Hepatoprotective effect of commercial herbal extracts on carbon tetrachloride-induced liver damage in Wistar rats

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ABSTRACT

Background: Various hepatoprotective herbal products from plants are available in Mexico, where up to 85% of patients with liver disease use some form of complementary and alternative medicine. However, only few studies have reported on the biological evaluation of these products. **Objective**: Using a model of carbon tetrachloride (CCl_4)-induced hepatotoxicity in rats, we evaluated the effects of commercial herbal extracts used most commonly in the metropolitan area of Monterrey, Mexico. **Materials and Methods**: The commercial products were identified through surveys in public areas. The effect of these products given with or without CCl_4 in rats was evaluated by measuring the serum concentrations of aspartate amino transferase (AST) and alanine amino transferase (ALT), and histopathological analysis. Legalon® was used as the standard drug. **Results**: The most commonly used herbal products were Hepatisan® capsules, Boldo capsules, Hepavida® capsules, Boldo infusion, and milk thistle herbal supplement (80% silymarin). None of the products tested was hepatotoxic according to transaminase and histological analyses. AST and ALT activities were significantly lower in the Hepavida + CCl_4-treated group as compared with the CCl_4-only group. AST and ALT activities in the silymarin, Hepatisan, and Boldo tea groups were similar to those in the CCl_4 group. The CCl_4 group displayed submassive confluent necrosis and mixed inflammatory infiltration. Both the Hepatisan + CCl_4 and Boldo tea + CCl_4 groups exhibited ballooning degeneration, inflammatory infiltration, and lytic necrosis. The silymarin + CCl_4 group exhibited microvesicular steatosis. The Hepavida + CCl_4- and Legalon + CCL_4-treated groups had lower percentages of necrotic cells as compared with the CCl_4-treated group; this treatment was hepatoprotective against necrosis. **Conclusion**: Only Hepavida had a hepatoprotective effect.

Key words: Alanine transferase, aspartate transferase, hepatoprotection, liver injury, natural products

INTRODUCTION

Interest in complementary and alternative medicine (CAM) is increasing throughout the world probably because there are only few universally effective and available options for the treatment of common liver diseases such as cirrhosis, fatty liver, and chronic hepatitis.[1-6] In recent years, researchers have used scientific methods to evaluate the effects of plants used in traditional CAM for the treatment of liver ailments.[7-10] In many cases, the mechanisms and modes of action of these plants as well as their therapeutic effectiveness have been confirmed in clinical studies. Several hundred plants have been examined so far, but only a handful have been studied thoroughly. Among these are *Silybum marianum* (milk thistle), *Picrorhiza kurroa* (kutki), *Curcuma longa* (turmeric), *Camellia sinensis* (green tea), and *Glycyrrhiza glabra* (licorice).[11,12]

The increasing use of herbal medicines reflects their perceived effectiveness in the treatment and prevention of disease, and the belief that these treatments are safe because

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they are "natural." In addition, most countries do not impose strict regulations on herbal preparations, and, therefore, access to these kinds of therapies is almost unrestricted.[13,14]

The use of CAM in the treatment of liver disease varies among different geographic regions. It is estimated that 20–65% of the population in Europe, 39% in the United States, and 85% in Mexico have used CAM for this purpose.[15,16] Several recent surveys from Europe and the United States have demonstrated sharp increases in the use of botanical drugs.[15,17] For example, 21% of patients attending an outpatient liver clinic took herbal preparations and 13% used herbs to treat liver diseases as per a previous study.[15]

The risks of toxicity associated with several commercially available herbal preparations have surfaced recently, challenging the notion that these therapies are innocuous.[18,19] This association between herbal remedies and hepatotoxicity is not a novel finding. It is well known that some plants, particularly the species containing pyrrolizidine derivatives, may be hepatotoxic.[13,20] The risks are particularly high when the therapeutic preparation contains a combination of numerous plants.

