostasis to restore normal hepatic mass and structure. Virtually, all of the surviving hepatocytes undergo cellular proliferation due to tissue remodeling. PHx

is a delayed physiological response during liver regeneration. We want to determine whether silymarin will accelerate the cell cycle to return to normal conditions during liver regeneration. Therefore, we detected cell cycle check proteins, cyclin D1/pRb in G1 phase and cyclin E/E2F in S phase, in sham, PHx, and silymarin treatment with PHx by western blot analysis. After partial hepatectomy, liver regeneration began to proliferate and cell cycle prolonged. We found G1 phase extended, from 6 hrs to 24 hrs and into S phase at 72 hrs during liver regeneration. Therefore, we could find cyclin D1 and pRb protein expression increased at 6 hrs PHx, but not significantly in cyclin E and E2F, when compared with sham, respectively ($^{*}p < 0.05$ versus sham). Partial hepatectomy treatment with silymarin has strongly enhanced cyclin D1, pRb, cyclin E, and E2F protein expression levels ($^{*}p < 0.05$ versus sham, $^{\#}p < 0.05$ versus PHx) (Figure 1(a)). After 24 hrs of liver regeneration, we found the strongest regeneration; cyclin D1, pRb, cyclin E, and E2F protein expression all were increased after PHx. We also could find silymarin improved this stage ($^{*}p < 0.05$ versus sham, $^{\#}p < 0.05$ versus PHx) (Figure 1(b)). On the other hand, during long term 72 hrs PHx, cyclin D1, and pRb protein expression were decreased compared with sham, respectively. Silymarin also was induced ($^{*}p < 0.05$ versus sham, $^{\#\#}p < 0.05$ versus PHx). In turn, cyclin E and E2F had increased. During this time, silymarin may has been losing functions to improve cell cycle, when compared with PHx ($^{\#\#}p < 0.05$ versus PHx); however, compared with the sham, silymarin also increased ($^{*}p < 0.05$ versus sham) (Figure 1(c)). After hepatic growth and restructuring, DNA synthesis was completed by 72 hrs and liver regeneration eventually stops. We focused on liver regeneration initiation and compared termination with it. Thus, we examined the cell cycle check point protein at different times. We examined cyclin D1/pRb in G1 phase and cyclin E/E2F in S phase protein expression by western blot analysis (Figures 2(a) and 2(b)). We found cyclin D1 was decreased at 24 hrs sham but increased at 72 hrs ($^{a}p < 0.05$ versus 6 hrs sham, $^{b}p < 0.05$ versus 24 hrs sham). When compared with PHx at 6 hrs, 24 hrs, and 72 hrs, we found cyclin D1 at 24 hrs PHx was increased ($^{c}p < 0.05$ versus 6 hrs PHx) but at 72 hrs PHx was decreased ($^{c}p < 0.05$ versus 6 hrs PHx, $^{d}p < 0.05$ versus 24 hrs PHx). However, pRb protein expression levels were increased only at 72 hrs sham ($^{a}p < 0.05$ versus 6 hrs sham, $^{b}p < 0.05$ versus 24 hrs sham). However, after 24 hrs PHx, pRb protein expression was increased ($^{c}p < 0.05$ versus 6 hrs PHx). After 72 hrs PHx, pRb was decreased ($^{d}p < 0.05$ versus 24 hrs PHx). No significant difference compared with 6 hrs PHx was shown. Notwithstanding, silymarin also enhanced cyclin D1/pRb protein expression at all the three PHx times, 6 hrs, 24 hrs, and 72 hrs PHx, respectively ($^{*}p < 0.05$ versus sham, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ versus PHx) (Figures 1 and 2(a)). On the other hand, in cell cycle S phase, we found cyclin E was increased at 24 hrs sham ($^{a}p < 0.05$ versus 6 hrs sham), but at 72 hrs sham has conversely to 6 hrs sham. After hepatectomy, we found cyclin E protein expression increased at 24 hrs PHx and more at 72 hrs PHx ($^{c}p < 0.05$, $^{cc}p < 0.01$ versus 6 hrs PHx, $^{d}p < 0.05$ versus 24 hrs PHx) (Figure 2(b)). On the other hand, E2F increased expression at 24 hrs and 72 hrs sham compared with 6 hrs sham ($^{a}p < 0.05$, $^{aa}p < 0.01$ versus 6 hrs sham, $^{b}p < 0.05$ versus 24 hrs sham). When compared with 6 hrs PHx, we found E2F protein expression increased at 24 hrs PHx but at 72 hrs PHx has more than 6 hrs and 24 hrs PHx ($^{c}p < 0.05$, $^{cc}p < 0.01$ versus 6 hrs PHx; $^{d}p < 0.05$, $^{dd}p < 0.01$ versus 24 hrs PHx). However, silymarin also has strong functions to induce cyclin E/E2F protein expression after PHx ($^{*}p < 0.05$ versus sham, $^{\#}p < 0.05$, versus PHx) (Figures 1 and 2(b)). Therefore, we could determine silymarin stimulated cell cycle protein expression for entry into S phase. After hepatic growth and restructuring, DNA synthesis is mostly complete by 72 hrs and liver regeneration eventually stops. We focused on liver regeneration initiation and compared termination with it. Furthermore, we detected whether silymarin regulated the cell cycle and accelerated it to become normal. We examined G2 and M phase check point protein, cyclin B and cyclin A, protein, and mRNA expression using western and RT-PCR. It is generally believed that the external controls have three important control points in the cell cycle at the end of G2 phase (G2/M transition), in mitosis, and in G1 phase. Intrinsic control mechanisms ensure that the cycle is executed completely. Therefore, we determine whether silymarin also improved the cell cycle at the end of G2 phase. We used western blot analysis and RT-PCR to detect protein and mRNA expression in the sham and PHx to determine the cell cycle. The results showed that the G2 phase check point protein, cyclin B, increased at 24 hrs and 72 hrs in the sham ($^{a}p < 0.05$ versus 6 hrs sham). However, cyclin B at 24 hrs in the PHx decreased compared with the sham at 24 and 6 hrs in the PHx, respectively. After 72 hrs in the PHx, cyclin B increased compared with the PHx at 24 hrs ($^{d}p < 0.05$, versus 24 hrs PHx) (Figure 3(a)). Cyclin B mRNA also decreased at 24 hrs in the PHx but increased at 72 hrs in the PHx (Figures 3(b) and 3(c)). Silymarin presented significant effects at 6 hrs, 24 hrs, and 72 hrs in the PHx to improve G1 phase into M phase. We also detected cyclin A protein and mRNA in the mitosis phase. We found that protein and mRNA expression increased at 24 hrs and 72 hrs in the sham. When compared with the sham, we found that cyclin A protein decreased at 6 hrs and 24 hrs in the PHx, respectively ($^{*}p < 0.05$ versus sham). However, cyclin A mRNA decreased at 24 hrs in the PHx and increased at 72 hrs in the PHx, when compared with 6 hrs PHx (Figures 4(a) and 4(b)). Interestingly, during liver regeneration after PHx, we found cyclin A mRNA and protein expression increased at 72 hrs. PHx led cyclin A expression to delay to 72 hrs (Figure 4(c)). Interestingly, silymarin had no significant beneficial effect on cell cycle functions after 72 hrs PHx. To determine whether growth factors are the primary accelerated cell cycle effects, we examined liver regeneration related growth factors, HGF and TGFα, expression. We found that HGF protein and mRNA expression decreased at 6 hrs in the PHx, but this was not found at 72 hrs in the PHx. We also found that HGF increased after treatment with silymarin comparing the sham and PHx at 6 hrs or 72 hrs (Figures 5(a) and 5(b)). TGFβ1 seems to be inhibited hepatocyte DNA synthesis that is negative regulator of liver growth.

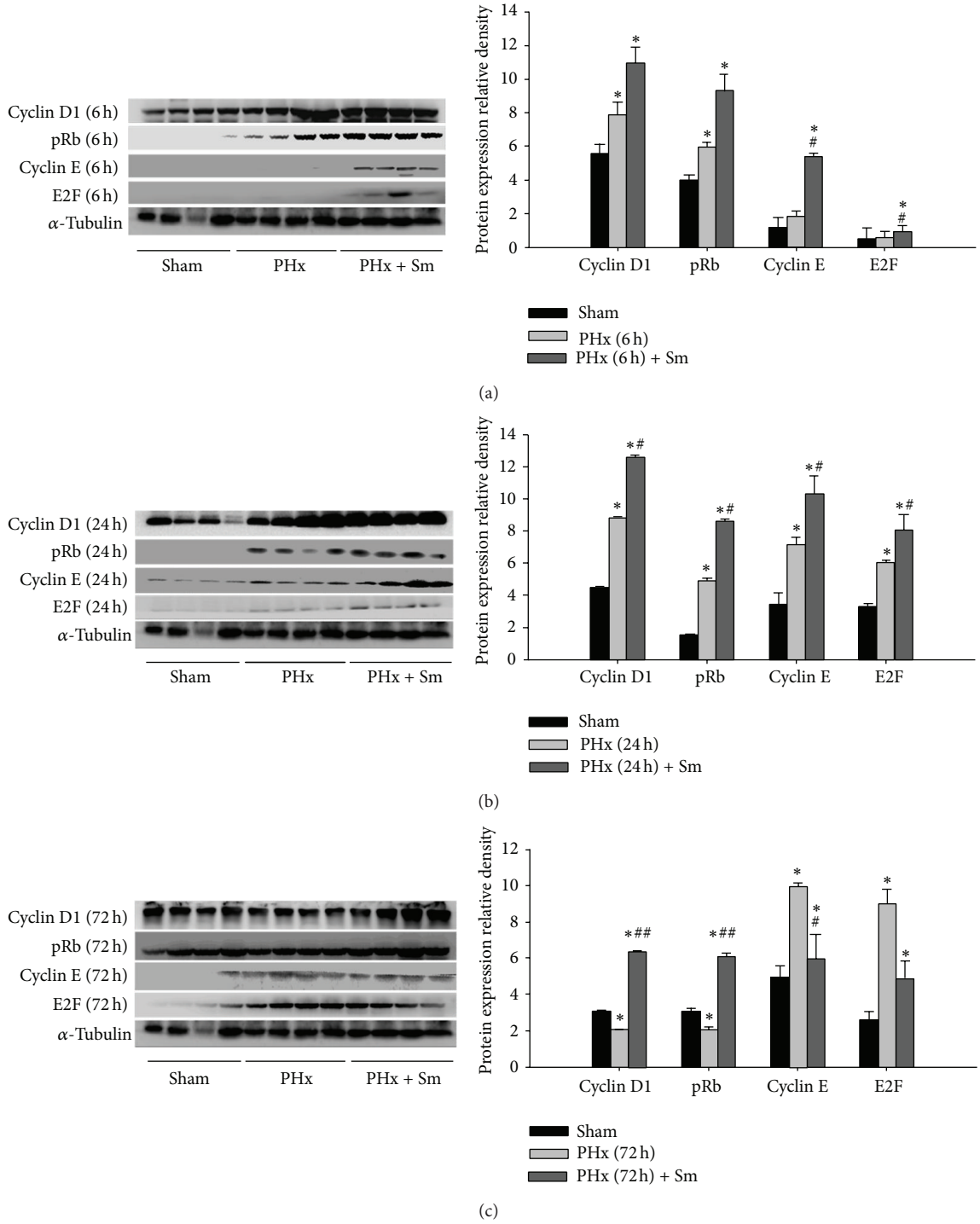

Figure 1: Silymarin accelerates cell cycle from G1 phase to S phase during liver regeneration. Expression protein levels for G1 and S phase checkpoint proteins, cyclin D1, pRb, cyclin E, and E2F in Sham, PHx, and silymarin (Sm) treatment PHx by western blot. (a) At 6 hrs PHx, (b) at 24 hrs PHx, and (c) at 72 hrs PHx. Quantification of densitometry analysis of protein expression levels. All data are presented as means ± SEM, $^{*}p < 0.05$, $^{**}p < 0.01$, as compared with the corresponding sham group. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, as compared with the corresponding PHx. α-Tubulin was used as a loading control for western blotting.

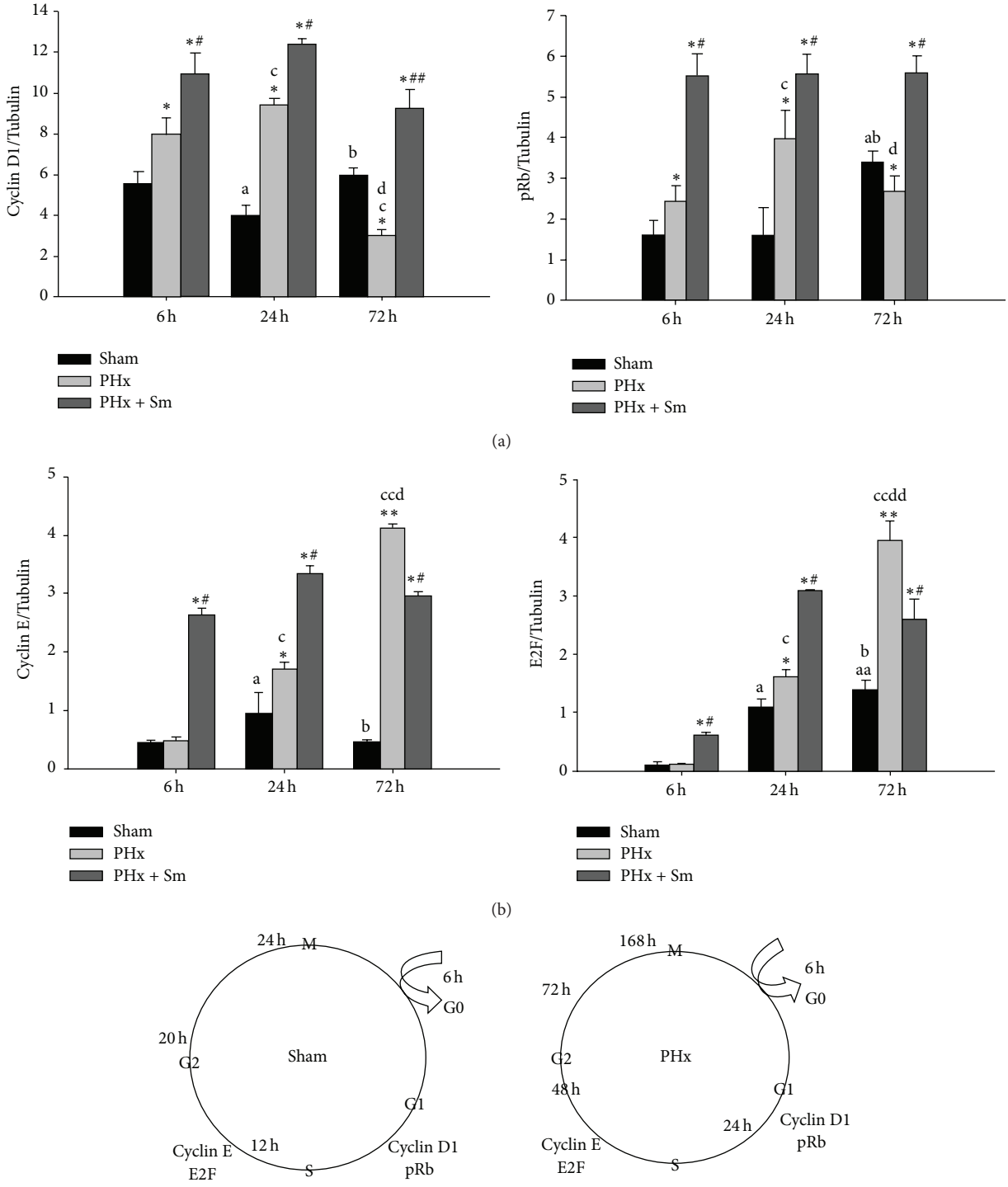

Figure 2: Partial hepatectomy is a delayed cell cycle physiological response. (a) Quantification of western blot densitometry analysis of G1 phase checkpoint proteins, cyclin D1 and pRb, expression levels in Sham, PHx, and silymarin (Sm) treatment partial hepatectomy at 6 hrs, 24 hrs, and 72 hrs. All data are presented as means ± SEM, $^{*}p < 0.05$, as compared with the corresponding sham group. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, as compared with the corresponding PHx. $^{a}p < 0.05$ as compared with 6 hrs sham group. $^{b}p < 0.05$ as compared with 24 hrs sham group. $^{c}p < 0.05$ as compared with 6 hrs PHx group. $^{d}p < 0.05$ as compared with 24 hrs PHx group. (b) Quantification of western blot densitometry analysis of S phase checkpoint proteins, cyclin E and E2F, expression levels in Sham, PHx, and silymarin (Sm) treatment partial hepatectomy at 6 hrs, 24 hrs, and 72 hrs. All data are presented as means ± SEM; $^{*}p < 0.05$, $^{**}p < 0.01$ as compared with the corresponding sham group. $^{\#}p < 0.05$, as compared with the corresponding PHx. $^{a}p < 0.05$, $^{aa}p < 0.01$ as compared with 6 hrs sham group. $^{b}p < 0.05$ as compared with 24 hrs sham group. $^{c}p < 0.05$, $^{cc}p < 0.01$, as compared with 6 hrs PHx group. $^{d}p < 0.05$, $^{dd}p < 0.01$ as compared with 24 hrs PHx group. (c) Representative cell cycle in sham and PHx at 6 hrs, 24 hrs, and 72 hrs three different times.