The present study was undertaken to evaluate the effects of commercial herbal extracts in the normal liver and in a rat model of acute carbon tetrachloride (CCl_4)-induced hepatotoxicity. CCl_4 is a chemical used widely to induce liver damage in experimental studies, and its toxicity has been studied extensively. CCl_4-induced hepatic injury is characterized by leakage of cellular enzymes into the bloodstream and by centrilobular necrosis.[21,22]

MATERIALS AND METHODS

Identification of commercial herb products
To identify the five most commonly used commercial herbal products employed in the treatment of liver diseases in the region, we administered surveys in various public areas around the metropolitan area of Monterrey, Nuevo León, Mexico. The survey asked the respondents to list all the commercial herbal products of which they were aware.

Chemicals
Sodium chloride solution (0.9%) was obtained from PISA (Guadalajara, Jal, Mexico), and olive oil was obtained from Ybarra SA (Seville, Spain). CCl_4 (99.9%) was purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Legalon® 70 mg silymarin) was obtained from Nycomed (Naucalpan de Juárez, Edo. México, Mexico). Hepatisan capsules (400 mg) were obtained from Herbital (Puebla, Pue, Mexico). Boldo capsules (500 mg) and Hepavida® capsules (400 mg) were obtained from Botnatura (Irapuato, Gto,

Mexico). Boldo infusion (1 g) was obtained from Therbal (Mexico City, Mexico). Milk thistle herbal supplement (80% silymarin) was obtained from GNC Herbal Plus (Pittsburg, PA, USA). Pentobarbital sodium was purchased from Holland S.A. de C.V. (Jiutepec, Mor, Mexico).

Animal procedures
Animal procedures were performed in accordance with the Guiding Principles for the Production, Care, and Use of Laboratory Animals of the Mexican Official Norm NOM-062-Z00-1999 published in the Federal Official Diary on August 22, 2001. All protocols used in this study were approved by the ethics committee of our institution. Experiments were performed on male Wistar rats weighing 200–300 g. The animals were maintained under standard conditions, such as stable room temperature (24 ± 3°C), a 12 h light/dark cycle, and access to commercial rat pellets and water *ad libitum*.

Assessment of toxicity *in vivo*
The rats were divided into eight groups ($n = 5$). One group received physiological saline (1 ml/kg, p.o.) and served as the normal control (NC). One group received a CCl_4/oil preparation (1:1, 1 ml/kg, i.p.) and served as the acute toxicity control; this group was sacrificed after 24 h to obtain liver and blood samples. One group received Legalon (70 mg/kg, p.o.), a silymarin preparation that was used as the hepatoprotective standard drug. The other groups received one of the following: Hepatisan (800 mg/kg, p.o.), Boldo capsules (1500 mg/kg, p.o.), Hepavida (1200 mg/kg, p.o.), Boldo tea (0.1 ml/kg, p.o.), or silymarin (200 mg/kg, p.o.). The dose of each product was established based on the dose stated for human consumption in the product insert.

To evaluate the potential hepatotoxicity, the six commercial products mentioned above were administered daily for 1 week.[23] This duration of treatment was selected to confirm that the dose to be tested for hepatoprotection was not toxic. At the end of the week, blood and liver samples were collected, and the serum activities of aspartate amino transferase (AST) and alanine amino transferase (ALT) were measured, and liver samples were analyzed histopathologically. Rats were anesthetized with pentobarbital sodium (35 mg/kg, i.p.), and 3 ml of blood was obtained from the abdominal aorta of each rat. Serum was obtained after centrifugation at 3500 rpm for 10 min and was stored at −20°C until enzyme analysis. The livers were dissected carefully and transferred immediately to a 10% formalin solution for fixation. Histopathological analysis was performed on the fixed tissue.[24,25]

Assessment of hepatoprotection *in vivo*
To evaluate the possible hepatoprotective effect, an additional six groups ($n = 5$ each) were included: Legalon+CCl_4, Hepatisan+CCl_4, Boldo capsules+CCl_4,

Hepavida+CCl_4, Boldo tea+CCl_4, and silymarin+CCl_4. To study the effect of the commercial products on CCl_4-induced toxicity, each product was administered at the dose described above for 3 consecutive days, and then CCl_4/oil (1:1, 1 ml/kg, i.p.) was administered.[26,27] The rats were sacrificed 24 h after injection of CCl_4, and the samples were obtained as described above.

Measurement of serum transaminases

The activities of AST and ALT were measured using an automatic biochemical detection system (DTII Vitro Systems Chemistry; module DTSCII, Johnson and Johnson Ortho-Clinical Diagnostics, New Brunswick, NJ, USA). In the AST assay (21CFR 862.1100), the amino group of L-aspartate is transferred to a-ketoglutarate in the presence of pyridoxal-5-phosphate to produce glutamate and oxaloacetate. The oxaloacetate formed in the deamination of the L-aspartate is converted to malate by malate dehydrogenase in the presence of reduced nicotinamide adenine dinucleotide (NADH), which is oxidized to NAD^+. The rate of oxidation of NADH is monitored by reflectance spectrophotometry at 37°C. The rate of change is then used to calculate enzyme activity.[28] In the ALT assay (21 CFR 862.1030), the amino group of L-alanine is transferred to a-ketoglutarate in the presence of pyridoxal-5-phosphate to produce glutamate and pyruvate. The pyruvate formed in the deamination of L-alanine is converted to lactate by lactate dehydrogenase in the presence of NADH, which is oxidized to NAD^+. The rate of oxidation of NADH is monitored by reflectance spectrophotometry at 37°C. The rate of change is then used to calculate enzyme activity.[29]

Histology

Liver tissues were embedded in paraffin blocks using conventional methods. The blocks were sectioned at 5 μm and stained with hematoxylin–eosin. The slides were observed under light microscopy to identify histopathological changes including ballooning degeneration, lytic necrosis, confluent necrosis, submassive necrosis, microvesicular steatosis, macrovesicular steatosis, and lymphocytic or mixed inflammatory infiltration. We also calculated the percentage of necrotic cells from the number of necrotic or apoptotic cells observed at three different high power fields (X400); from zones 1–3, 50 cells were counted in each zone.

Statistical analysis

The data are expressed as mean and standard error. The data were analyzed using the Mann–Whitney–Wilcoxon test, Dunnett's test, and Pearson correlation where appropriate. The SPSS statistical software package (v. 16.0; SPSS Inc., Chicago, IL, USA) was used for all analyses. $P < 0.05$ was considered significant.

RESULTS

The five most commonly used herbal products for the treatment of liver diseases in the Monterrey metropolitan area are listed in Table 1. Four products were manufactured in Mexico and one in the United States. There was a wide diversity in the constituents of these products. However, four contained Boldo, two of which contained almost exactly the same ingredients [Table 1].

Assessment of *in vivo* toxicity

After 7 days of administration, none of the products tested increased ALT and AST activities significantly as compared with the NC group. The ALT and AST activities were significantly lower in the Legalon group than in the NC group ($P = 0.0251$ and $P = 0.0006$, respectively) [Table 2]. This

Table 1: Constituents and sources of commercial products

Commercial Product	Ingredients	Country
Hepavida	*Rhamnus purshiana, Brickellia grandiflora, Tagetes floridas, Arctostaphylos pungens, Amphipterygium adstringens, Selaginella spp., Peumus boldus, Cynara scolymus, Taraxacum officinale, Foeniculum vulgare, Solidago virgaurea, Lantana trifolia, Kohleria deppeana*	Mexico
Boldo capsules	*Peumus boldus*	Mexico
Hepatisan	*Rhamnus purshiana, Brickellia grandiflora, Miconia argentea, Tagetes floridas, Arctostaphylos pungens, Amphipterygium adstringens, Selaginella spp., Peumus boldus, Cynara scolymus, Taraxacum officinale, Foeniculum vulgare, Solidago virgaurea, Lantana trifolia, Kohleria deppeana.*	Mexico
Boldo tea	100% (*Peumus boldus*)	Mexico
Silymarin	80% silymarin	USA