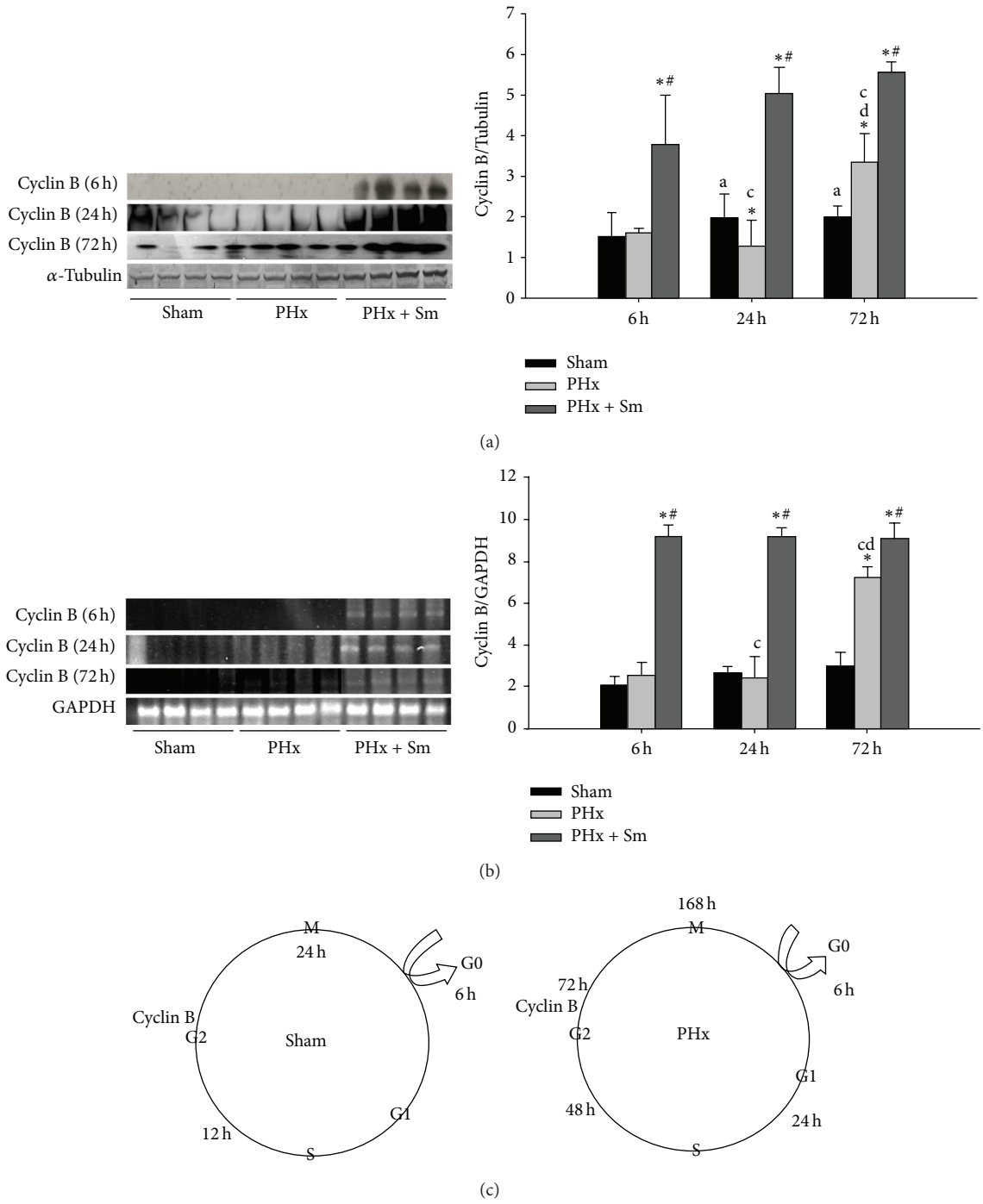

Figure 3: Silymarin accelerates liver regeneration after partial hepatectomy in rats by a mechanism related to G2 phase in cell cycle. (a) Expression protein levels of G2 phase checkpoint protein, cyclin B, were increased in treatment Sm after 6, 24, and 72 hrs of partial hepatectomy (PHx). Quantification of densitometry analysis of protein expression levels. All data are presented as means ± SEM, $^{*}p < 0.05$, as compared with the corresponding sham group. $^{\#}p < 0.05$ as compared with the corresponding PHx. $^{a}p < 0.05$ as compared with 6 hrs sham group. $^{c}p < 0.05$ as compared with 6 hrs PHx group. $^{d}p < 0.05$ as compared with 24 hrs PHx group. α-Tubulin was used as a load control for western blotting. (b) Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis of cyclin B mRNA expression that was increased in treatment Sm after 6, 24, and 72 h PHx. Quantification of densitometry analysis of mRNA expression levels. All data are presented as means ± SEM, $^{*}p < 0.05$, as compared with the corresponding sham group. $^{\#}p < 0.05$ as compared with the corresponding PHx. $^{c}p < 0.05$ as compared with 6 hrs PHx group. $^{d}p < 0.05$ as compared with 24 hrs PHx group. GAPDH was used as a load control for RT-PCR. (c) Representative cell cycle in sham and PHx at various times.

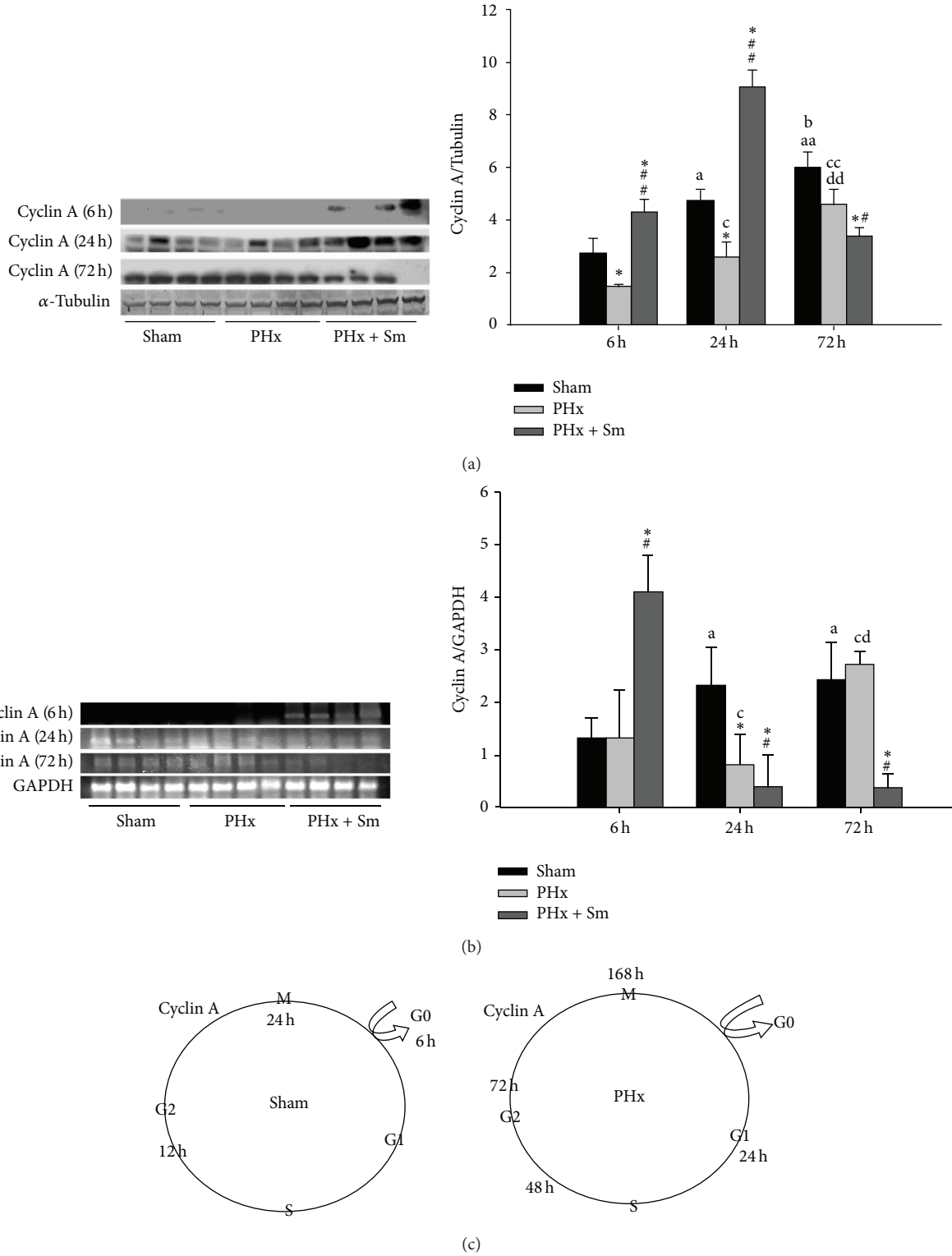

Figure 4: Hepatic expression of cell cycle progression associated with silymarin (Sm) in liver regeneration by western blot and RT-PCR analysis. (a) Silymarin cannot hold cell cycle life in rat liver regeneration after partial hepatectomy in 72 hrs. Equal amounts of proteins lysate were separated by 12.5% SDS-PAGE by western blotting with antibodies to cyclin A. α-Tubulin was used as a load control for western blotting. Quantification of densitometry analysis of protein expression levels. All data are presented as means ± SEM, $^{*}p < 0.05$, as compared with the corresponding sham group. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ as compared with the corresponding PHx. $^{a}p < 0.05$, $^{aa}p < 0.01$ as compared with 6 hrs sham group. $^{b}p < 0.05$ as compared with 24 hrs sham group. $^{c}p < 0.05$, $^{cc}p < 0.01$ as compared with 6 hrs PHx group. $^{d}p < 0.05$, $^{dd}p < 0.01$ as compared with 24 hrs PHx group. (b) RT-PCR cyclin A mRNA expression analysis showed decrease after silymarin treatment after PHx at 24 hrs and 72 hrs. GAPDH was used as a load control for RT-PCR. Quantification of densitometry analysis of mRNA levels. All data are presented as means ± SEM, $^{*}p < 0.05$, as compared with the corresponding sham group. $^{\#}p < 0.05$, as compared with the corresponding PHx. $^{a}p < 0.05$ as compared with 6 hrs sham group. $^{c}p < 0.05$ as compared with 6 hrs PHx group. $^{d}p < 0.05$ as compared with 24 hrs PHx group.

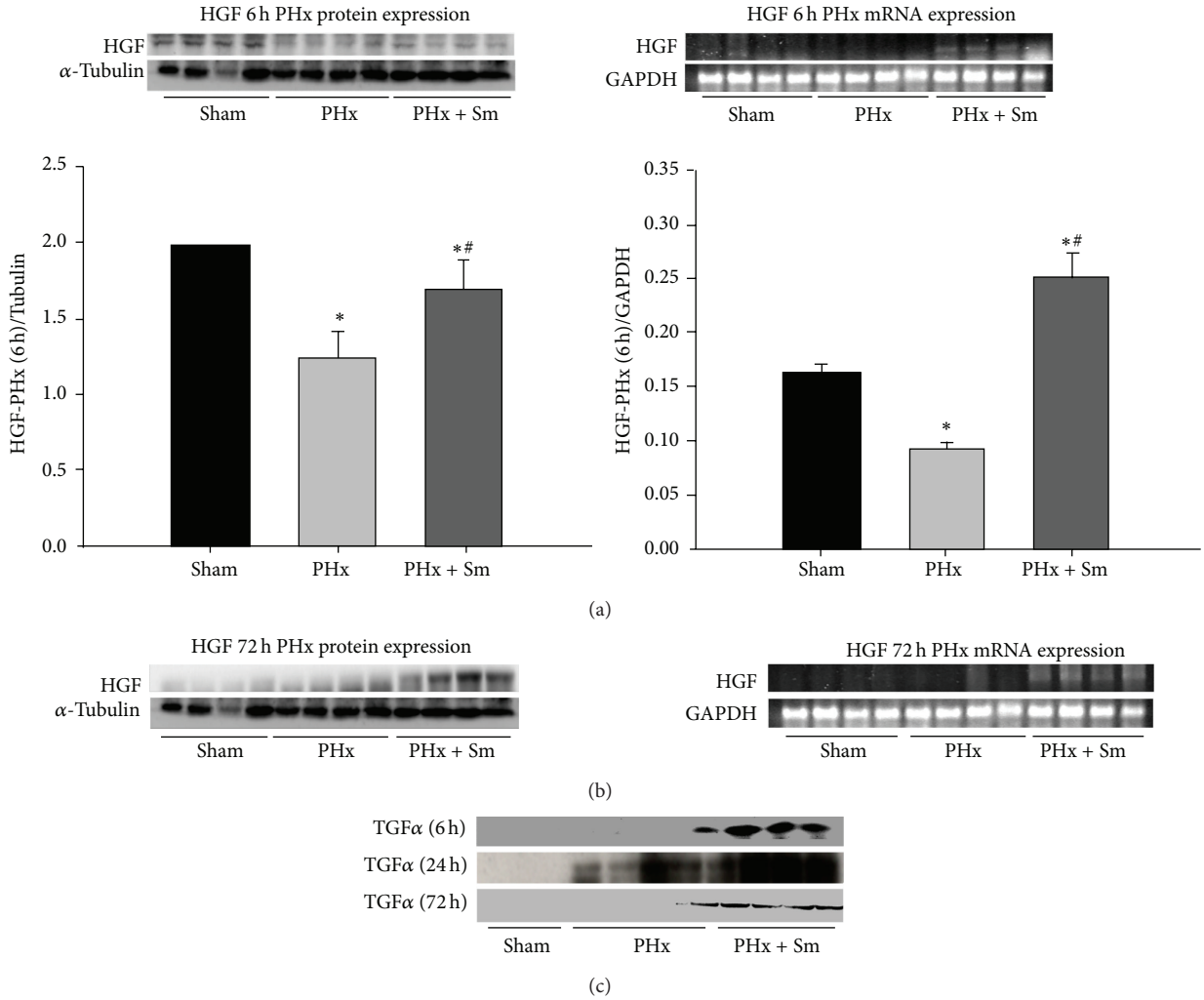

Figure 5: The effect of silymarin (Sm) on hepatocyte proliferation after PHx. (a) Silymarin (Sm) induced HGF protein and mRNA expression at 6 hrs PHx using western blot and RT-PCR analysis. Densitometry analysis quantification of protein and mRNA expression levels; all data are presented as mean ± SEM, $^{*}p < 0.05$ as compared with the corresponding sham group. $^{\#}p < 0.05$, as compared with the corresponding PHx. α-Tubulin was used as a loading control for western blotting. GAPDH was used as a load control for RT-PCR. (b) Silymarin (Sm) induced HGF protein and mRNA expression at 72 hrs PHx using western blot and RT-PCR analysis. Quantification of densitometry analysis of protein and mRNA expression levels; all data are presented as mean ± SEM, $^{*}p < 0.05$, as compared with the corresponding sham group. $^{\#}p < 0.05$ as compared with the corresponding PHx. α-Tubulin was used as a load control for western blotting. GAPDH was used as a loading control for RT-PCR. (c) Silymarin (Sm) induced TGFα protein expression from 6 to 72 h PHx by western blot.

In order to determine whether cell proliferation and cell cycle are coordinately regulated by TGFβ1 in regeneration, the result showed that TGFβ1 protein was not significantly different in sham rats. However, after hepatectomy TGFβ1 protein expression levels increased at 24 hrs PHx ($^{c}p < 0.05$ versus 6 hrs PHx), with the greatest expression at 72 hrs PHx ($^{cc}p < 0.01$ versus 6 hrs PHx, $^{d}p < 0.05$, versus 24 hrs PHx) (Figure 6(a)). On the other hand, mRNA expression increased at 24 hrs and 72 hrs sham (Figure 6(b)). After partial hepatectomy, we also found mRNA expression increased at 24 hrs and 72 hrs PHx ($^{c}p < 0.05$, $^{cc}p < 0.01$ versus 6 hrs PHx; $^{d}p < 0.05$ versus 24 hrs PHx). One possibility is that TGFβ1 is a mitoinhibitor that causes the end of regeneration. Therefore, this pointed out that liver regeneration proceeds to completion. Obviously, hepatocytes proceed through regeneration despite the TGFβ1 increase. A further possibility is TGFβ1 mitoinhibitory effects return by 96 hrs and hepatocyte proliferation stops between 48 and 72 hrs. Resistance to TGFβ1 by regenerating hepatocytes may allow hepatocytes to proliferate even through TGFβ1 concentrations increasing. Silymarin improved TGFβ1 protein and mRNA expression ($^{*}p < 0.05$, $^{**}p < 0.01$ versus sham; $^{\#}p < 0.01$, $^{\#\#}p < 0.05$ versus PHx) (Figure 6). Growth factors in the G1 phase have stronger expression. TGFα presented increased protein expression at 24 hrs in the PHx. This was not found at 6 and 72 hrs. After treatment with silymarin TGFα protein expression increased at 6 hrs, 24 hrs, and 72 hrs in the PHx. We used the Ki-67 antibody to

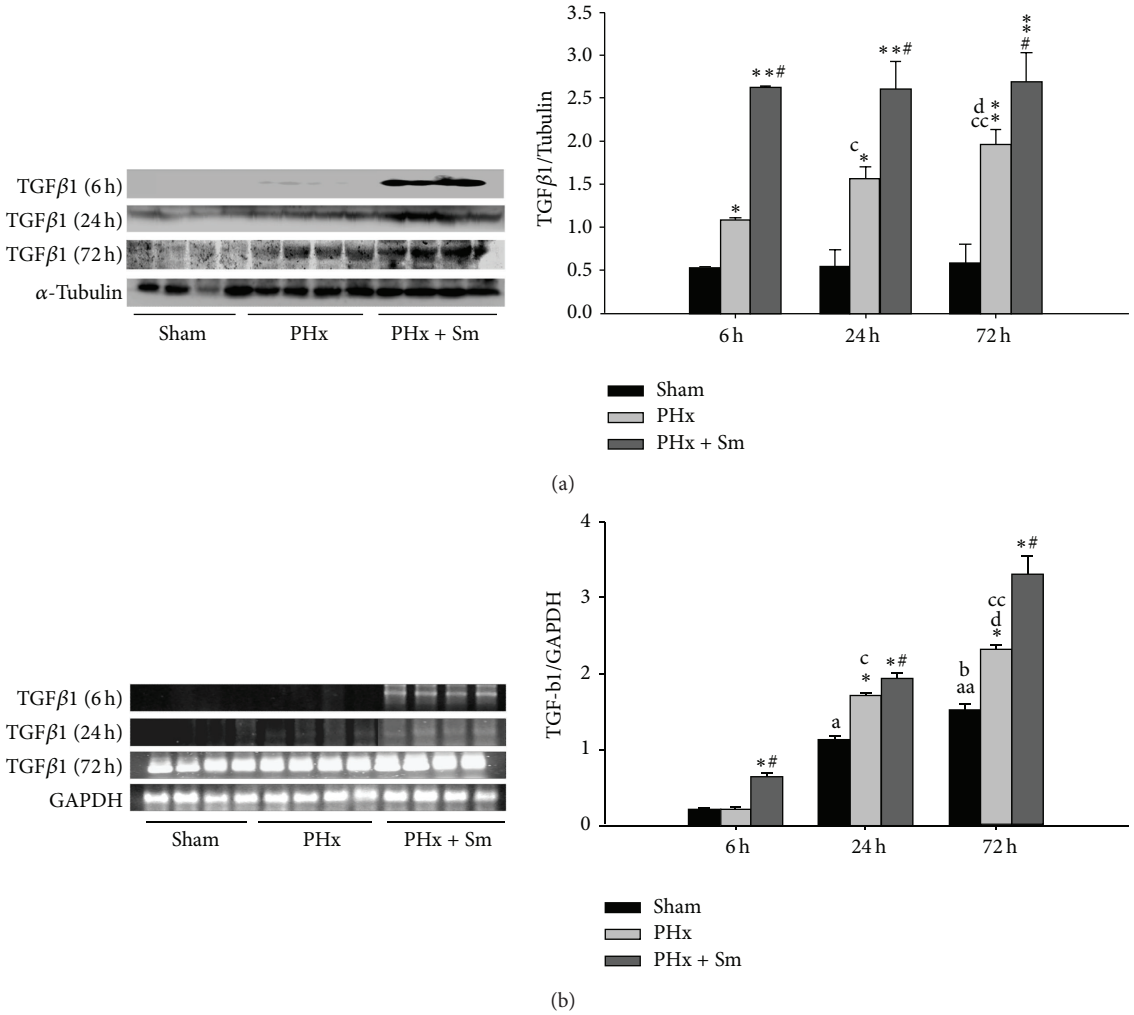

Figure 6: TGFβ1 contributed in some way to the events leading hepatocytes from the G0 phase to the G1 phase of the cell cycle in liver regeneration after partial hepatectomy. (a) Equal amounts of protein lysates were separated by 12% SDS-PAGE by western blotting with antibodies to TGFβ1. Protein expression levels were increased in treatment silymarin (Sm) after 6 hrs, 24 hrs, and 72 hrs PHx. Quantification of densitometry analysis of protein expression levels. All data are presented as means ± SEM, $^{*}p < 0.05$, $^{**}p < 0.01$, as compared with the corresponding sham group. $^{\#}p < 0.05$ as compared with the corresponding PHx. $^{c}p < 0.05$, $^{cc}p < 0.01$ as compared with 6 hrs PHx group. $^{d}p < 0.05$ as compared with 24 hrs PHx group. α-Tubulin was used as a load control for western blotting. (b) RT-PCR analysis of TGFβ1 mRNA expression during liver regeneration. Quantification of densitometry analysis of mRNA expression levels. All data are presented as means ± SEM, $^{*}p < 0.05$, as compared with the corresponding sham group. $^{\#}p < 0.05$ as compared with the corresponding PHx. $^{a}p < 0.05$, $^{aa}p < 0.01$ as compared with 6 hrs sham group. $^{b}p < 0.05$ as compared with 24 hrs sham group. $^{c}p < 0.05$, $^{cc}p < 0.01$ as compared with 6 hrs PHx group. $^{d}p < 0.05$ as compared with 24 hrs PHx group. GAPDH was used as a loading control for RT-PCR.