Table 2: AST (IU/L) and ALT (IU/L) activities in commercial herbal products compared with the normal control

Treatment	AST (IU/L)		ALT (IU/L)	
	Mean	Standard error	Mean	Standard error
Normal control	285	31.9	69	10.4
Legalon	98*	41.2	59	13.4
Hepavida	187	41.2	92	13.4
Boldo capsules	307	41.2	108	13.4
Hepatisan	397	41.2	111	13.4
Boldo tea	344	41.2	104	13.4
Silymarin	163	35.7	59	11.6
Significance	0.0006**		0.0251**	

$n = 5$ rats in each group. The data are expressed as mean and standard error. AST, aspartate aminotransferase; ALT, alanine aminotransferase. *: Significantly different from the normal control, Dunnett's test, $P < 0.05$. **: Significant, Dunnett's test of the equality of effects for all treatments.

suggests that none of the products tested was hepatotoxic, at least when given subacutely.

The livers of the CCl_4 group showed severe inflammatory infiltration and necrosis. No other group showed abnormalities in the liver architecture, necrosis, or steatosis. These results indicate that none of the products studied produced hepatotoxicity. Thus, they were all considered for further evaluation of their possible hepatoprotective effects.

Assessment of hepatoprotection *in vivo*

AST and ALT activities were significantly lower in the Hepavida+CCl_4 and Legalon+CCl_4 groups as compared with the CCl_4-only group, and these levels were comparable to those of the NC. The enzyme activities were significantly higher in the groups treated with silymarin, Hepatisan, and Boldo tea than in the NC group; the enzyme activities in the silymarin, Hepatisan, and Boldo tea groups did not differ significantly from those in the CCl_4 group [Table 3].

All animals exhibited hepatocyte ballooning degeneration independent of the treatment received. The CCl_4 group showed submassive and confluent necrosis with mixed inflammatory infiltration. The Legalon+CCl_4 group exhibited microvesicular steatosis without necrosis, particularly in the perivenular region. Both the Hepatisan+CCl_4 and Boldo tea+CCl_4 groups exhibited ballooning degeneration, lymphocytic or mixed inflammatory infiltration, and lytic necrosis [Figure 1]. The silymarin group exhibited microvesicular steatosis irrespective of CCl_4 administration.

The total percentages of necrotic cells differed significantly between the treatments. The percentages were significantly higher in the Hepatisan, Boldo tea, and silymarin groups than in the NC. The percentages of necrotic cells were significantly lower in the Legalon and Hepavida groups than in the CCl_4 group, indicating a hepatoprotective effect against necrosis [Table 4].

Table 3: Comparison of AST (IU/L) and ALT (IU/L) activities between treatments with different commercial herbal products as compared with the normal control and CCl_4

Treatment	AST (IU/L)			ALT (IU/L)		
	Mean	Standard error		Mean	Standard error	
Normal control	285	1920		69	22	
CCl_4	8421	2479		1439	329	
Legalon+CCl_4	399	2147	B	102	285	B
Hepavida+CCl_4	858	2147	B	246	285	B
Boldo capsules+CCl_4	1318	2147		525	285	
Hepatisan+CCl_4	1485	2147	A,B	478	285	A
Boldo tea+CCl_4	12757	2147	A	1459	285	A
Silymarin+CCl_4	7577	2147	A	1309	285	A
Significance	0.0015*			0.0034*		