examine immunofluorescence and Brdu antibodies using the immunohistochemistry assay to determine cell proliferation. The Ki-67 protein is present during all active phases in the cell cycle (G1, S, G2, and mitosis) but is absent from resting cells (G0). Therefore, both PHx and after PHx treatment Sm presented higher positive Ki-67 antibody expression from immunofluorescence analysis. Lower 10% was expressed as lower proliferation. PHx is a hyperplasia considered to be a physiological response to a specific stimulus. PHx and Sm-treated cells with hyperplastic growth remained subject to normal regulatory control mechanisms (Figure 7(a)) (green color). And Ki-67 staining is different and BrdU staining is detected only in the S phase of the cell, which is in the DNA replication phase of the cell. BrdU is a uridine derivative that will replace thymidine incorporated into the DNA of cells in S phase (Figure 7(b), yellow arrows). Brown color BrsU nuclear cells were observed at 24 hrs and 72 hrs PHx and Sm-treated cells. We suggest that silymarin could lead cell cycle accelerated and made delated cell cycle run fastly to feedback normal (Figure 8). During 72 hrs PHx, silymarin may deliver in the aggregate a set of signals that lead to regeneration termination in the cyclin A phase. Silymarin can function as a hepatoprotectant agent. In the future, silymarin may be useful as an adjuvant for the treatment of specific liver diseases.

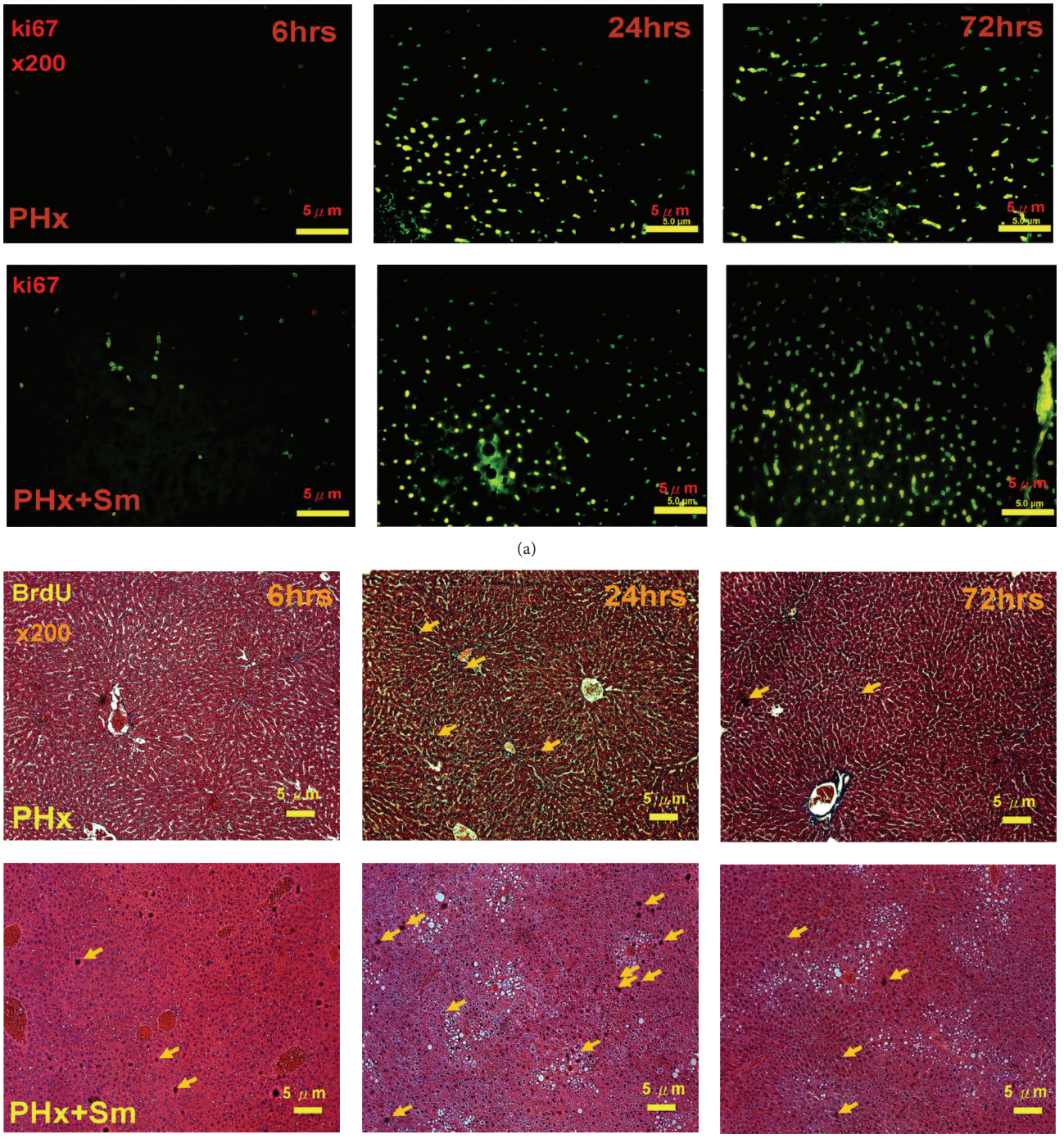

Figure 7: Silymarin accelerates cell cycle from G1 phase to S phase during liver regeneration. (a) Immunofluorescence analysis of Ki-67 antibody after PHx at 6 hrs, 24 hrs, and 72 hrs and presence of silymarin treatment at 6 hrs, 24 hrs, and 72 hrs. Green points express Ki-67 stained hepatocytes. (b) Immunohistochemical staining of BrdU after PHx at 6 hrs, 24 hrs, and 72 hrs and presence of silymarin treatment at 6 hrs, 24 hrs, and 72 hrs. Yellow arrows point to positively BrdU stained hepatocytes nuclei.

4. Discussion

Silymarin, a flavonoid complex extracted from Milk Thistle seeds, has been used for centuries to treat liver diseases and it is still used today as an important contributor to the support of healthy liver function [22, 23]. The liver is one of the most complex organs, playing an important role in digestion, detoxification, blood sugar regulation, and fat metabolism. Liver regeneration induced by surgical injury is an orchestrated response [24–26]. Silymarin is currently

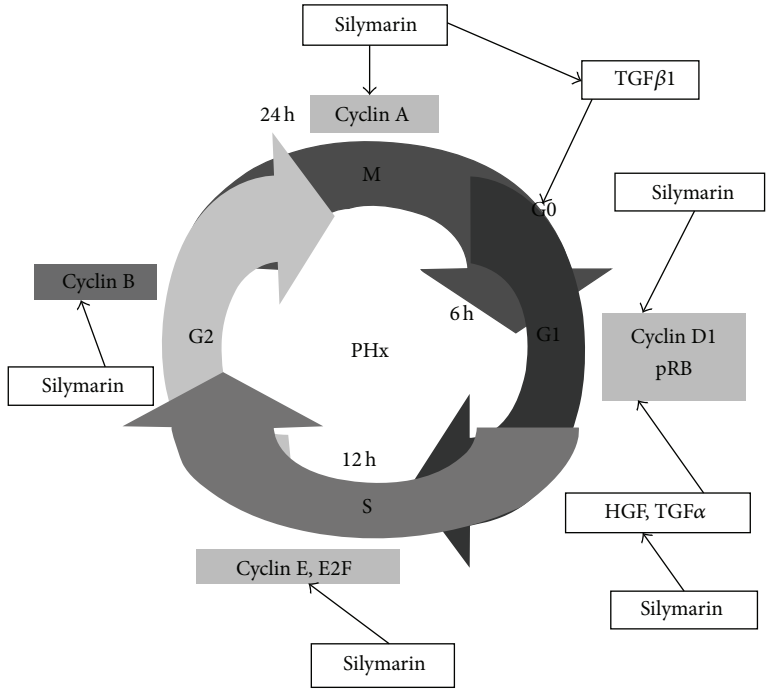

Figure 8: Silymarin improves liver regeneration. Silymarin accelerated cell cycle in liver regeneration after partial hepatectomy (PHx). Growth factors, HGF, TGFα, and TGFβ1, leading to the cell cycle running fast after silymarin treatment during liver regeneration. Silymarin makes the prolonged PHx cell cycle become normal. G1 phase check point protein: cyclin D1 and pRb. S phase check point protein: cyclin E and E2F. G2 phase check point protein: cyclin B, and M phase check point protein: cyclin A.

the world's most effective treatment for liver disease. It has shown positive effects in treating nearly every known form of liver disease including cirrhosis, hepatitis, necrosis, and liver damage due to drug and alcohol abuse. Many medical studies have shown that liver damage from alcohol, drugs, pesticides, some poisons, and hepatitis can be prevented or repaired with silymarin extract. In order to set in the optimal mass in relationship to its body size, the liver induces compensatory hyperplasia mechanisms. The herbal medicine, silymarin, has been used to treat liver disorders for thousands of years in the East. Silymarin has shown positive effects in treating nearly every known form of liver disease. The prognosis for a patient with a hepatic tumor after surgery is improved with silymarin treatment. The sooner the tumor growth is caught depending on the size and type, the more likely the patient will survive. Partial hepatectomy (PHx) has now become a promising therapy internationally for pathological liver conditions [27]. After hepatectomy, silymarin induces cellular proliferation and inhibits apoptosis resulting in tissue remodeling and restoration to normal hepatic mass [28, 29]. Our previous report reveals that silymarin effectively cyclin D1 and E1 thereby potentially induce cellular proliferation. However, their effect on other cyclins and on how the improvement in cellular proliferation translates into effective hepatic health is not known yet. This study investigated the silymarin action mechanisms that improve cell cycle machinery regulation after partial hepatectomy (PHx). Similar to our previous findings, silymarin enhanced cyclin D1 and cyclin E1. Further, the results show that silymarin upregulated cyclin A and cyclin B at various time intervals. Following hepatectomy, liver regeneration triggers postponed cell cycle. We determined that PHx delayed cell cycle comprised at least 2 critical phases: (a) the transition of the quiescent hepatocytes into G1 phase [30, 31], (b) the transition of the restriction point in the G1 to S phase of the cycle [32–34]. We found that, from 6 hrs to 24 hrs, the liver remains in the G1 phase, until 72 hrs liver regeneration into S phase; however, one cell cycle run presumably 24 hrs. In other words, 3 cycles are run within 72 hrs. Recent accumulating evidence indicates that silymarin may act as a cell cycle progression agent to trigger regeneration after PHx from G1 phase into S phase (Figures 1 and 2). Silymarin (Sm) has the ability to influence partial hepatectomy programmed cell cycle. Overall, silymarin induced the strongest effects at 24 hrs; however, an oral dose achieved in experimentation half-life is approximately at 72 hrs. Furthermore, we found silymarin induced them at stopped for long-term 72 hr PHx. Protein synthesis is also stimulated, thereby accelerating the regeneration process and the production of liver cells. Efficacy in the restoration of liver function and liver regeneration has been established. Our previous report highlights the potential of silymarin to enhance the growth factors such as the HGF at various time points [35]. Similarly, it should be noted, from the present study, that HGF as well as other growth factors such as TGFα and TGFβ1 is also stimulated by silymarin after PHx in a similar fashion. Silymarin is a protective drug for several liver diseases. It presents only minor side effects and a good safety profile and most importantly is affordable for

patients. The results from this study suggest that silymarin has considerable therapeutic potential, protecting intact liver cells, or cells not yet irreversibly damaged, by acting on the cell membranes to prevent the entry of toxic substances (Figure 7). Silymarin was recently reported to present the best liver regeneration effects. A toxicity study on silymarin observed that the seed extract is nontoxic and caused no death up to a dose of 3.2 g/kg orally. It is safe and was used in doses for further studies. The whole silymarin plant is rich in novel anticarcinogen substances and sesquiterpene lactones [36]. The present study addresses an area of special interest, liver regeneration treatment after partial hepatectomy. All of the liver cells proliferate to rebuild the lost hepatic tissue. The initiation of synthesis occurs in hepatocytes after surgery. Silymarin accelerates the cell cycle in the G1 to S phases.

Abbreviations

HGF: Hepatocyte growth factor
PHx: Partially hepatectomized
Sm: Silymarin
ERK: Extracellular signal-regulated kinase.

Conflict of Interests

The authors who have taken part in this study declare that they do not have anything to disclose regarding funding or conflict of interests with respect to this paper.

Authors' Contribution

Jia-Ping Wu and Chin-Chuan Tsai equally contributed to this work.

Acknowledgments

Jia-Ping Wu operated all the experiments and Dr Chih-Yang Huang set up the idea and all the experimental design. The authors thank the staff of Chin-Chuan Tsai for Chinese medicine, silymarin, preparation. The authors thank Yu-Lan Yeh and Yueh-Min Lin for the help and use of microscope facility and Chien-Chung Lin for western blot analysis and RT-PCR instruction. The authors are grateful to Cecilia Hsuan Day and Chia-Yao Shen for antibodies supply and allowing them to discuss work before publication. The authors would like to thank Lung-Fa Pan for his assistance with the statistical analyses. The authors gratefully thank V. Vijaya Padma for excellent technical assistance and critical reading of the paper. This study is supported in part by the Taiwan Ministry of Health and Welfare Clinical Trial and Research Center of Excellence (MOHW104-TDU-B-212-113002).

References

[1] K. Wellington and B. Jarvis, "Silymarin: a review of its clinical properties in the management of hepatic disorders," *BioDrugs*, vol. 15, no. 7, pp. 465–489, 2001.

[2] C. Loguercio and D. Festi, "Silybin and the liver: from basic research to clinical practice," *World Journal of Gastroenterology*, vol. 17, no. 18, pp. 2288–2301, 2011.

[3] M. Cengiz, H. M. Kutlu, D. D. Burukoglu, and A. Ayhanci, "A comparative study on the therapeutic effects of silymarin and silymarin-loaded solid lipid nanoparticles on d-GaIN/TNF-α-induced liver damage in balb/c mice," *Food and Chemical Toxicology*, vol. 77, pp. 93–100, 2015.

[4] P. V. Kiruthiga, R. B. Shafreen, S. K. Pandian, and K. P. Devi, "Silymarin protection against major reactive oxygen species released by environmental toxins: exogenous H_2O_2 exposure in erythrocytes," *Basic & Clinical Pharmacology & Toxicology*, vol. 100, no. 6, pp. 414–419, 2007.

[5] R. Saller, R. Meier, and R. Brignoli, "The use of silymarin in the treatment of liver diseases," *Drugs*, vol. 61, no. 14, pp. 2035–2063, 2001.

[6] S. C. Pradhan and C. Girish, "Hepatoprotective herbal drug, silymarin from experimental pharmacology to clinical medicine," *Indian Journal of Medical Research*, vol. 124, no. 5, pp. 491–504, 2006.

[7] G. K. Michalopoulos and M. C. DeFrances, "Liver regeneration," *Science*, vol. 276, no. 5309, pp. 60–66, 1997.

[8] L. Fan, Y. Ma, Y. Liu, D. Zheng, and G. Huang, "Silymarin induces cell cycle arrest and apoptosis in ovarian cancer cells," *European Journal of Pharmacology*, vol. 743, pp. 79–88, 2014.

[9] N. M. El-Lakkany, O. A. Hammam, W. H. El-Maadawy, A. A. Badawy, A. A. Ain-Shoka, and F. A. Ebeid, "Anti-inflammatory/anti-fibrotic effects of the hepatoprotective silymarin and the schistosomicide praziquantel against Schistosoma mansoni-induced liver fibrosis," *Parasites and Vectors*, vol. 5, no. 1, article 9, 2012.

[10] Z. Yang, L. Zhuang, Y. Lu, Q. Xu, and X. Chen, "Effects and tolerance of silymarin (milk thistle) in chronic hepatitis C virus infection patients: a meta-analysis of randomized controlled trials," *BioMed Research International*, vol. 2014, Article ID 941085, 9 pages, 2014.

[11] N. Kumar, A. Rai, N. D. Reddy et al., "Silymarin liposomes improves oral bioavailability of silybin besides targeting hepatocytes, and immune cells," *Pharmacological Reports*, vol. 66, no. 5, pp. 788–798, 2014.

[12] N. Fausto, A. D. Laird, and E. M. Webber, "Liver regeneration. 2. Role of growth factors and cytokines in hepatic regeneration," *The FASEB Journal*, vol. 9, no. 15, pp. 1527–1536, 1995.

[13] T. Ishikawa, V. M. Factor, J. U. Marquardt et al., "Hepatocyte growth factor/c-met signaling is required for stem-cell-mediated liver regeneration in mice," *Hepatology*, vol. 55, no. 4, pp. 1215–1226, 2012.

[14] Y. Gui, M. Yeganeh, S. Ramanathan et al., "SOCS1 controls liver regeneration by regulating HGF signaling in hepatocytes," *Journal of Hepatology*, vol. 55, no. 6, pp. 1300–1308, 2011.

[15] N. Okumura, T. Seki, and T. Ariga, "Cell surface-bound plasminogen regulates hepatocyte proliferation through a uPA-dependent mechanism," *Bioscience, Biotechnology and Biochemistry*, vol. 71, no. 6, pp. 1542–1549, 2007.

[16] K. Shanmukhappa, G. E. Sabla, J. L. Degen, and J. A. Bezerra, "Urokinase-type plasminogen activator supports liver repair independent of its cellular receptor," *BMC Gastroenterology*, vol. 6, article 40, 2006.

[17] M. Bueno, S. Salgado, C. Beas-Zárate, and J. Armendariz-Borunda, "Urokinase-type plasminogen activator gene therapy in liver cirrhosis is mediated by collagens gene expression

down-regulation and up-regulation of MMPs, HGF and VEGF," *Journal of Gene Medicine*, vol. 8, no. 11, pp. 1291–1299, 2006.

[18] J.-I. Okano, G. Shiota, K. Matsumoto et al., "Hepatocyte growth factor exerts a proliferative effect on oval cells through the PI3K/AKT signaling pathway," *Biochemical and Biophysical Research Communications*, vol. 309, no. 2, pp. 298–304, 2003.

[19] M. Khoshnoodi, N. Fakhraei, and A. R. Dehpour, "Possible involvement of nitric oxide in antidepressant-like effect of silymarin in male mice," *Pharmaceutical Biology*, vol. 53, no. 5, pp. 739–745, 2015.

[20] C.-Y. Chiu, K.-K. Kuo, T.-L. Kuo, K.-T. Lee, and K.-H. Cheng, "The activation of MEK/ERK signaling pathway by bone morphogenetic protein 4 to increase hepatocellular carcinoma cell proliferation and migration," *Molecular Cancer Research*, vol. 10, no. 3, pp. 415–427, 2012.

[21] J. F. Barreto, S. N. Wallace, D. J. Carrier, and E. C. Clausen, "Extraction of nutraceuticals from milk thistle: I. Hot water extraction," *Applied Biochemistry and Biotechnology—Part A: Enzyme Engineering and Biotechnology*, vol. 108, no. 1-3, pp. 881–890, 2003.

[22] L. Duan, S. N. Wallace, A. Engelberth et al., "Extraction of co-products from biomass: example of thermal degradation of silymarin compounds in subcritical water," *Applied Biochemistry and Biotechnology*, vol. 158, no. 2, pp. 362–373, 2009.

[23] K. N. Tzirogiannis, K. T. Kourentzi, S. Zyga et al., "Effect of 5-HT 7 receptor blockade on liver regeneration after 60–70% partial hepatectomy," *BMC Gastroenterology*, vol. 14, no. 1, article 201, 2014.

[24] A. N. Kandilis, J. Koskinas, I. Vlachos et al., "Liver regeneration: immunohistochemichal study of intrinsic hepatic innervation after partial hepatectomy in rats," *BMC Gastroenterology*, vol. 14, no. 1, article 202, 2014.

[25] H. J. Eo, G. H. Park, H. M. Song et al., "Silymarin induces cyclin D1 proteasomal degradation via its phosphorylation of threonine-286 in human colorectal cancer cells," *International Immunopharmacology*, vol. 24, no. 1, pp. 1–6, 2015.

[26] L. H. Li, L. J. Wu, S. I. Tashiro, S. Onodera, F. Uchiumi, and T. Ikejima, "The roles of Akt and MAPK family members in silymarin's protection against UV-induced A375-S2 cell apoptosis," *International Immunopharmacology*, vol. 6, no. 2, pp. 190–197, 2006.

[27] T. Benko, S. Frede, Y. Gu et al., "Glycine pretreatment ameliorates liver injury after partial hepatectomy in the rat," *Journal of Investigative Surgery*, vol. 23, no. 1, pp. 12–20, 2010.

[28] H. O. El Mesallamy, N. S. Metwally, M. S. Soliman, K. A. Ahmed, and M. M. Abdel Moaty, "The chemopreventive effect of *Ginkgo biloba* and *Silybum marianum* extracts on hepatocarcinogenesis in rats," *Cancer Cell International*, vol. 11, no. 1, article 38, 2011.

[29] N. Reisinger, S. Schaumberger, V. Nagl, S. Hessenberger, and G. Schatzmayr, "Milk thistle extract and silymarin inhibit lipopolysaccharide induced lamellar separation of hoof explants in vitro," *Toxins*, vol. 6, no. 10, pp. 2962–2974, 2014.

[30] M. Vaid, R. Prasad, Q. Sun, and S. K. Katiyar, "Silymarin targets β-catenin signaling in blocking migration/invasion of human melanoma cells," *PLoS ONE*, vol. 6, no. 7, Article ID e23000, 2011.

[31] H.-J. Kwon, Y.-S. Won, Y.-D. Yoon et al., "Vitamin D3 up-regulated protein 1 deficiency accelerates liver regeneration after partial hepatectomy in mice," *Journal of Hepatology*, vol. 54, no. 6, pp. 1168–1176, 2011.

[32] J. Fehér and G. Lengyel, "Silymarin in the prevention and treatment of liver diseases and primary liver cancer," *Current Pharmaceutical Biotechnology*, vol. 13, no. 1, pp. 210–217, 2012.

[33] F. J. Burczynski, G. Wang, D. Nguyen, Y. Chen, H. J. Smith, and Y. Gong, "Silymarin and hepatoprotection," *Zhong Nan Da Xue Xue Bao Yi Xue Ban*, vol. 37, no. 1, pp. 6–10, 2012.

[34] K. Ramasamy and R. Agarwal, "Multitargeted therapy of cancer by silymarin," *Cancer Letters*, vol. 269, no. 2, pp. 352–362, 2008.

[35] C.-C. Tsai, J.-P. Wu, Y.-M. Lin et al., "The effect of *Elephantopus scaber* L. on liver regeneration after partial hepatectomy," *Evidence-Based Complementary and Alternative Medicine*, vol. 2013, Article ID 369180, 11 pages, 2013.

[36] R. T. Atawia, H. H. Mosli, M. G. Tadros, A. E. Khalifa, H. A. Mosli, and A. B. Abdel-Naim, "Modulatory effect of silymarin on inflammatory mediators in experimentally induced benign prostatic hyperplasia: emphasis on PTEN, HIF-1α, and NF-κB," *Naunyn-Schmiedeberg's Archives of Pharmacology*, vol. 387, no. 12, pp. 1131–1140, 2014.

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Research Article

Network Pharmacology-Based Antioxidant Effect Study of Zhi-Zi-Da-Huang Decoction for Alcoholic Liver Disease

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Zhi-Zi-Da-Huang decoction (ZZDHD), a classic traditional Chinese medicine (TCM) formula, has been used for centuries to treat alcoholic liver disease. Reliable therapeutics of ZZDHD has also been validated in clinical practice. In this study, molecular docking and network analysis were carried out to explore the antioxidative mechanism of ZZDHD as an effective therapeutic approach to treat alcoholic liver disease. Multiple active compounds of ZZDHD were screened based on four key original enzymes (cytochrome P450 2E1, xanthine oxidase, inducible nitric oxide synthase, and cyclooxygenase-2) involved in ethanol-induced oxidative stress damage. A drug-target network was constructed through network pharmacology analysis, which predicted the relationships of active ingredients to the targets. Some results had been verified by the previous experimental pharmacological studies; meanwhile, it was first reported that xanthine oxidase and eriocitrin, neoeriocitrin, isorhoifolin, and poncirin had interactions. The network pharmacology strategy used provided a forceful tool for searching the mechanism of action of TCM formula and novel bioactive ingredients.

1. Introduction

Alcoholic liver disease (ALD) is one of the major diseases threatening human health and is also the leading cause of liver-related morbidity and mortality worldwide [1, 2]. Although no single process or underlying mechanism can account for all the effects of alcohol on an organism or even on one specific organ [3], it has been proven that reactive oxygen species (ROS) and oxidative stress play central roles in the early stage of the disease process [4]. In ethanol-induced liver damage, ethanol can increase the generation of reactive oxygen species (ROS) such as $O_2^{-\bullet}$, H_2O_2, and excessive oxygen free radicals disrupt the balance between the oxidation and antioxidation systems, further causing oxidative damage. Moreover, ROS cause the lipid peroxidation of cellular membranes [5], and lipid peroxidation in turn further aggravates ROS generation [6]. Thus, oxidative stress results from excessive oxygen free radical formation and antioxidant depletion due to the ROS generated by ethanol administration [7].

In early-stage alcoholic liver damage, these two enzymes, cytochrome P450 2E1 (CYP2E1) and xanthine oxidase (XO), have been proven to generate ROS in liver as a consequence of alcohol exposure [8, 9]. Ethanol mainly increases the activities of CYP2E1 and XO, which can generate excessive oxygen free radicals and lead to lipid peroxidation, further causing oxidative stress damage. Oxidative stress mainly increases the activities of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2), further aggravating lipid peroxidation and oxidative stress [10, 11]. In ethanol-induced liver damage, four enzymes including CYP2E1, XO, COX-2, and iNOS are the original proteins described as generating ROS or oxidative stress, and they play important roles in the damage due to oxidative stress. Hence, the timely and effective control of high activities of these enzymes, which would eliminate ROS or relieve oxidative stress, is beneficial for the treatment of alcoholic liver injury.

Zhi-Zi-Da-Huang decoction (ZZDHD), a traditional Chinese medicine (TCM) formula, was first described in

the book of Jin-Kui-Yao-Lue (*Synopsis of Golden Chamber*). ZZDHD, consisting of four crude herbs: *Gardenia jasminoides* Ellis (Zhi-Zi), *Rheum officinale* Baill (Da-Huang), *Citrus aurantium* L. (Zhi-Shi), and Semen Sojae Preparatum (Dan-Dou-Chi), has been reported to have the ability to treat or alleviate the symptoms of alcoholic jaundice, alcoholic liver disease, and acute hepatitis [12]. Wang et al. [13] reported that ZZDHD had a significant protective effect by reversing biochemical parameters and histopathological changes, and its antioxidant function ameliorated the hepatic injury induced by alcohol. Thirty active components of ZZDHD have also been reported [14]; however, it remains unclear how the active ingredients function as antioxidants.

Network pharmacology, a key technology of system biology, has attracted much attention by researching the molecular mechanisms of TCM formula for complicated diseases [15–17]. Zhang et al. [18] studied an integrative platform of TCM network pharmacology and its application on a herbal formula. Li et al. [19] also determined active compounds and action mechanisms of Ge-Gen-Qin-Lian decoction for treatment of type 2 diabetes by network pharmacology method. Many active chemical compositions of TCM target multiple proteins in the biological network of some disease. Molecular docking is available for modeling interactions between small molecules and proteins. Thus, research of TCM based on network pharmacology, which is a holistic understanding of the molecular mechanisms responsible for the pharmacological effects of herbal medicines [17, 20], is well worth exploration.

In this study, a network pharmacology study of ZZDHD was established through molecular docking and network analysis based on thirty identified active components of ZZDHD and four potential targets including cytochrome P450 2E1 (CYP2E1), xanthine oxidase (XO), inducible nitric oxide synthase (iNOS), and cyclooxygenase-2 (COX-2). The study provides a powerful tool for explaining the antioxidant mechanism of TCM formula and discovering novel bioactive ingredients.

2. Materials and Methods

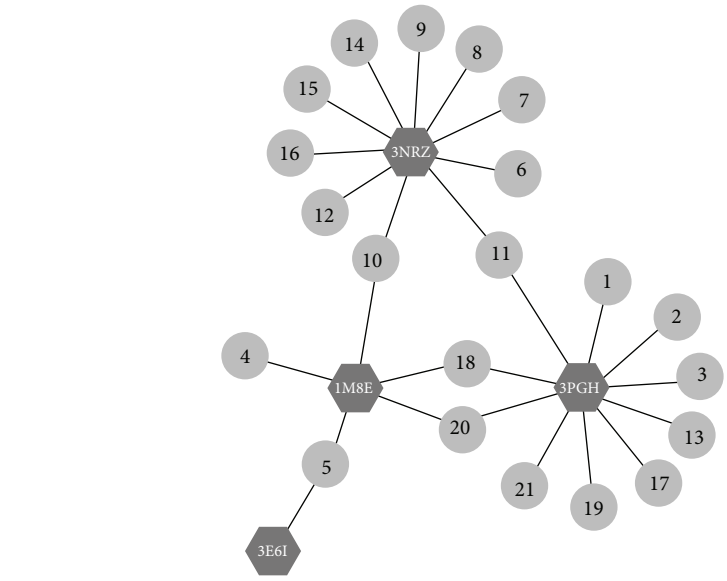
2.1. Potential Targets and Ligand Structures Preparation. Reactive oxygen species are the major prooxidant agents in oxidative stress-induced lipid peroxidation, which results in oxidative damage to various types of cell components including lipids, proteins, and DNA [21–23]. The crystal structures of all candidate targets were retrieved from RCSB Protein Data Bank (http://www.pdb.org/), and relevant proteins including CYP2E1 (PDB ID code 3E6I), XO (PDB ID code 3NRZ), COX-2 (PDB ID code 3PGH), and iNOS (PDB ID code 1M8E) were chosen as potential targets of oxidative-stress damage. All proteins were performed using the CHARMm Force Field with the help of the software package Discovery Studio 2.5 (Accelrys, USA). A total of 30 chemical structures of ZZDHD were identified by HPLC-PDA-ESI-MS/MS in our early laboratory work (see Figure S1, available online at http://dx.doi.org/10.1155/2014/492470), and all the structures of these compounds were optimized by MMFF94 Force Field in DS 2.5.

2.2. Molecular Docking and Network Building. Molecular docking was conducted with the LibDock protocol based on the CHARMm Force Field in DS2.5. LibDock is considered to balance speed and accuracy and is based on the matching of polar and apolar binding site features of the protein-ligand complex. In the docking procedure, high quality was set for the docking preference parameter, and the best was set for the conformation method. The other parameters were used as the default. In general, the protein-ligand docking active site is defined by the location of the original ligand. A compound of ZZDHD was considered to be a potentially active ingredient if the LibDockScore of the compound was higher than the original ligand. Conversely, the compound was not considered if it was not shown at the binding site of the protein-ligand complex. The drug-target network was then constructed using Cytoscape 3.0.2 software (http://www.cytoscape.org/) based on the top 10 of the molecular docking rank. In the network, nodes stand for compounds and targets, and edges represent the compound-target interactions.

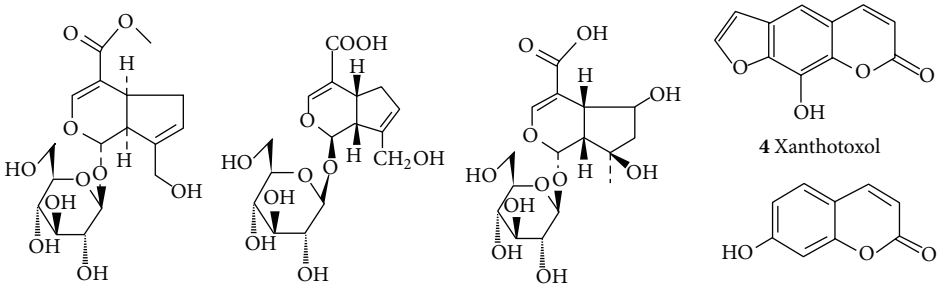
3. Results and Discussion

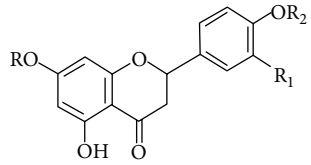
3.1. Network Construction of Molecular Docking-Based Pharmacology. The active ingredients of ZZDHD were predicted through molecular docking. To further illuminate the relationship between effective compounds and potential targets, a drug-target network was built through network analysis (Figure 1). Multiple active pharmaceutical ingredients of ZZDHD were found to affect different targets.

The network showed the interactions of XO with 10 compounds such as hesperetin and naringenin, COX-2 with 10 compounds such as aloe-emodin and hesperetin, and iNOS with 5 compounds such as aloe-emodin and naringenin; CYP2E1 showed interactions with only umbelliferone. Previous laboratory pharmacological studies have provided much information about the chemical compounds screened and the corresponding targets. Park et al. reported [24] that aloe-emodin dose-dependently inhibited iNOS mRNA expression and nitric oxide (NO) production at 5–40 μM. In addition, the levels of cyclooxygenase-2 (COX-2) mRNA and prostaglandin E2 (PEG_2) production were suppressed by 40 μM aloe-emodin. This result indicated that aloe-emodin was an effective inhibitor for these two targets. Jayaraman et al. [25] have proved that naringenin decreased the expression of iNOS and COX-2 in the liver of ethanol fed rats. Moreover, Park et al. [26] investigated the inhibiting mechanism of naringenin at the molecular level, finding that naringenin inhibited iNOS and COX-2 mRNA expression and reduced the production of NO and PGE_2. Chao et al. [27] considered that naringenin had a stronger inhibitory effect toward iNOS and COX-2 than did an equal concentration of vitamin C. Another study showed the possible interaction between naringenin and XO. Naoghare et al. [28] proposed that XO was also inhibited by naringenin because the concomitant hydrophilicity and hydrophobicity within the naringenin molecule helped it to bind the active site of XO more strongly, thereby reducing the activity of XO. In the present study, the pharmacological network built showed

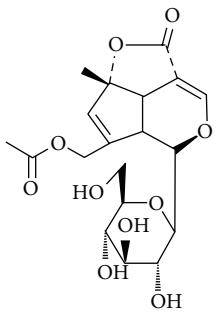


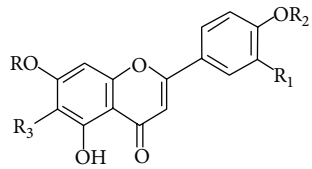
(a)



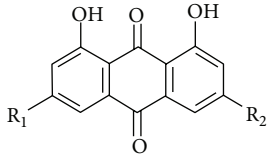


R	R_1	R_2	Compound
6 Rutinose	OH	H	Eriocitrin
7 Neohesperidose	OH	H	Neoeriocitrin
8 Neohesperidose	H	H	Naringin
9 Rutinose	OH	CH_3	Hesperidin
10 H	H	H	Naringenin
11 H	OH	CH_3	Hesperetin
12 Neohesperidose	H	CH_3	Poncirin





R	R_1	R_2	R_3	Compound
14 Rutinose	H	H	H	Isorhoifolin
15 Neohesperidose	H	H	H	Rhoifolin
16 Rutinose	OH	CH_3	H	Diosmin
17 H	CH_3O	CH_3	CH_3O	Eupatilin



	R_1	R_2	Compound
18	H	CH_2OH	Aloe-emodin
19	H	COOH	Rhein
20	OH	CH_3	Emodin
21	OCH_3	CH_3	Physcion

(b)

Figure 1: The drug-target network related to antioxidative mechanism of ZZDHD in ALD was shown in (a), CYP2E1 (PDB ID code 3E6I), XO (PDB ID code 3NRZ), COX-2 (PDB ID code 3PGH), and iNOS (PDB ID code 1M8E). (b) displayed the corresponding chemical structures of the 21 active components from ZZDHD.