$n=5$ rats in each group. Results given as mean and standard error. AST, aspartate aminotransferase; ALT, alanine aminotransferase; CCl_4, carbon tetrachloride.
*:Significant, Mann–Whitney–Wilcoxon test of the equality of effects for all treatments. A, significantly different from the normal control. B, significantly different from the toxicity control (CCl_4). Mann–Whitney–Wilcoxon test, $P<0.05$
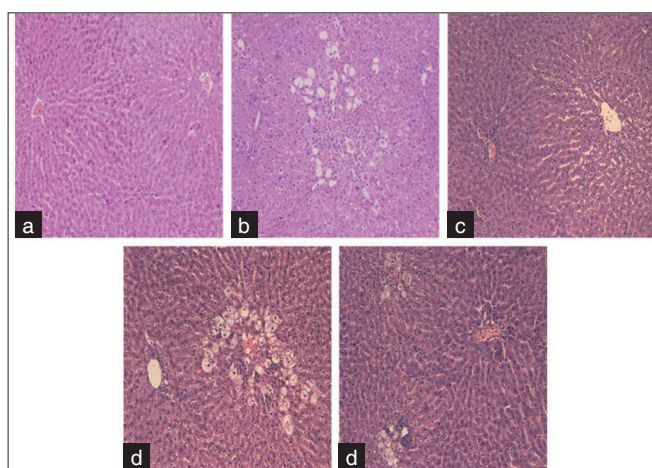

Figure 1: Hematoxylin and eosin staining, X40. (a) Normal control, normal histological findings in the liver parenchyma. (b) CCl_4, submassive confluent necrosis and mixed inflammatory infiltration. (c) Legalon+CCl_4, perivenular microvesicular steatosis without necrosis. (d) Hepatisan+CCl_4, ballooning inflammatory infiltration and lytic necrosis. (e) Boldo tea+CCl_4, balllooning degeneration, inflammatory infiltration, and lytic necrosis

Table 4: Comparison of the percentages of necrotic cells between different commercial herbal products and controls

Treatment	Percentage of necrotic cells			
	Median range		Mean	Median
Normal control	6		0	0
CCl_4	22		47	50
Legalon+CCl_4	22		47	50
Hepavida+CCl_4	9	B	10	0
Boldo capsule+CCl_4	11	B	15	10
Hepatisan+CCl_4	12		12	9
Boldo tea+CCl_4	25	A	55	55
Silymarin+CCl_4	26	A	60	65
Significance	0.0012*			

$n=5$ rats in each group. The mean, median, and median range are shown. CCl_4, carbon tetrachloride.
*:Significant, Mann–Whitney–Wilcoxon test of the equality of effects for all treatments. A, significantly different from the normal control. B, significantly different from the toxicity control (CCl_4). Mann–Whitney–Wilcoxon test, $P<0.05$

For treatments given without CCl$_4$, the histological changes correlated significantly only with AST and ALT activities (Pearson correlation = 0.739, $P < 0.0001$). For treatments given with CCl$_4$, ALT and AST activities were positively associated with the presence of confluent necrosis ($P = 0.0019$ and $P = 0.0033$, respectively) [Table 5]. AST activity was also positively associated with submassive necrosis ($P = 0.030$) [Table 5].

DISCUSSION

Principal findings
We tested the hepatoprotective effects of five commercial herbal products using a rat model of CCl$_4$-induced hepatotoxicity, comparing them to the reference drug Legalon. We found that none showed hepatotoxic effects in our experimental model and that only Boldo tea caused slight histological inflammation after 7 days of administration. However, only one of these products (Hepavida) showed consistent hepatoprotective effects against CCl$_4$-induced damage. The reference drug Legalon was also hepatoprotective.

Boldo, which comes from a tree in the Monimiaceae family, is believed to be a regulator of hepatic function and an antispasmodic, digestive stimulant, and sedative.[30,31] Four of the products tested contained Boldo, and, of these, only one had a beneficial effect, suggesting that Boldo has poor hepatoprotective properties. Silymarin, a mixture of flavonolignans extracted from seeds of *S. marianum*, is composed primarily of silibinin, silidianin, and silychristin.[9,32] A survey in patients in a hepatology clinic found that 31% were using over-the-counter "alternative agents" for their liver diseases, the most common being milk thistle (silymarin).[33] In our study, the silymarin extract did not show toxicity at the doses administered, but it failed to show a hepatoprotective effect against CCl4-induced damage in rats. Legalon, a standardized extract of silymarin, which we used as a positive control for hepatoprotection,[34-36] showed no toxicity and attenuated the increase in liver enzyme activities in our CCl4 toxicity model. Although the silymarin concentrations in both preparations differed, this suggests that the extract should be analyzed to determine whether it contains the amounts of silymarin stated on the label and how these differ from the amount contained in Legalon.