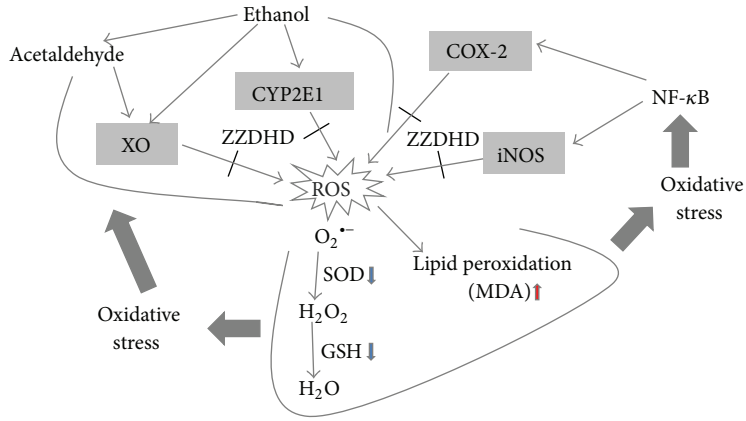


Figure 2: Schematic illustrating proposed action mechanism by which ZZDHD prevents ALD. That ethanol induces CPY2E1, and XO causes oxidative stress in liver, due to overproduction of ROS, antioxidants reduction, and lipid peroxidation. These results, in turn, improve NF-κB activation and then increase the expression such as in COX-2 and iNOS, further aggravating oxidative injury. Multiple components from ZZDHD (e.g., aloe-emodin and naringenin) inhibit the activation of CYP2E1, XO, COX-2, and iNOS, thereby protecting liver against the damage.

that naringenin interacted with iNOS and XO. In fact, COX-2 also exhibits interactions with naringenin, but as the rank was not in the top 10, the interaction was not shown in our network.

Although the working mechanism was described from molecular point of view, it had a strong theoretical and scientific implication. Partial predicted results have been reported in some literatures, serving as a good reference value for verifying our network. Moreover, we first reported that XO had interactions with eriocitrin, neoeriocitrin, isorhoifolin, and poncirin. The antioxidant activities of eriocitrin, neoeriocitrin, isorhoifolin, and poncirin and their inhibiting effects on oxidative or ROS damages had been investigated previously [29–32], but their inhibitory effects on XO were little reported. Hence, this screening method also provided important theoretical guidance for exploring XO inhibitors from TCM. Drug-target network indicated the molecular mechanism of action of ZZDHD with regard to antioxidant effects. In our future studies, the details and experimental verification for novel screened active compounds will be elucidated.

3.2. Antioxidative Molecular Mechanism of ZZDHD. The antioxidation mechanisms of ZZDHD were predicted using network pharmacology. Much research has revealed that CYP2E1 plays a role in ethanol-induced liver steatosis. Ethanol initially induces CYP2E1 activation followed by increases in oxidative stress in the liver [33], and the ROS generated by CYP2E1 promotes oxidative stress. ROS leads to the production of reactive aldehydes, also with potent proinflammatory properties [8]. Oxidative stress increases NF-κB activation, thus enhancing the expression of COX-2 and iNOS [34]. The upregulation of COX-2 expression causes the enhanced production of PGE_2, which functions through its receptors (EP_2 and/or EP_4) in hepatocytes to increase the accumulation of triglycerides [11], with further development in fatty liver. Recent studies have also shown that specific iNOS inhibitors or iNOS knockout protects against ethanol-caused oxidative stress [35, 36]. Other sources of prooxidant agent include acetaldehyde, an excellent substrate for the enzyme XO, which generates more toxic oxygen radicals during its oxidative catalysis [37]. Indeed, the inhibition of XO decreases ethanol- and acetaldehyde-induced lipid peroxidation.

Overproduction of ROS disrupts the balance between the oxidation and antioxidation systems, further causing oxidative stress [7]. Radical scavenging system is the body's first line of defense against oxidation, and SOD and GSH are the main components of the antioxidant protection system. SOD is a basic antioxidant enzyme responsible for catalyzing the dismutation of superoxide anion radical ($O_2^{-\bullet}$), and its activity indirectly indicates the body's ability of scavenging free radical [38]. GSH is a major nonprotein thiol and plays a central role in coordinating the antioxidant defense process [39]. Depletion of GSH may be associated with aggravation of oxidative damage, which usually reflects the level of the organism's antioxidant ability. MDA, an end-product of lipid peroxidation, has been widely used as an indicator for the degree of lipid peroxidation [40] and it also indirectly incarnates the formation of free radicals and the degree of cell damage. Previous studies had reported that ZZDHD markedly improved SOD, GSH, and MDA, which indicated ZZDHD had contributed to the elimination of excessive oxygen free radicals and the reduction of lipid peroxidation [13].

ZZDHD modulated the activity of ethanol-metabolizing enzymes such as CYP2E1 and XO and inhibited the expression of enzymes such as COX-2 and iNOS. The above result showed that the antioxidant effect of ZZDHD may be an important influence factor for relieving oxidative stress as well as reducing the generation of lipid peroxidation induced by ethanol (Figure 2).

4. Conclusion

In this paper, a drug-target network was constructed through molecular docking and network analysis. The network predicted the underlying antioxidant mechanism of ZZDHD as an effective therapeutic approach to treat alcoholic liver disease. This study demonstrated that a network pharmacology-based approach was useful for elucidating the interrelationship between complex diseases such as ALD and TCM formula interventions. Therefore, network pharmacology is a forceful tool for exploring the potential mechanism of action of TCM formula and new active ingredients. As further steps, experimental verification of the potential effective compounds after candidate screening is needed to validate the interactions between drugs and proteins based on theoretical predictions.

Conflict of Interests

The authors declare no competing financial interests.

Acknowledgment

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References

[1] A. Cruz and J. Muntane, "Alcoholic liver disease, oxidative stress, and antioxidants," *Revista Espanola de Enfermedades Digestivas*, vol. 103, no. 8, pp. 393–395, 2011.

[2] B. Gao and R. Bataller, "Alcoholic liver disease: pathogenesis and new therapeutic targets," *Gastroenterology*, vol. 141, no. 5, pp. 1572–1585, 2011.

[3] D. Wu and A. I. Cederbaum, "Alcohol, oxidative stress, and free radical damage," *Alcohol Research and Health*, vol. 27, no. 4, pp. 277–284, 2003.

[4] X. Lv, Z. Chen, J. Li et al., "Caffeine protects against alcoholic liver injury by attenuating inflammatory response and oxidative stress," *Inflammation Research*, vol. 59, no. 8, pp. 635–645, 2010.

[5] I. Kurose, H. Higuchi, S. Kato et al., "Oxidative stress on mitochondria and cell membrane of cultured rat hepatocytes and perfused liver exposed to ethanol," *Gastroenterology*, vol. 112, no. 4, pp. 1331–1343, 1997.

[6] R. M. Adibhatla and J. F. Hatcher, "Phospholipase A 2, reactive oxygen species, and lipid peroxidation in cerebral ischemia," *Free Radical Biology and Medicine*, vol. 40, no. 3, pp. 376–387, 2006.

[7] E. Albano, "Alcohol, oxidative stress and free radical damage," *Proceedings of the Nutrition Society*, vol. 65, no. 3, pp. 278–290, 2006.

[8] H. Tilg, A. R. Moschen, and N. C. Kaneider, "Pathways of liver injury in alcoholic liver disease," *Journal of Hepatology*, vol. 55, no. 5, pp. 1159–1161, 2011.

[9] S. Kato, T. Kawase, J. Alderman, N. Inatomi, and C. S. Lieber, "Role of xanthine oxidase in ethanol-induced lipid peroxidation in rats," *Gastroenterology*, vol. 98, no. 1, pp. 203–210, 1990.

[10] X.-S. Deng and R. A. Deitrich, "Ethanol metabolism and effects: nitric oxide and its interaction," *Current Clinical Pharmacology*, vol. 2, no. 2, pp. 145–153, 2007.

[11] N. Enomoto, K. Ikejima, S. Yamashina et al., "Kupffer cell-derived prostaglandin E2 is involved in alcohol-induced fat accumulation in rat liver," *The American Journal of Physiology—Gastrointestinal and Liver Physiology*, vol. 279, no. 1, pp. G100–G106, 2000.

[12] J. C. Chen, "Hypothesis and clinical study of modular formulology," *Journal of Chinese Medicine*, vol. 12, pp. 69–80, 2001.

[13] H. Wang, F. Feng, B.-Y. Zhuang, and Y. Sun, "Evaluation of hepatoprotective effect of Zhi-Zi-Da-Huang decoction and its two fractions against acute alcohol-induced liver injury in rats," *Journal of Ethnopharmacology*, vol. 126, no. 2, pp. 273–279, 2009.

[14] H. Wang and F. Feng, "Identification of components in Zhi-Zi-Da-Huang decoction by HPLC coupled with electrospray ionization tandem mass spectrometry, photodiode array and fluorescence detectors," *Journal of Pharmaceutical and Biomedical Analysis*, vol. 49, no. 5, pp. 1157–1165, 2009.

[15] S. Li, T. P. Fan, W. Jia, A. Lu, and W. Zhang, "Network pharmacology in traditional Chinese medicine," *Evidence-Based Complementary and Alternative Medicine*, vol. 2014, Article ID 138460, 2 pages, 2014.

[16] W. Tao, X. Xu, X. Wang et al., "Network pharmacology-based prediction of the active ingredients and potential targets of Chinese herbal Radix Curcumae formula for application to cardiovascular disease," *Journal of Ethnopharmacology*, vol. 145, no. 1, pp. 1–10, 2013.

[17] J. Gu, H. Zhang, L. Chen, S. Xu, G. Yuan, and X. Xu, "Drug-target network and polypharmacology studies of a Traditional Chinese Medicine for type II diabetes mellitus," *Computational Biology and Chemistry*, vol. 35, no. 5, pp. 293–297, 2011.

[18] X. Liang, H. Li, and S. Li, "A novel network pharmacology approach to analyse traditional herbal formulae: the Liu-Wei-Di-Huang pill as a case study," *Molecular BioSystems*, vol. 10, no. 5, pp. 1014–1022, 2014.

[19] H. Li, L. Zhao, B. Zhang et al., "A network pharmacology approach to determine active compounds and action mechanisms of ge-gen- qin-lian decoction for treatment of type 2 diabetes," *Evidence-Based Complementary and Alternative Medicine*, vol. 2014, Article ID 495840, 12 pages, 2014.

[20] J. Zhao, P. Jiang, and W. Zhang, "Molecular networks for the study of TCM pharmacology," *Briefings in Bioinformatics*, vol. 11, no. 4, pp. 417–430, 2009.

[21] I. S. Young and J. V. Woodside, "Antioxidants in health and disease," *Journal of Clinical Pathology*, vol. 54, no. 3, pp. 176–186, 2001.

[22] Z.-M. Lu, W.-Y. Tao, X.-L. Zou, H.-Z. Fu, and Z.-H. Ao, "Protective effects of mycelia of Antrodia camphorata and Armillariella tabescens in submerged culture against ethanol-induced hepatic toxicity in rats," *Journal of Ethnopharmacology*, vol. 110, no. 1, pp. 160–164, 2007.

[23] S.-L. Liu, C.-C. Cheng, C.-C. Chang et al., "Discovery of serum biomarkers of alcoholic fatty liver in a rodent model: C-reactive protein," *Journal of Biomedical Science*, vol. 18, no. 1, article 52, 2011.

[24] M.-Y. Park, H.-J. Kwon, and M.-K. Sung, "Evaluation of aloin and aloe-emodin as anti-inflammatory agents in aloe by using murine macrophages," *Bioscience, Biotechnology and Biochemistry*, vol. 73, no. 4, pp. 828–832, 2009.

[25] J. Jayaraman, V. A. S. Jesudoss, V. P. Menon, and N. Namasivayam, "Anti-inflammatory role of naringenin in rats with ethanol induced liver injury," *Toxicology Mechanisms and Methods*, vol. 22, no. 7, pp. 568–576, 2012.

[26] H. Y. Park, G.-Y. Kim, and Y. H. Choi, "Naringenin attenuates the release of pro-inflammatory mediators from lipopolysaccharide-stimulated BV2 microglia by inactivating nuclear factor-κB and inhibiting mitogen-activated protein kinases," *International Journal of Molecular Medicine*, vol. 30, no. 1, pp. 204–210, 2012.

[27] C.-L. Chao, C.-S. Weng, N.-C. Chang, J.-S. Lin, S.-T. Kao, and F.-M. Ho, "Naringenin more effectively inhibits inducible nitric oxide synthase and cyclooxygenase-2 expression in macrophages than in microglia," *Nutrition Research*, vol. 30, no. 12, pp. 858–864, 2010.

[28] P. K. Naoghare, H. T. Kwon, and J. M. Song, "On-chip assay for determining the inhibitory effects and modes of action of drugs against xanthine oxidase," *Journal of Pharmaceutical and Biomedical Analysis*, vol. 51, no. 1, pp. 1–6, 2010.

[29] D. N. Olennikov, N. K. Chirikova, Z. M. Okhlopkova, and I. S. Zulfugarov, "Chemical composition and antioxidant activity of Tánara Ótó (Dracocephalum palmatum Stephan), a medicinal plant used by the North-Yakutian nomads," *Molecules*, vol. 18, no. 11, pp. 14105–14121, 2013.

[30] S. Tan, M. Li, X. Ding et al., "Effects of Fortunella margarita fruit extract on metabolic disorders in high-fat diet-induced obese C57BL/6 mice," *PLoS ONE*, vol. 9, no. 4, Article ID e93510, 2014.

[31] K.-I. Minato, Y. Miyake, S. Fukumoto et al., "Lemon flavonoid, eriocitrin, suppresses exercise-induced oxidative damage in rat liver," *Life Sciences*, vol. 72, no. 14, pp. 1609–1616, 2003.

[32] A. C. E. Graziano, V. Cardile, L. Crascì et al., "Protective effects of an extract from Citrus bergamia against inflammatory injury in interferon-gamma and histamine exposed human keratinocytes," *Life Sciences*, vol. 90, no. 25-26, pp. 968–974, 2012.

[33] L. Yang, D. Wu, X. Wang, and A. I. Cederbaum, "Cytochrome P4502E1, oxidative stress, JNK, and autophagy in acute alcohol-induced fatty liver," *Free Radical Biology and Medicine*, vol. 53, no. 5, pp. 1170–1180, 2012.

[34] M. Tahir, M. U. Rehman, A. Lateef et al., "Diosmin protects against ethanol-induced hepatic injury via alleviation of inflammation and regulation of TNF-α and NF-κB activation," *Alcohol*, vol. 47, no. 2, pp. 131–139, 2013.

[35] A. A. Nanji, S. S. Greenberg, S. R. Tahan et al., "Nitric oxide production in experimental alcoholic liver disease in the rat: role in protection from injury," *Gastroenterology*, vol. 109, no. 3, pp. 899–907, 1995.

[36] S. E. McKim, E. Gäbele, F. Isayama et al., "Inducible nitric oxide synthase is required in alcohol-induced liver injury: studies with knockout mice," *Gastroenterology*, vol. 125, no. 6, pp. 1834–1844, 2003.

[37] C. Guerri, C. Montoliu, and J. Renau-Piqueras, "Involvement of free radical mechanism in the toxic effects of alcohol: implications for fetal alcohol syndrome," *Advances in Experimental Medicine and Biology*, vol. 366, pp. 291–305, 1994.

[38] J. M. Matés, "Effects of antioxidant enzymes in the molecular control of reactive oxygen species toxicology," *Toxicology*, vol. 153, no. 1–3, pp. 83–104, 2000.

[39] V. Damodara Reddy, P. Padmavathi, and N. C. Varadacharyulu, "Emblica officinalis protects against alcohol-induced liver mitochondrial dysfunction in rats," *Journal of Medicinal Food*, vol. 12, no. 2, pp. 327–333, 2009.

[40] M. Zhao, Y.-Q. Du, L. Yuan, and N.-N. Wang, "Protective effect of puerarin on acute alcoholic liver injury," *The American Journal of Chinese Medicine*, vol. 38, no. 2, pp. 241–249, 2010.

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Research Article

Lipid Lowering Effect of *Punica granatum* L. Peel in High Lipid Diet Fed Male Rats

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Many herbal medicines have been recommended for the treatment of dyslipidemia. The antilipidemic effect of hydroethanolic extract of pomegranate peel (*Punica granatum* L.) was investigated in high lipid diet fed male rats. Intraperitoneally administration of pomegranate peel extract (50, 100, 200, and 300 mg/kg body weight) for 23 days on the levels of serum cholesterol, triglycerides, LDL, HDL, alkaline phosphatase (AP), aspartate aminotransferase (AST), and alanine aminotransferase (ALT) in high lipid diet fed male rats was evaluated. Treatment of pomegranate extract decreased body weight in treated rats, significantly. Administration of the plant extract significantly decreased serum total cholesterol, triglycerides, LDL-C, alkaline phosphatise, AST, and ALT levels, whereas it increased serum HDL-C in high lipid diet fed rats in comparison to saline control group. Also, histopathological study showed that treatment of pomegranate peel extract attenuates liver damage in high lipid diet fed rats in comparison to saline group. It is concluded that the plant should be considered as an excellent candidate for future studies on dyslipidemia.

1. Introduction

Dyslipidemia is generally characterized by elevated levels of total cholesterol, triglycerides, low density lipoprotein cholesterol, and decreased levels of high density lipoprotein cholesterol [1]. Dyslipidemia as an independent preventable risk factor of coronary heart disease has been shown to increase the risk of cardiovascular mortality [2–7]. Therefore, the study on the various indicators and risk factors of dyslipidemia appears to be significant in future health outcomes.

Punica granatum Linn. (Punicaceae) is a shrub or small tree and considered to be a native of Iran and Afghanistan. It is also found growing wild in the warm valleys and outer hills of the Himalayas [8]. The pomegranate fruit consists of the peel, seeds, and the arils. The peel makes up about 50% of the fruit, whereas the arils and seeds make up 40% and 10%, respectively. The peel is rich in many compounds such as phenolics, flavonoids, ellagitannins and proanthocyanidin compounds, complex polysaccharides, and many minerals including potassium, nitrogen, calcium, magnesium, phosphorus, and sodium [9].

The different parts of pomegranate (*Punica granatum* L.) have been known as a reservoir of bioactive compounds with potential biological activities. Pomegranate decreased the dyslipidemia of obesity and cardiovascular risk factors [10]. Antiparasitic, antimicrobial, and antioxidant activities of pomegranate leaves extracts were reported [11–13]. Several papers were reported on the ability of pomegranate leaves extracts to fight obesity [14], cancer, and other human diseases [15].

It is reported that 6-week treatment with pomegranate flower extract ameliorated fatty liver, reflected by diminishment of relative and total hepatic triglyceride contents and

fatty droplet deposit in the livers of Zucker diabetic fatty rats [16].

In traditional Chinese medicine, different pomegranate extracts and preparations including the bark, root, and juice of the fruit, especially the dried peels, have been used to treat many conditions [10].

The aim of the present study was to investigate the antihyperlipidemic effects of pomegranate extract peel in high lipid diet fed male rats.

2. Materials and Methods

2.1. Plant Material. Fresh *Punica granatum* L. peels were collected from Saveh area (October 2013). Voucher specimens (Farabi Herbarium number GUE 7321) were authenticated by Associate Professor Ali Mazooji, Department of Biology, Faculty of Biology, Islamic Azad University. The plant material was dried under shade and powdered using Ultra-Torax. The powder (60 g) was extracted with 300 mL aqueous 80% ethanol in a Soxhlet apparatus for 72 hours. The extract was filtered and concentrated to dryness under reduced pressure in a rotary evaporator at 40–50°C yielding 15.3% (w/w) plant extract. The extract yield was 19%. The obtained pomegranate alcoholic extract was stored at −20°C until usage. Plant extract was suspended in saline (doses 50, 100, 200, and 300 mg/kg body weight) prior to intraperitoneal administration to the experimental animals.

2.2. Experimental Animals and Induction of Hyperlipidemia. Male Wistar rats initially weighing 200 to 250 g purchased from the Pasteur Institute (Karaj, Iran) were used in the experiments. The diet was purchased from Pars-Dam food service, Tehran, Iran. The animal room was maintained at 22°C ± 2°C with timed lighting on from 7 AM to 19 PM and relative air humidity of 40% to 60%. Each animal was used once only. The animal protocol was approved by the Ethics Committee of Islamic Azad University, Tehran, Iran, and conforms to the guidelines of the Committee for the Purpose of Control and Supervision on Experiments on Animals, Iran, and also to international guidelines. Accordingly, five rats were housed per cage of size 50 cm × 23 cm. Hyperlipidemia was induced by feeding 10% lipid supplemented in the basal diet. The basal diet contained (g% of final diet), casein 15.0, sucrose 68.3, hydrogenated coconut oil 10.0, cellulose 2.0, salt mixture 4.0, vitamin mixture 0.5, and choline chloride 0.2. The animals were distributed into six groups each containing 8 rats. The control group was fed on the basal diet and given water *ad libitum*. Extract was dissolved in saline and administered intraperitoneally (i.p.) for 23 days. Animals in the control group received only 0.5 mL saline as vehicle.

Experimental groups were as follows: Group 1: normal control, fed on basal diet; Group 2: untreated control, fed on 10% lipid diet and given saline 0.5 mL/rat (i.p.); Groups 3, 4, 5, and 6, fed on 10% lipid in diet and administered extract at doses 50, 100, 200, and 300 mg/kg/day (i.p.).

The initial body weights of all the animals in each group were measured. After 23 days, the rats were fasted for 12 h and their final body weights were determined. Then, rats were fasted overnight, and blood samples were drawn from heart under light ether anaesthesia. The animals were removed after blood collection. Serum cholesterol, triglyceride, LDL, HDL, aspartate aminotransferase (AST), and alanine aminotransferase (ALT) levels were determined by kit (Parsazmoon Company, Iran).

2.3. Histopathological Studies in the Liver. For qualitative analysis of liver histology, the tissue samples were fixed for 48 h in 10% formalin-saline and dehydrated by passing successfully in different mixtures of ethyl alcohol-water, cleaned in xylene, and embedded in paraffin. Sections of the tissue were prepared by using a rotary microtome and stained with haematoxylin and eosin dye, which was mounted in a neutral deparaffinated xylene medium for microscopic observations. Histological damage including fatty change in hepatocyte, dilation of sinozoid, and congestion in high lipid diet fed. Each damage is given 1 score.

2.4. Statistical Analysis. Statistical analyses and representations were performed in Microsoft Excel. All data was analyzed by one-way ANOVA and presented as the mean value ± S.E.M. of eight rats ($n = 8$). The results of the lipid fed untreated control group were compared to normal control group and those of extract treated groups were compared to untreated control group. P values were checked at three levels of significance, namely, 0.05, 0.01, and 0.001. P value less than 0.05 was considered "significant" and P less than 0.01 and 0.001 as "highly significant."

3. Results

3.1. General Improvement in the Hyperlipidemic State. Changes in initial and final body weights in control and experimental groups are shown in Figure 1. The results showed treatment of extract decreased final body weight elevations in comparison to control saline group ($P < 0.01$).

The results showed treatment of extract decreased liver and kidney coefficients (liver weight/body weight and kidney weight/body weight) in comparison to control saline group, insignificantly. The administration of the pomegranate peel extract (50, 100, 200, and 300 mg/kg body wt) significantly decreased serum triglycerides, cholesterol, LDL, AP, ALT, and AST levels, while increasing serum HDL level in high lipid diet fed rats compared with saline group (Table 1).

Histopathological study shows that the administration of the pomegranate peel extract (50, 100, 200, and 300 mg/kg body wt) significantly decreased histopathological damage of liver including fatty change in hepatocyte, dilation of sinusoid, and congestion (Figure 2) in high lipid diet fed rats compared with saline group (Table 1).

4. Discussion

Dyslipidemia is a multifactorial and polygenic disorder resulting from an interaction between an individual's genetic background and multiple environmental factors including behavioural and social risk factors [10].

Table 1: Effect of i.p. administration of hydroethanolic extract of *Punica granatum* peel at doses 50, 100, 200, and 300 mg/kg on liver and kidney coefficients, serum parameters, and histopathological damage of liver in high lipid diet fed rats.

Parameters	Control	Saline	Extract (mg/kg)			
			50	100	200	300
Liver coefficient	0.028	0.042	0.037	0.035	0.039	0.039
Kidney coefficient	0.0027	0.0036	0.0033	0.0033^{+}	0.0035	0.0036
Triglycerides (mg/dL)	146 ± 21	$475 \pm 11^{***}$	381 ± 23	325 ± 43	302 ± 31	$210 \pm 27^{+++}$
Cholesterol (mg/dL)	73 ± 8	$110 \pm 11^{***}$	$87 \pm 9^{++}$	$82 \pm 5^{++}$	$80 \pm 9^{++}$	$81 \pm 7^{++}$
LDL (mg/dL)	92 ± 6	$321 \pm 11^{**}$	209 ± 23	$145 \pm 29^{+}$	$79 \pm 8^{++}$	$61 \pm 7^{+++}$
HDL (mg/dL)	98 ± 9	$28 \pm 3^{*}$	89 ± 11	$128 \pm 5^{+++}$	$179 \pm 18^{+++}$	$185 \pm 20^{+++}$
AST (UI/L)	1234 ± 34	$1538 \pm 47^{***}$	$1219 \pm 39^{+++}$	$1232 \pm 71^{+++}$	$1170 \pm 49^{+++}$	$1233 \pm 36^{+++}$
ALT (UI/L)	1267 ± 56	$1553 \pm 32^{***}$	$1130 \pm 44^{+++}$	$1246 \pm 51^{+++}$	$1233 \pm 68^{+++}$	$1290 \pm 54^{+++}$
AP (UI/L)	983 ± 21	$1362 \pm 71^{***}$	1311 ± 39	1248 ± 48	$1100 \pm 59^{++}$	$1049 \pm 78^{+++}$
Histopathological damage of liver	0 ± 0	$1.5 \pm 0.11^{*}$	0.5 ± 0.23	0.4 ± 0.23	$0.17 \pm 0.31^{+}$	$0.09 \pm 0.27^{+}$

$^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ different from control group.
$^{+}P < 0.05$, $^{++}P < 0.01$, $^{+++}P < 0.001$ different from saline group.

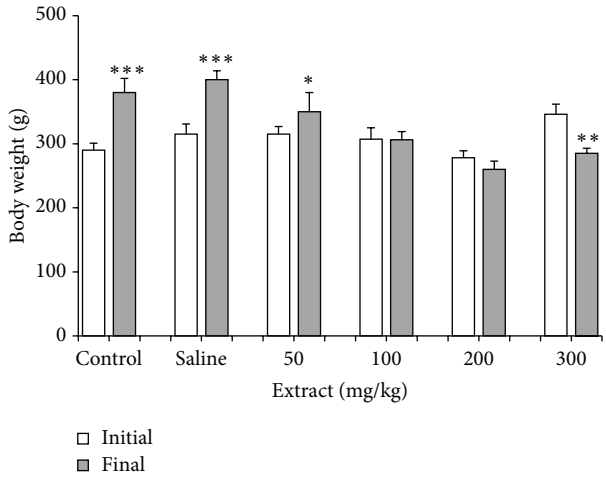

Figure 1: Effect of i.p. administration of pomegranate peel hydroethanolic extract at doses of 50, 100, 200, and 300 mg/kg body wt on body weight in high lipid diet fed rats. Each column represents mean ± SEM for 8 rats. Control saline group was administrated with saline as vehicle. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ different from initial body weight in each group.

Fruits are rich sources of vitamins, minerals, and biologically active compounds. However, very often they are consumed without the peels despite the fact that some fruit peels are rich in polyphenolic compounds, flavonoids, ascorbic acid, and other biologically active components that have positive influence on health [2, 17].

Our results demonstrated that administration of hydroethanolic extract from *Punica granatum* peel showed marked antihyperlipidemic effects in high lipid diet fed rats. Pomegranate extract decreased serum cholesterol, triglycerides, LDL, ALT, AST, and AP, while increasing serum HDL levels in high lipid diet fed rats in comparison to saline treated rats. Also, the extract attenuated liver damage including fatty change in hepatocyte, dilation of sinusoid, and congestion in high lipid diet fed rats compared with saline group.

In agreement, it is reported that the different parts of pomegranate (*Punica granatum* L.) have been known as a reservoir of bioactive compounds with potential biological activities. Pomegranate, especially the leaves of pomegranate, decreased the dyslipidemia of obesity and cardiovascular risk factors [10]. The ability of pomegranate leaves extracts to fight obesity is shown [14].

On the other hand, pomegranate flower has been demonstrated to ameliorate hyperlipidemia and decrease excess cardiac lipid accumulation in Zuker diabetic fatty rats [18] and to attenuate atherosclerosis in apolipoprotein E deficient mice [19]. Moreover, oleanolic acid and ursolic acid, two of the active components contained in pomegranate flower [20], have been long-recognized to have antihyperlipidemic properties [21]. Gallic acid, another important component in pomegranate flower [20], has been demonstrated to improve high fat diet induced hyperlipidemia and fatty liver in mice [22].

Also, Parmar and Kar reported pomegranate peel extract ameliorated biochemical and histopathologic alterations induced by the atherogenic diet [23]. The protective role of the fruit peel could be related to its flavonoids and polyphenolic contents, which possess antioxidative activity [24]. Moreover, the juice of *P. granatum* is also known to prevent atherosclerosis, which further supports its antiatherogenic potential [25].

It is reported that addition of pomegranate juice to simvastatin in a macrophage cell culture model system improves the statin ability to inhibit cellular cholesterol biosynthesis and to protect the cells from oxidative stress. These effects could be related to the antioxidant hydrolyzable tannin punicalagin and to the phytosterol β-sitosterol, which are both present in pomegranate [26]. Moreover, phytosterols of pomegranate consumption decreased serum cholesterol levels in dyslipidemic patients, as well as their cardiovascular risk [27, 28].

As a result, it may be concluded that pomegranate peel seeds extract possesses antilipidemic activities in high lipid

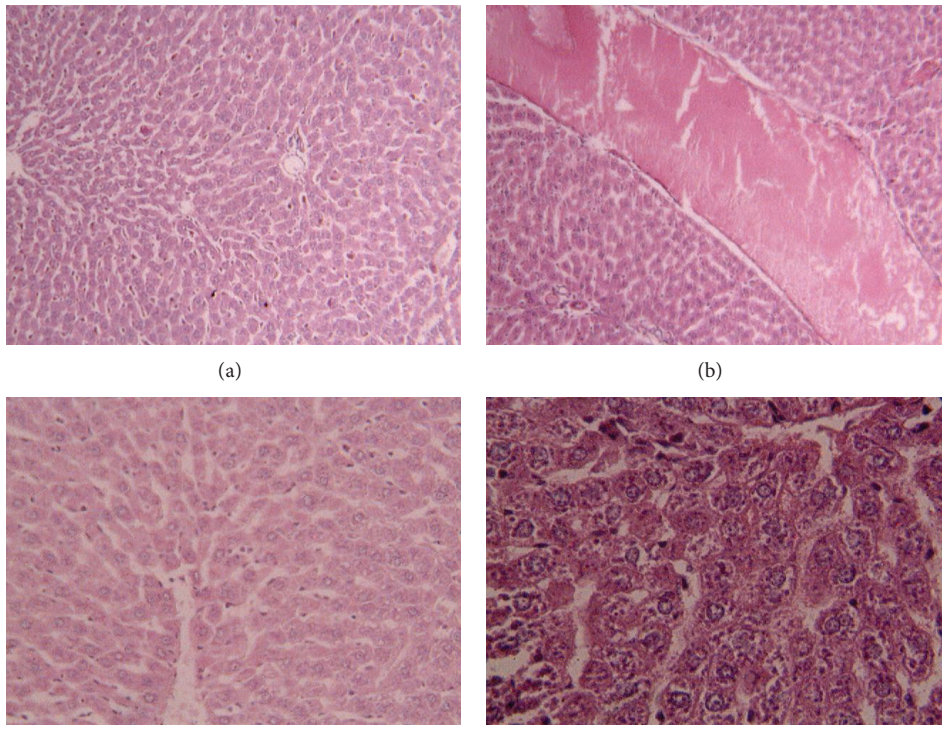

Figure 2: Histopathology of liver tissue in high lipid diet and normal diet fed rats (hematoxylin-eosin). (a) Control liver tissue (×100), (b) congestion damage in high lipid diet fed rats (×100), (c) dilation of sinusoid (×400), and (d) fatty change in hepatocyte (×100).

diet fed rats and that the pomegranate peel extract may be of use as an antidyslipidemic agent. It is concluded that the plant should be considered as an excellent candidate for future studies on dyslipidemia. In addition, further comprehensive pharmacologic investigations, including experimental chronic studies, should be carried out.

Conflict of Interests

The authors declare that there is no conflict of interests.

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References

[1] Z. Yan-Ling, Z. Dong-Qing, H. Chang-Quan, and D. Bi-Rong, "Cigarette smoking and its association with serum lipid/lipoprotein among Chinese nonagenarians/centenarians," *Lipids in Health and Disease*, vol. 11, no. 1, article 94, 2012.

[2] W. P. Castelli, R. J. Garrison, and P. W. F. Wilson, "Incidence of coronary heart disease and lipoprotein cholesterol levels. The Framingham Study," *The Journal of the American Medical Association*, vol. 256, no. 20, pp. 2835–2838, 1986.

[3] Lipid Research Clinics Program, "The lipid research clinics coronary primary prevention trial results. I. Reduction in incidence of coronary heart disease," *Journal of the American Medical Association*, vol. 251, no. 3, pp. 351–364, 1984.

[4] Lipid Research Clinics Program, "The Lipid Research Clinics Coronary Primary Prevention Trial results. II. The relationship of reduction in incidence of coronary heart disease to cholesterol lowering," *Journal of the American Medical Association*, vol. 251, 365, no. 3, p. 374, 1984.

[5] T. Okamura, "Dyslipidemia and cardiovascular disease: a series of epidemiologic studies in Japanese populations," *Journal of Epidemiology*, vol. 20, no. 4, pp. 259–265, 2010.

[6] A. R. Sharrett, C. M. Ballantyne, S. A. Coady et al., "Coronary heart disease prediction from lipoprotein cholesterol levels, triglycerides, lipoprotein(a), apolipoproteins A-I and B, and HDL density subfractions: the Atherosclerosis Risk in Communities (ARIC) Study," *Circulation*, vol. 104, no. 10, pp. 1108–1113, 2001.

[7] P. P. Toth, "Subclinical atherosclerosis: what it is, what it means and what we can do about it," *International Journal of Clinical Practice*, vol. 62, no. 8, pp. 1246–1254, 2008.

[8] G. V. Satyavati, A. K. Gupta, and N. Tandon, *Medicinal Plants of India*, vol. 2, Indian Council of Medical Research, New Delhi, India, 1978.

[9] M. Viuda-Martos, J. Fernández-López, and J. A. Pérez-Álvarez, "Pomegranate and its many functional components as related to human health: a review," *Comprehensive Reviews in Food Science and Food Safety*, vol. 9, no. 6, pp. 635–654, 2010.

[10] F. Lei, X. N. Zhang, W. Wang et al., "Evidence of anti-obesity effects of the pomegranate leaf extract in high-fat diet induced obese mice," *International Journal of Obesity*, vol. 31, no. 6, pp. 1023–1029, 2007.

[11] H. O. Egharevba, O. F. Kunle, I. Iliya et al., "Phytochemical analysis and antimicrobial activity of *Punica granatum* L. (fruit bark and leaves)," *New York Science Journal*, vol. 3, no. 3, pp. 91–98, 2010.

[12] A. El-Shennawy, E. Ali, W. El-Komy et al., "Evaluation of ponytail antiparasitic activity of pomegranate juice, peels and leaves against Giardia lamblia," *International Journal of Infectious Diseases*, vol. 14, no. 2, p. S84, 2010.

[13] C. Wang, L. Shi, L. Fan et al., "Optimization of extraction and enrichment of phenolics from pomegranate (*Punica granatum* L.) leaves," *Industrial Crops and Products*, vol. 42, no. 1, pp. 587–594, 2013.

[14] M. N. Al-Muammar and F. F. Khan, "Obesity: the preventive role of the pomegranate (*Punica granatum*)," *Nutrition*, vol. 28, no. 6, pp. 595–604, 2012.

[15] E. P. Lansky and R. A. Newman, "*Punica granatum* (pomegranate) and its potential for prevention and treatment of inflammation and cancer," *Journal of Ethnopharmacology*, vol. 109, no. 2, pp. 177–206, 2007.

[16] K. Z. Xu, C. Zhu, M. S. Kim, J. Yamahara, and Y. Li, "Pomegranate flower ameliorates fatty liver in an animal model of type 2 diabetes and obesity," *Journal of Ethnopharmacology*, vol. 123, no. 2, pp. 280–287, 2009.

[17] M. Leontowicz, S. Gorinstein, H. Leontowicz et al., "Apple and pear peel and pulp and their influence on plasma lipids and antioxidant potentials in rats fed cholesterol-containing diets," *Journal of Agricultural and Food Chemistry*, vol. 51, no. 19, pp. 5780–5785, 2003.

[18] T. H. W. Huang, Q. Yang, M. Harada et al., "Pomegranate flower extract diminishes cardiac fibrosis in zucker diabetic fatty rats: modulation of cardiac endothelin-1 and nuclear factor-kappaB pathways," *Journal of Cardiovascular Pharmacology*, vol. 46, no. 6, pp. 856–862, 2005.

[19] M. Aviram, N. Volkova, R. Coleman et al., "Pomegranate phenolics from the peels, arils, and flowers are antiatherogenic: studies in vivo in atherosclerotic apolipoprotein E-deficient (E0) mice and in vitro in cultured macrophages and lipoproteins," *Journal of Agricultural and Food Chemistry*, vol. 56, no. 3, pp. 1148–1157, 2008.