Strenghts and weaknesses of the study
We used a well-studied model of hepatotoxicity (CCl$_4$) and used both biochemical and histological markers of liver injury. In addition, we compared the effects of the commercial products against the reference drug Legalon, a drug with established hepatoprotective properties.

There are some weaknesses in our study. Our sample size was small (5 animals per group), a fact that could account for the large standard errors we obtained in our data. We also did not objectively test the commercial products in order to confirm that they possessed the contents that were reported in the label by the manufacturer. There are also inherent complications in identifying which of the many components in a commercial mixture of herbs are responsible for its biological effects, if any, are found.

Relation to other studies
We know of no other studies that have analyzed the hepatoprotective effects of these commercial products. As mentioned above, Legalon is of established efficacy. Several studies have reported that the major histopathological parameters altered with CCl4-induced damage are degeneration in hepatocytes, the presence of necrosis and inflammatory infiltration, and elevated ALT and AST activities.[37,38] Our histology analyses showed a positive association between elevated ALT and AST activities and the presence of confluent necrosis and submassive necrosis in the groups that received commercial products, which were ineffective in attenuating the increase in enzyme activities caused by CCl4 treatment.

Implications
A wide variety of herbal products are available in Mexico, many without the official approval of the Secretary of Health. It has been reported that 60–80% of the general population uses these products, and up to 85% of patients with liver disease use some form of CAM.[39] There are reported cases of acute liver disease, at times severe, associated with the consumption of herbal products, and some products have been withdrawn from the market based on recommendations

Table 5: Associations between biochemical and histological variables

Variable	Factor	Level	Median	Level	Median	Significant
ALT (UI/L)	Confluent necrosis	NO	64	Yes	836	0.0019
AST (UI/L)	Dominant hitologic image	Global degeneration	659	Confluent necrosis	3684	0.0229
AST (UI/L)	Confluent necrosis	NO	245	Yes	3278	0.0033
AST (UI/L)	Submassive necrosis	NO	988	Yes	10464	0.0304

n = 5 rats in each group. AST, aspartate aminotransferase; ALT, alanine aminotransferase. Significant: Pearson correlation, $P < 0.05$

made by national pharmacovigilance agencies.[13,40] However, consumption of these products is increasing.

In this paper, we studied five herbal products used commonly in the region.

Consistency in biological activity and composition are essential to ensuring the safety of therapeutic agents. However, botanical preparations rarely meet this standard because of problems identifying plants, variable growing conditions, differences in the processing of extracts, and lack of information about the pharmacologically active ingredients. The use of marker compounds to standardize herbal preparations promotes batch-to-batch consistency but does not ensure consistent pharmacological activity. Moreover, analyses of purportedly standardized herbal preparations show that botanical products often do not contain the amounts of the compounds stated on the label.[41] It is possible that some of these factors contributed to the results obtained in this study. For example, both Hepavida and Hepatisan supposedly contain the same compounds, yet only Hepavida was hepatoprotective. A similar finding was obtained with products that contain Boldo; only one was hepatoprotective in our model. This suggests that the presence of the active ingredient (boldine) in this plant should be confirmed in the commercial products, given the variability of the effects of the different preparations.

As suggested above, there is a need for quality control in laboratories that produce botanical products used in the treatment of diseases, and the presence of active pharmacological components should be confirmed. Reporting and regulatory systems should be created to monitor adverse effects of herbal products, and care should be taken in the identification of compounds and in the evaluation of the potential benefits of phytotherapy. In conclusion, only Hepavida had a hepatoprotective effect in our model.

For future research

Simple methods could be used to test commercial products for their advertised hepatoprotective effects, given that they are usually poorly regulated. However, our results only apply to relatively short periods of administration. Further research is needed to establish if the products tested here might have hepatoprotective effects after chronic use (months to years) or if they have liver regenerative properties. Evidently, their clinical value could only be established with human studies.

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