[20] Y. Li, Y. Qi, T. H. W. Huang, J. Yamahara, and B. D. Roufogalis, "Pomegranate flower: a unique traditional antidiabetic medicine with dual PPAR-α/-γ activator properties," *Diabetes, Obesity and Metabolism*, vol. 10, no. 1, pp. 10–17, 2008.

[21] J. Liu, "Oleanolic acid and ursolic acid: research perspectives," *Journal of Ethnopharmacology*, vol. 100, no. 1-2, pp. 92–94, 2005.

[22] A. Jang, P. Srinivasan, N. Y. Lee et al., "Comparison of hypolipidemic activity of synthetic gallic acid-linoleic acid ester with mixture of gallic acid and linoleic acid, gallic acid, and linoleic acid on high-fat diet induced obesity in C57BL/6 Cr Slc mice," *Chemico-Biological Interactions*, vol. 174, no. 2, pp. 109–117, 2008.

[23] H. S. Parmar and A. Kar, "Protective role of *Citrus sinensis*, *Musa paradisiaca*, and *Punica granatum* peels against diet-induced atherosclerosis and thyroid dysfunctions in rats," *Nutrition Research*, vol. 27, no. 11, pp. 710–718, 2007.

[24] K. N. Chidambara Murthy, G. K. Jayaprakasha, and R. P. Singh, "Studies on antioxidant activity of pomegranate (*Punica granatum*) peel extract using in vivo models," *Journal of Agricultural and Food Chemistry*, vol. 50, no. 17, pp. 4791–4795, 2002.

[25] P. Sestili, C. Martinelli, D. Ricci et al., "Cytoprotective effect of preparations from various parts of Punica granatum L. fruits in oxidatively injured mammalian cells in comparison with their antioxidant capacity in cell free systems," *Pharmacological Research*, vol. 56, no. 1, pp. 18–26, 2007.

[26] M. Rosenblat, N. Volkova, and M. Aviram, "Pomegranate phytosterol (β-sitosterol) and polyphenolic antioxidant (punicalagin) addition to statin, significantly protected against macrophage foam cells formation," *Atherosclerosis*, vol. 226, no. 1, pp. 110–117, 2013.

[27] M. Rocha, C. Bañuls, L. Bellod, A. Jover, V. M. Víctor, and A. Hernández-Mijares, "A review on the role of phytosterols: new insights into cardiovascular risk," *Current Pharmaceutical Design*, vol. 17, no. 36, pp. 4061–4075, 2011.

[28] K. G. Moruisi, W. Oosthuizen, and A. M. Opperman, "Phytosterols/stanols lower cholesterol concentrations in familial hypercholesterolemia subjects: a systematic review with meta-analysis," *Journal of the American College of Nutrition*, vol. 25, no. 1, pp. 41–48, 2006.

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Research Article

Camellia sinensis (L.) Kuntze Extract Ameliorates Chronic Ethanol-Induced Hepatotoxicity in Albino Rats

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The goal of this study was to investigate the hepatoprotective effects of aqueous extract of *Camellia sinensis* or green tea extract (AQGTE) in chronic ethanol-induced albino rats. All animals were divided into 4 groups in the study for a 5-week duration. 50% ethanol was given orally to the rats with two doses (5 mg/kg bw and 10 mg/kg bw) of AQGTE. Ethanol administration caused a significant increase in the levels of plasma and serum enzymatic markers, alanine aminotransferase (ALT), aspartate aminotransferase (AST), and alkaline phosphatase (ALP), and nonenzymatic markers (cholesterol and triglycerides), lipid peroxidation contents, malondialdehyde (MDA), and glutathione-S-transferase (GST), and decreased the activities of total proteins, albumin, and cellular antioxidant defense enzymes such as superoxide dismutase (SOD). The elevation and reduction in these biochemical enzymes caused the damage in hepatocytes histologically due to the high production of ROS, which retards the antioxidant defense capacity of cell. AQGTE was capable of recovering the level of these markers and the damaged hepatocytes to their normal structures. These results support the suggestion that AQGTE was able to enhance hepatoprotective and antioxidant effects *in vivo* against ethanol-induced toxicity.

1. Introduction

Alcohol is widely consumed in alcoholic drinks in modern society, and ethanol is one of the main causes of a variety of medical problems and liver diseases worldwide [1]. The liver is the major target organ of ethanol toxicity [2]. Chronic ethanol feeding causes a decrease in the major antioxidant factors in the liver, including enzymes [3, 4] and nonenzymatic antioxidants [5, 6]. This is due to the generation of an excessive amount of reactive oxygen species (ROS), which results in the detrimental effects of the cellular antioxidant defense system [7, 8]. Thus, excess alcohol consumption may accelerate an oxidative mechanism directly or indirectly, which eventually produces cell death and tissue damage [9–13]. Therefore, alternative treatments for liver disorders are needed to replace the existing synthetic drugs.

The plants having antioxidants prevent the cell death and tissue damage resulting from chronic alcohol consumption [14].

Green tea (*Camellia sinensis*, Theaceae) is the second most popular beverage worldwide [15]. It contains six primary catechins or polyphenol compounds. These constituents have potent antioxidant action and their putative disease preventive effects [16]. These polyphenols prevent oxygen-free radicals-induced hepatocyte lethality, reduce the risk of liver disease, and protect against liver injury, that is, fibrosis and liver cirrhosis in rats [17–19]. In the current study, we evaluated the influence of green tea extract on liver specific enzymatic and nonenzymatic markers, lipid peroxidation, antioxidants in blood, and liver histology associated with chronic ethanol consumption.

2. Materials and Methods

2.1. Chemicals. Ethanol (purity (GC) ≥ 99.9%) and all other chemicals were from Merck (Merck KGaA, Darmstadt, Germany). All chemicals used were of analytical grade.

2.2. Animal Treatment. Twenty-four healthy male Wistar strain rats (100–140 g; 14–16 weeks old) were obtained from animal house of C.C.S, University, Meerut, U.P., India. All animals were acclimatized for laboratory conditions at room temperature and kept on normal diet. The tap water ad libitum was also provided for two weeks before starting the experiment. All animals were cared for according to guidelines of the Institutional Animal Ethics registered by IAEC (384/PO/a/01/CPCSEA 27-03-2015). Committee (IAEC) and experiments were also approved. The experiment duration was 5 weeks. All animals were divided into 4 groups: controls (received distilled water as drinking source), ethanol control (0.5 mL C_2H_5OH/100 gm body weight), ethanol + GTE (0.5 mL C_2H_5OH + 5 mg GTE/100 gm body weight), and ethanol + GTE (0.5 mL C2H5OH + 10 mg GTE/100 gm body weight). The animals of alcohol control group were administered oral dose of ethanol everyday between 10:00 AM and 11:00 AM. Experimental animals of group 3 and group 4 were given orally 5 mg and 10 mg/100 gm body weight dose of GTE, respectively, after 1 hour of feeding of alcohol.

Camellia sinensis was procured from Tea State of Tata Group of Company, TALAT, Assam, H.P. (India). Preparation of GTE was done according to the method described by [20]. Green tea is prepared by picking the leaves, lightly steaming them, and allowing them to dry. The dried material was ground into powder using mortar and pestle and sieved with a sieve. 100 g of the powdered plant material was steeped in 600 mL of distilled water and heated in water bath for 3 h at 90°C. The mixture was allowed to cool to room temperature, filtered, and dried. Total 3 gm of yield of dried GTE was obtained. This GTE was used to treat rats.

At the end of study, body weights of all animals were recorded, then sacrificed under light ether anesthesia, and dissected. The blood samples were collected by retroorbital plexus in fluoride and plain glass tubes. Liver was removed, weighed, and processed for biochemical studies. Blood samples from rats were immediately centrifuged at 3000 rpm for 10 min at 4°C for serum samples. The supernatant of serum was separated from the pellet and used for biochemical analysis.

2.3. Histopathological Examination. After scarification of rats, the samples of liver tissues were collected from all groups. For histological study, they were fixed in 10% neutral buffered formalin, dehydrated in ascending grades of ethanol alcohols, cleared in xylol, casting, blocking, cutting at 5 μm thickness, and stained [21]. For the homogenate, liver was removed quickly and kept in iced 0.15 M NaCl solution, for removing the blood cells, blotted on filter paper, weighed, and homogenized. The supernatant was kept on ice until assayed after centrifugation of homogenates at 10,000 g for 15 min at 4°C.

2.4. Biochemical Analysis

2.4.1. Measurement of Blood Alcohol. Blood was taken from the tail vein 1 h after gavage, 2 weeks after initiation of alcohol. Blood alcohol levels (BAL) were measured using the alcohol dehydrogenase kit from Sigma Chemical Co., India [22].

2.4.2. Determination of Liver Enzymatic Markers. The levels of liver enzymes were measured using commercial kits (Sigma Chemical Co., India). The plasma levels of alanine transaminase (ALT) and aspartate transaminase (AST) were estimated according to [23] and alkaline phosphatase (ALP) was according to [24].

2.4.3. Determination of Liver Nonenzymatic Markers. The levels of cholesterol and triglycerides were measured by using standard assay kits (Sigma Chemical Co., India).

2.4.4. Determination of Total Proteins Levels. Estimation of total proteins levels in plasma and in liver tissue homogenates was measured by the method previously described using bovine serum albumin as the standard [25]. The plasma levels of albumin were determined using commercial kit (Sigma Chemical Co., India) according to Pinnell and Northam colorimetric method [26].

2.4.5. Determination of Liver MDA Contents. The tissues of liver were thawed, weighed, and homogenized 1 : 9 w : v in 0.9% saline. Then the homogenates were centrifuged at 3000 rpm for 10 min at 4°C in a high-speed centrifuge and the supernatant was taken for the assays of MDA (a measure of lipid peroxidation) contents. MDA was assayed by the measurement of thiobarbituric acid-reactive substances (TBARS) levels spectrophotometrically at 532 nm. Results were expressed as nmol·mg^{-1} protein [27].

2.4.6. Determination of Antioxidants. The level of superoxide dismutase (SOD) in liver tissue homogenate was assayed according to [28]. Level of GST in liver homogenates was measured according to Habig et al. [29].

2.5. Statistical Analysis. The results were presented as mean ± SD. Statistical significance and differences from control and test values were evaluated by Student's t-test. Statistical probability $P < 0.05$ was considered as statistically significant. Statistical analysis was conducted by using Sigma Plot software (Version 11).

3. Results

3.1. Measurement of Blood Alcohol. The rats increased their weight at a constant rate in each of the groups studied; there was no difference in weight gain among the groups. At week 2, BAL 1 h after ethanol administration by gavage was similar in alcohol/GTE group (376.6 ± 68.1 mg/100 mL) and alcohol group (387.3 ± 51.9 mg/100 mL).

3.2. GTE Effect on C_2H_5OH Induced Changes in Liver Enzymatic Markers. The levels of ALT, AST, and ALP were evaluated in albino rats serum. As shown in Tables 1 and 2, a single dose of EtOH (0.5 mL/100 gm body weight) caused hepatotoxicity. The levels of these enzymatic markers in ethanol control group were increased significantly as compared to control groups. These increased levels were due to the liver cell injuries induced by EtOH. Administration of GTE significantly prevented the EtOH-induced elevation of serum ALT, AST, and ALP levels.

Table 1: Effect of AQGTE on concentrations of nonenzymatic biochemical parameters in blood in chronic ethanol-induced hepatic damage in rats.

Analyzed parameters	Controls	EtOH group **0.5 mL/kg B wt.**	EtOH + GTE group **0.5 mL + 5 mg/kg B wt.**	EtOH + GTE group **0.5 mL + 10 mg/kg B wt.**
AST (IU/L)	22.00 ± 3.139	46.50 ± 2.715***	42.50 ± 2.184****	28.16 ± 2.119***
ALT (IU/L)	39.00 ± 2.678	100.00 ± 5.319***	54.83 ± 4.148***	24.83 ± 1.678***
ALP (KA Units/100 mL)	104.00 ± 8.871	144.00 ± 3.551***	130.50 ± 3.303***	105.83 ± 4.548***

Values represent mean ± SEM; $n = 6$; significance as per Student's t-test.
*$P < 0.01$, **$P < 0.005$, ***$P < 0.001$, **** nonsignificant, ***** no change.

Table 2: Effect of AQGTE on concentrations of nonenzymatic biochemical parameters in liver tissue in chronic ethanol-induced hepatic damage in rats.

Analyzed parameters	Controls	EtOH group **0.5 mL/kg B wt.**	EtOH + GTE group **0.5 mL + 5 mg/kg B wt.**	EtOH + GTE group **0.5 mL + 10 mg/kg B wt.**
AST (IU/L)	1458 ± 119.5	2564 ± 116.42***	24150 ± 111.20****	2398 ± 87.29***
ALT (IU/L)	2230 ± 146.1	2564 ± 116.42***	2415 ± 111.45***	2398 ± 87.89***
ALP (KA Units/100 mL)	108.00 ± 8.867	136.16 ± 1.490***	129.66 ± 0.98	124.01 ± 1.06***

Values represent mean ± SEM; $n = 6$; significance as per Student's t-test.
*$P < 0.01$, **$P < 0.005$, ***$P < 0.001$, **** nonsignificant, ***** no change.

Table 3: Effect of AQGTE on concentrations of nonenzymatic biochemical parameters in liver tissue in chronic ethanol-induced hepatic damage in rats.

Analyzed parameters	Controls	EtOH group **0.5 mL/kg B wt.**	EtOH + GTE group **0.5 mL + 5 mg/kg B wt.**	EtOH + GTE group **0.5 mL + 10 mg/kg B wt.**
Cholesterol (mg/dL)	56.21 ± 5.279	76.54 ± 2.364*	67.65 ± 1.89****	57.30 ± 1.115***
Triglycerides (U/L)	4.70 ± 0.218	32.50 ± 1.453***	21.26 ± 0.459****	6.03 ± 0.490***
Total protein (g/dL)	4.06 ± 0.816	2.52 ± 0.159***	3.55 ± 0.216****	3.91 ± 0.131***
Albumin g/dL	2.7 ± 0.196	1.38 ± 0.106***	1.88 ± 0.217****	2.07 ± 0.216***
Globulin g/dL	1.38 ± 0.186	1.12 ± 0.077****	1.68 ± 0.245****	1.86 ± 0.216****
Urea (mg/dL)	22.01 ± 1.261	23.00 ± 0.633*****	24.00 ± 1.63	26.33 ± 2.85

Values represent mean ± SEM; $n = 6$; significance as per Student's t-test.
*$P < 0.01$, **$P < 0.005$, ***$P < 0.001$, **** nonsignificant, ***** no change.

3.3. GTE Effect on C_2H_5OH Induced Changes in Liver Nonenzymatic Markers. The cholesterol and triglycerides level of untreated ethanol control group was significantly higher than the other experimental groups (Table 3). In contrast, the levels of cholesterol and triglycerides of the 5 mg/kg body weight of GTE with EtOH groups were significantly lower than the ethanol control group. Added to this, 10 mg/kg body weight of GTE with EtOH was able to reduce the level of these nonenzymatic markers to near the normal value, whereas the level of urea was normal till the duration of experiments.

3.4. Determination of Total Proteins Levels in Liver. The level of total proteins depends upon the addition of albumin and globulin levels. The level of albumin in ethanol group was significantly lower than the control group, whereas the globulin level was normal. The level of albumin in 5 mg/kg body weight of GTE with EtOH groups was significantly higher, whereas 10 mg/kg body weight dose of GTE was able to increase the level of albumin to near the normal value when compared with ethanol and control groups.

3.5. GTE Effect on C_2H_5OH Induced Changes in Liver MDA Contents. The level of MDA (Table 4) in ethanol control group was significantly increased as compared to control groups. The increased level of MDA in ethanol control group indicated the presence of lipid peroxidation of liver cells, which was due to the toxic effect of EtOH. Furthermore, all of the GTE treatment groups were significantly different as compared to ethanol control group, and the MDA content was normalized as compared to normal control and ethanol control groups. Both doses of GTE were able to reduce the level of MDA as compared to normal control.

3.6. GTE Effect on C_2H_5OH Induced Changes in Activity of Antioxidant Enzymes. Table 4 summarizes the activities of hepatic antioxidant enzymes. Chronic EtOH administration to rats caused a significant decrease in the activities of SOD and a significant increase in the level of GST. 5 mg doses of GTE were able to increase the level of SOD and decrease the level of GST as compared to ethanol control. 10 mg doses of GTE were able to normalize the levels of SOD and GST as compared to control groups and showed a spectacular restoration of hepatic SOD and GST activities.

3.7. Histopathological Examination. The liver sections from different experimental groups were used to observe

Table 4: Effect of AQGTE on concentrations of MDA content and activity of antioxidant enzymes in liver tissue in chronic ethanol-induced hepatic damage in rats.

Analyzed parameters	Controls	EtOH group **0.5 mL/kg B wt.**	EtOH + GTE group **0.5 mL + 5 mg/kg B wt.**	EtOH + GTE group **0.5 mL + 10 mg/kg B wt.**
MDA (nmol/mg protein)[1]	4.20 ± 0.068	10.20 ± 0.483***	6.00 ± 0.266****	3.90 ± 0.085***
SOD (U/mg protein)[1]	16.15 ± 0.054	11.44 ± 0.168***	14.54 ± 0.145****	18.36 ± 0.131***
GST (μg/min/mg protein)	3.23 ± 0.154	6.96 ± 0.205***	4.20 ± 0.169****	3.46 ± 0.023***

Values represent mean ± SEM; $n = 6$; significance as per Student's t-test.
$^{*}P < 0.01$, $^{**}P < 0.005$, $^{***}P < 0.001$, **** nonsignificant, ***** no change.
[1]Number of nmol per 1.

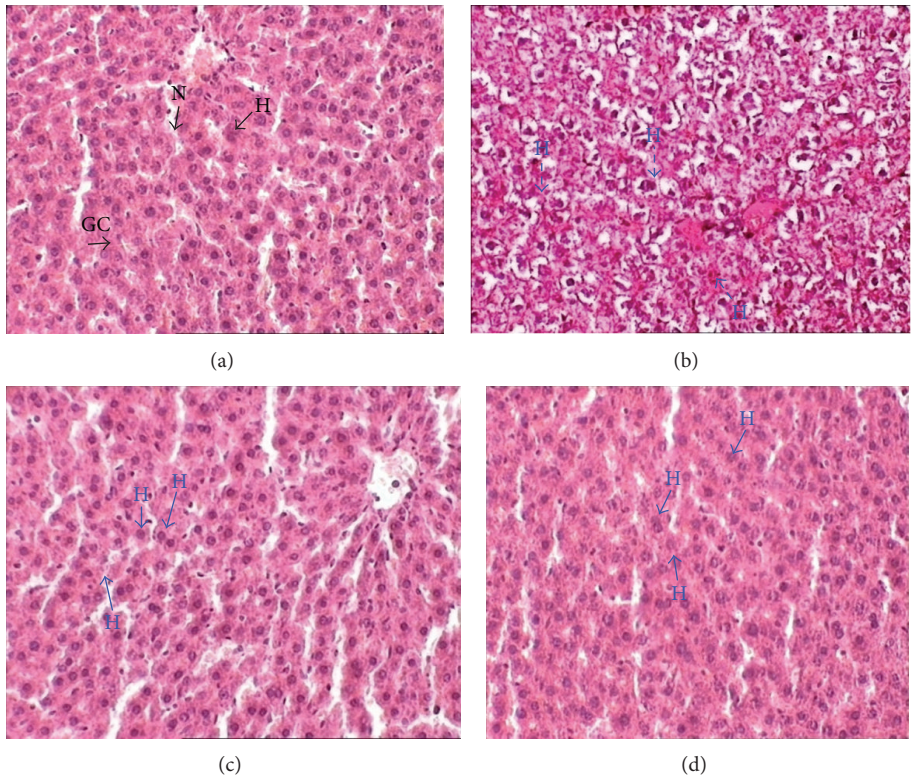

Figure 1: Microsection of liver of different experimental groups showing normal structure having hepatocytes (H) with granular cytoplasm (GC), and clear nucleus (N) was stained with hematoxylin and eosin with the magnification of 400x. (a) Normal control with normal structure of liver cells. (b) Ethanol control with several microvacuolations of hepatocytes condition as indicated by the dashed line arrows. (c) 0.5 mL EtOH + 5 mg/100 gm AQGTE-treated group with recovery effect from microvacuolation of hepatocytes. (d) 0.5 mL EtOH + 10 mg/100 gm AQGTE-treated group with recovery effect from microvacuolation of hepatocytes. H = hepatocyte, S = sinusoidal space, hepatocyte developed with microvacuolation.

histopathological changes which were stained with H and E staining and observed via a light microscope (Figure 1). In normal control group (Figure 1(a)), normal hepatocytes were observed, while, in ethanol control (Figure 1(b)), the hepatocytes showed fatty changes which were also known as microvesicular steatosis where the hepatocytes developed an open space around the nuclei. The administration of 5 mg/kg and 10 mg/kg body weight of GTE with EtOH showed a recovery effect of hepatocytes with minimal to no microvesicular steatosis. These results showed that GTE treatment resulted in a protective effect on EtOH-induced hepatotoxicity.

4. Discussion

Ethanol is a hepatotoxicant to induce liver damage since it is clinically relevant [30]. This liver damage is associated with several reactions of free radicals such as reactive oxygen species (ROS) which causes elevation in MDA and GST content while reducing the level of SOD. The elevation and reduction of enzymatic and non-enzymatic markers of serum were also associated with this condition. Alcoholic liver disease was normally found in liver histology. GTE was enriched with antioxidants that could revert and lower the free radicals level. It had shown that the beneficial effects

of this phytochemical in preventing the ethanol-induced hepatotoxicity are mediated by the antioxidant effects.

In the present study, we evaluated the protective effects of GTE against ethanol-induced hepatotoxicity. Ethanol administration increased the BAL, which caused the changes in the behavior of rats. Both doses of green tea extract normalized this change in BALs [22]. Ethanol administration elevated the concentrations of key cellular enzymes like AST, ALT, and ALP present in the liver cells leak into the serum during liver damage [31–35]. Elevated activities of these enzymes indicate hepatocytes damage where the leakage of cell membrane participated in the accumulation of these enzymes into the plasma [36, 37]. This is because of higher concentration of alcohol dehydrogenase in liver, which catalyzes alcohol to its corresponding aldehyde [38]. GTE had the ability to reduce the level of these enzyme markers. Therefore, prolonged treatment of 10 mg/100 gm body weight of GTE administration could help to normalize the ALT, AST, and ALP enzyme levels. Our results are also consistent with protective effects of different extracts with antioxidant ability against alcohol-induced hepatocyte cells of liver [20, 35, 39–41]. Ethanol administration decreased (serum protein and albumin) and increased (cholesterol and TG) the levels of nonenzymatic markers which caused the liver damage. This damage is attributed to the higher concentration of alcohol dehydrogenase enzyme which catalyses alcohol to aldehyde and accumulation of export type proteins due to inhibition of the secretion of the proteins from the liver of alcoholics [38, 42]. Both doses of GTE restored the low level of protein in a dose dependent manner to normal level [43–45]. Level of cholesterol was increased with ethanol and decreased with both doses of GTE in all treatment groups [46].

Liver histology of such experimental animals also showed improvement (Figure 1). In this study these biochemical tests were supported by histopathological observations of liver sections [47].

The level of MDA and GST in cirrhotic rats was found to be high as compared to the controls [48, 49]. The levels of MDA and GST content were low in GTE treated group (Table 4) as polyphenol rich green tea extracts inhibit lipid peroxidation in experimental rats [50]. During this study, the antioxidant system of cirrhotic rats was severely impaired, causing a high level of MDA and GST. The oxidative tissue damage in cirrhosis causes a significantly low level of catalase. During the process of inflammation, oxidative stress occurs which leads to a significant decrease in antioxidant enzyme system. The main target of oxidative stress is the poly unsaturated fatty acids in cell membranes causing lipid peroxidation and excessive formation of MDA and GST which may lead to damage of the cell structure and function [51]. The low level of SOD indicates that the high risks of cell injuries and the treatment of GTE doses tend to increase or normalize the level of SOD. The level of SOD in the liver of alcohol-induced mice treated with both doses of GTE was observed to be increased. In addition, this signified that GTE could provide the elevated SOD enzyme to the injured liver cells which in turn could recover the liver cells to normal and eventually be able to produce the significant amount of SOD enzyme by itself as a protective action from the damage caused by toxic substance such as alcohol [47]. Chronic alcohol consumption not only activates free radical generation, but also alters the levels of both enzymatic and nonenzymatic endogenous antioxidant systems [14].

5. Conclusion

In conclusion, in the present study, we investigated that the natural antioxidants present in AQGTE ameliorate liver damage caused by chronic ethanol exposure. These results were more effective in reverting the enzymatic markers (AST, ALT, and ALP), nonenzymatic markers in liver homogenate (protein, cholesterol, and triglycerides), antioxidant activity in liver homogenate (MDA, GST, and SOD), and histological conditions back to normal. It shows that GTE has the capability to prevent this toxicity by inhibiting the hepatocyte damage, peroxidation of lipids, and improving the activity of antioxidant enzymes.

Conflict of Interests

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

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References

[1] E. Cebral, A. Lasserre, V. Rettori, and M. A. F. De Gimeno, "Impaired mouse fertilization by low chronic alcohol treatment," *Alcohol and Alcoholism*, vol. 32, no. 5, pp. 563–572, 1997.

[2] C. Loguercio, P. Piscopo, C. Guerriero, V. De Girolamo, D. Disalvo, and C. Del Vecchio Blanco, "Effect of alcohol abuse and glutathione administration on the circulating levels of glutathione and on antipyrine metabolism in patients with alcoholic liver cirrhosis," *Scandinavian Journal of Clinical and Laboratory Investigation*, vol. 56, no. 5, pp. 441–447, 1996.

[3] J. K. Reddy and M. S. Rao, "Lipid metabolism and liver inflammation—II. Fatty liver disease and fatty acid oxidation," *American Journal of Physiology. Gastrointestinal and Liver Physiology*, vol. 290, no. 5, pp. G852–G858, 2006.

[4] M. G. Shalan, M. S. Mostafa, M. M. Hassouna, S. E. H. El-Nabi, and A. El-Refaie, "Amelioration of lead toxicity on rat liver with Vitamin C and silymarin supplements," *Toxicology*, vol. 206, no. 1, pp. 1–15, 2005.

[5] B. F. Hagen, A. Bjorneboe, G.-A. Bjorneboe, and C. A. Drevon, "Effect of chronic ethanol consumption on the content of α-tocopherol in subcellular fractions of rat liver," *Alcoholism: Clinical and Experimental Research*, vol. 13, no. 2, pp. 246–251, 1989.

[6] O. H. Lowry, N. J. Rosebrough, A. L. Farr, and R. J. Randall, "Protein measurement with the Folin phenol reagent.," *The Journal of biological chemistry*, vol. 193, no. 1, pp. 265–275, 1951.

[7] S. A. Jewell, D. Di Monte, A. Gentile, A. Guglielmi, E. Altomare, and O. Albano, "Decreased hepatic glutathione in chronic

alcoholic patients," *Journal of Hepatology*, vol. 3, no. 1, pp. 1–6, 1986.

[8] S. Sherlock and J. Dooley, *Assessment of Liver Function in Diseases of the Liver and Biliary System*, Blackwell Scientific Publications, Oxford, UK, 1993.

[9] P. E. Hartman and D. W. Shankel, "Antimutagens and Anticarcinogens a survey of putative interceptor molecules," *Environmental and Molecular Mutagenesis*, vol. 15, no. 3, pp. 145–182, 1990.

[10] S. Kato, T. Kawase, J. Alderman, N. Inatomi, and C. S. Lieber, "Role of xanthine oxidase in ethanol-induced lipid peroxidation in rats," *Gastroenterology*, vol. 98, no. 1, pp. 203–210, 1990.

[11] S. M. Khan and G. Kour, "Subacute oral toxicity of chlorpyriphos and protective effect of green tea extract," *Pesticide Biochemistry and Physiology*, vol. 89, no. 2, pp. 118–123, 2007.

[12] S. Tokumaru, I. Tsukamoto, H. Iguchi, and S. Kojo, "Specific and sensitive determination of lipid hydroperoxides with chemical derivatization into 1-naphthyldiphenylphosphine oxide and high-performance liquid chromatography," *Analytica Chimica Acta*, vol. 307, no. 1, pp. 97–102, 1995.

[13] L. Tussey and M. R. Felder, "Tissue-specific genetic variation in the level of mouse alcohol dehydrogenase is controlled transcriptionally in kidney and posttranscriptionally in liver," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 86, no. 15, pp. 5903–5907, 1989.

[14] E. Albano, S. French, and M. Ingelman-Sundberg, "Cytochrome p450 2E1, hydroxyl ethyl radicals, and immune reaction associated alcoholic liver injury," *Alcoholism: Clinical and Experimental Research*, vol. 18, pp. 1057–1068, 1994.

[15] S. M. K. R. Zaidi, T. M. Al-Qirim, and N. Banu, "Effects of antioxidant vitamins on glutathione depletion and lipid peroxidation induced by restraint stress in the rat liver," *Drugs in R & D*, vol. 6, no. 3, pp. 157–165, 2005.

[16] J. King, "The transferase alanine and aspartate transaminase," in *Practical Clinical Enzymology*, pp. 363–395, Nostrand, London, UK, 1965.

[17] S. S. Bun, H. Bun, D. Guédon, C. Rosier, and E. Ollivier, "Effect of green tea extracts on liver functions in Wistar rats," *Food and Chemical Toxicology*, vol. 44, no. 7, pp. 1108–1113, 2006.

[18] Y. Cai, L. Ma, L. Hou, B. Zhou, L. Yang, and Z. Liu, "Antioxidant effects of green tea polyphenols on free radical initiated peroxidation of rat liver microsomes," *Chemistry and Physics of Lipids*, vol. 120, no. 1-2, pp. 109–117, 2002.

[19] X. Jin, R.-H. Zheng, and Y.-M. Li, "Green tea consumption and liver disease: a systematic review," *Liver International*, vol. 28, no. 7, pp. 990–996, 2008.

[20] D. Dahiru and O. Obidoa, "Pretreatment of albino rats with aqueous leaf extract of *Ziziphus mauritiana* protects against alcohol induced liver damage," *Tropical Journal of Pharmaceutical Research*, vol. 6, no. 2, article 705, 2007.

[21] J. D. Bancroft, *Histopathological Stains and their Diagnostic Uses*, Churchill Livingstone, New York, NY, USA, 1975.

[22] B. M. Walker and C. L. Ehlers, "Age-related differences in the blood alcohol levels of Wistar rats," *Pharmacology Biochemistry and Behavior*, vol. 91, no. 4, pp. 560–565, 2009.

[23] H. Kuper, A. Tzonou, E. Kaklamani, C. C. Hsieh, P. Lagiou, and H. O. Adami, "Tobacco smoking, alcohol consumption and their interaction in the causation of hepatocellular carcinoma," *International Journal of Cancer*, vol. 85, pp. 498–502, 2000.

[24] G. N. Bowers Jr. and R. B. McComb, "A continuous spectrophotometric method for measuring the activity of serum alkaline phosphatase," *Clinical Chemistry*, vol. 12, no. 2, pp. 70–89, 1966.

[25] L. A. Menahan, K. A. Sobocinski, and B. P. Austin, "The origin of plasma alkaline phosphatase activity in mice and rats," *Comparative Biochemistry and Physiology B: Comparative Biochemistry*, vol. 79, no. 2, pp. 279–283, 1984.

[26] R. Polavarapu, D. R. Spitz, J. E. Sim et al., "Increased lipid peroxidation and impaired antioxidant enzyme function is associated with pathological liver injury in experimental alcoholic liver disease in rats fed diets high in corn oil and fish oil," *Hepatology*, vol. 27, no. 5, pp. 1317–1323, 1998.

[27] A. E. Pinnell and B. E. Northam, "New automated dye-binding method for serum albumin determination with bromcresol purple," *Clinical Chemistry*, vol. 24, no. 1, pp. 80–86, 1978.

[28] P. E. Molina, C. McClain, D. Valla et al., "Molecular pathology and clinical aspects of alcohol-induced tissue injury," *Alcoholism: Clinical and Experimental Research*, vol. 26, no. 1, pp. 120–128, 2002.

[29] W. H. Habig, M. J. Pabst, and W. B. Jakoby, "Glutathione S transferases. The first enzymatic step in mercapturic acid formation," *Journal of Biological Chemistry*, vol. 249, no. 22, pp. 7130–7139, 1974.

[30] C. S. Lieber, "Role of oxidative stress and antioxidant therapy in alcoholic and nonalcoholic liver diseases," *Advances in Pharmacology*, vol. 38, pp. 601–628, 1997.

[31] A. Raj, K. V. Praveen, S. Varghese, J. K. Mukkadan, and P. K. Joseph, "Biochemical effects of feeding soft drink and ethanol," *Indian Journal of Experimental Biology*, vol. 47, no. 5, pp. 333–337, 2009.

[32] S. K. Das, L. Dhanya, S. Varadhan, S. Mukherjee, and D. M. Vasudevan, "Effects of chronic ethanol consumption in blood: a time dependent study on rat," *Indian Journal of Clinical Biochemistry*, vol. 24, no. 3, pp. 301–306, 2009.

[33] A. C. Deb, *Fundamentals of Biochemistry*, New Central Book Agency, Kolkata, India, 7th edition, 1998.

[34] V. Gujrati, N. Patel, V. N. Rao et al., "Hepatoprotective activity of alcoholic and aqueous extracts of leaves of *Tylophora indica* (Linn.) in rats," *Indian Journal of Pharmacology*, vol. 39, no. 1, pp. 43–47, 2007.

[35] A. Y. Sun, M. Ingelman-Sundberg, E. Neve et al., "Ethanol and oxidative stress," *Alcoholism: Clinical and Experimental Research*, vol. 25, no. 5, pp. 237–237, 2001.

[36] E. Baldi, P. Burra, M. Plebani, and M. Salvagnini, "Serum malondialdehyde and mitochondrial aspartate aminotransferase activity as markers of chronic alcohol intake and alcoholic liver disease," *The Italian Journal of Gastroenterology*, vol. 25, no. 8, pp. 429–432, 1993.

[37] N. J. Schisler and S. M. Singh, "Effect of ethanol in vivo on enzymes which detoxify oxygen free radicals," *Free Radical Biology and Medicine*, vol. 7, no. 2, pp. 117–123, 1989.

[38] C. S. Yang, J. M. Landau, M. T. Huang, and H. L. Newmark, "Inhibition of carcinogenesis by dietary polyphenolic compounds," *Annual Review of Nutrition*, vol. 21, pp. 381–406, 2001.

[39] B. Halliwell and J. M. C. Gutteridge, "Oxygen free radicals and iron in relation to biology and medicine: some problems and concepts," *Archives of Biochemistry and Biophysics*, vol. 246, no. 2, pp. 501–514, 1986.

[40] R. Nordmann, C. Ribière, and H. Rouach, "Implication of free radical mechanisms in ethanol-induced cellular injury," *Free Radical Biology and Medicine*, vol. 12, no. 3, pp. 219–240, 1992.

[41] H. Ohkawa, N. Ohishi, and K. Yagi, "Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction," *Analytical Biochemistry*, vol. 95, no. 2, pp. 351–358, 1979.

[42] E. Baraona and C. S. Lieber, "Effects of alcohol on hepatic transport of proteins," *Annual Review of Medicine*, vol. 33, pp. 281–292, 1982.

[43] H. P. Misra and I. Fridovich, "The role of superoxide anion in the autoxidation of epinephrine and a simple assay for superoxide dismutase.," *The Journal of Biological Chemistry*, vol. 247, no. 10, pp. 3170–3175, 1972.

[44] R. Nordmann, "Alcohol and antioxidant systems," *Alcohol and Alcoholism*, vol. 29, no. 5, pp. 513–522, 1994.

[45] S. Shaw, E. Jayatilleke, W. A. Ross, E. R. Gordon, and C. S. Leiber, "Ethanol-induced lipid peroxidation: potentiation by long-term alcohol feeding and attenuation by methionine," *Journal of Laboratory and Clinical Medicine*, vol. 98, no. 3, pp. 417–424, 1981.

[46] V. Arulmozhi, M. Krishnaveni, K. Karthishwaran, G. Dhamodharan, and S. Mirunalini, "Antioxidant and antihyperlipidemic effect of *Solanum nigrum* fruit extract on the experimental model against chronic ethanol toxicity," *Pharmacognosy Magazine*, vol. 6, no. 21, pp. 42–50, 2010.

[47] R. Saravanan, P. Viswanathan, and K. V. Pugalendi, "Protective effect of ursolic acid on ethanol-mediated experimental liver damage in rats," *Life Sciences*, vol. 78, no. 7, pp. 713–718, 2006.

[48] K. Husain, M. Vasquez Ortiz, and J. Lalla, "Physical training ameliorates chronic alcohol-induced hypertension and aortic reactivity in rat," *Alcohol and Alcoholism*, vol. 41, no. 3, pp. 247–253, 2006.

[49] M. Saoudi, S. Jebahi, K. Jamoussi, G. B. Salah, C. Kallel, and A. El Fek, "Haematological and biochemical toxicity induced by methanol in rats: ameliorative effects of *Opuntia vulgaris* fruit extract," *Human & Experimental Toxicology*, vol. 30, no. 12, pp. 1963–1971, 2011.

[50] Z. Zhou, X. Sun, and J. Y. Kang, "Metallothionein protection against alcoholic liver injury through inhibition of oxidative stress," *Experimental Biology and Medicine*, vol. 227, no. 3, pp. 214–222, 2002.

[51] M. A. Leo, M. Arai, M. Sato, and C. S. Lieber, "Hepatotoxicity of vitamin A and ethanol in the rat," *Gastroenterology*, vol. 82, no. 2, pp. 194–205, 1982.