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RESEARCH ARTICLE

Hepatoprotective activity of aqueous and ethanolic *Bixa orellana* L. leaf extracts against carbon tetrachloride-induced hepatotoxicity

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ABSTRACT

Background: There is an increasing incidence of liver failure and lack of effective drugs for liver diseases. **Aim and Objective:** This study identified the phytochemicals present in aqueous and ethanolic *Bixa orellana* L. leaf extracts and investigated the hepatoprotective activity against carbon tetrachloride (CCl_4)-induced hepatotoxicity in mice. Specifically, the study compared serum levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) and examined liver tissues histopathology. **Materials and Methods:** Aqueous and ethanolic *B. orellana* L. leaf extracts were prepared and screened for phytochemical contents. An oral dose of 500 mg/kg body weight was identified from toxicity test and administered to albino mice. Microscopic examination of the liver tissues assessed the extent of hepatic injury. Serum AST and ALT levels were compared using one-way analysis of variance with Bonferroni *post hoc* analysis at 5% level of significance using Stata/SE V12.0 software. **Results:** *B. orellana* L. leaves contained alkaloids, anthraquinones, sugars, and tannins. Aqueous and ethanolic leaf extracts of *B. orellana* L. did not show any toxicity up to 2000 mg/kg body weight oral dose in mice. Pre-treatment for 7 days before CCl_4 administration significantly prevented elevation of serum AST and ALT levels with histopathologic findings showing a protective effect on the hepatocytes. **Conclusion:** *B. orellana L.* leaves have potent hepatoprotective activity against oxidative damage.

KEY WORDS: *Bixa orellana* L.; Leaf Extract; Carbon Tetrachloride; Hepatotoxicity; Liver Enzymes; Oxidative Damage

INTRODUCTION

Liver is a large and complex organ primarily involved in metabolism.[1] An increase in the incidence of liver failure along with the lack of an effective drug that stimulates liver function has increased the need for new therapies.[2] Currently, many researchers are exploring the plant kingdom in search of new medicinal agents to alleviate and prevent liver diseases. Plants have been utilized by humans as sources of medicines. Several studies on the pharmacologic properties of different plants led to the discovery of potential therapeutic agents and precursors of complex substances.[3]

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Bixa orellana L.*,* locally known as atsuete, is a small tree that grows around 3-10 m.[4] Its leaves have antiseptic, antibacterial, and antiemetic effects, whereas the seeds were used for fever and buccal tumors.[5] Many pharmacologic studies have been conducted on its potential as a source of medicine.[6-8] Its methanolic leaf extract has anticonvulsant, analgesic, antidiarrheal, and radical scavenging properties,

whereas the methanolic seed extract has hepatoprotective properties against carbon tetrachloride (CCl_4)-induced hepatic damage.[9] With the lack of effective drugs for liver diseases and the abundance of *B. orellana* L. in the country render the study of hepatoprotective activity of its leaf extract important. Hence, this study determined the presence of phytochemicals in *B. orellana* L. leaf extract and assessed its hepatoprotective activity against CCl_4-induced hepatotoxicity in mice on their aspartate aminotransferase (AST) and alanine aminotransferase (ALT) serum levels. Moreover, the study investigated the effects of aqueous and ethanolic leaf extracts in preventing damage on hepatocytes through histopathologic examination of the liver tissues.

MATERIALS AND METHODS

Reagents

High-performance liquid chromatography grade ethanol was purchased from Harnwell Chemicals. Zoletil 50 was purchased from Provisions Marketing, Quezon City. CCl_4 (Merck & Co.) was used as hepatotoxic agent. Vitamin E used was Myra 400 E. All other chemicals used were of analytical grade and provided by the University of the Philippines Manila.

Plant Material

Fresh leaves of *B. orellana* L. were collected in January 2011 in Pototan, Iloilo, Philippines. Pressed, dried sample of it was brought to the Botany Department of the National Museum, Philippines for authentication. The leaves of *B. orellana* L were washed with distilled water, air-dried for a week, cut into small pieces, and pulverized using an electric blender (Hanabishi Superblender Model No. HJB-115).

Preparation of Aqueous and Ethanolic Extracts

Aqueous extract of the powdered leaves was prepared by macerating 25 g of the powder in 100 mL distilled water and subsequently heated in water bath (60°C) for 1 h and then vacuum filtered using Whatman No.42 paper. Ethanolic extract was prepared by soaking 25 g of the ground leaves to 80% ethanol for 24 h and then vacuum filtered. Both aqueous and ethanolic extracts were sent to the Tissue Bank of the Philippine General Hospital for sample freeze-drying.

Phytochemical Screening

Phytochemical screening was done using thin-layer chromatography method except for the froth test for saponins.[10]

Experimental Animals and Treatment Administration

A total of 45 albino mice, 4-5 weeks old, were obtained from the animal house of the Research Institute for Tropical Medicine, Alabang, Philippines. The animals were individually caged in a well-ventilated plastic container, subsequently housed under natural light and dark schedules, were given free access to food and water throughout the entire investigation. The study protocol adhered to the guidelines set for the Care and Use of Laboratory Animals. A 10-day acclimatization period was done before experimental treatment administrations.

Lethal dose of the ethanolic and aqueous extracts was tested with 300 mg/kg starting dose.[11] Each group which is comprised of 5 mice was acclimatized for 10 days before dosing. Dosages of 300 and 2000 mg/kg body weight were tested for toxicity of the aqueous and ethanolic extracts for 7 days. In the toxicity test, some mice were observed to be lethargic after administration of the test solutions at dosages of 300 and 2000 ppm, but no deaths were observed after the 7-day period suggesting that both the aqueous and ethanolic extracts had no acute toxicity up to 2000 ppm dosage. Hence, a concentration of 500 mg/kg body weight was chosen for the treatment groups based on the result of the toxicity test. The final experimental set up employed 7 groups, with 5 mice per group (Table 1).

Treatment was orally given once daily for 7 days. Test solutions were prepared by suspending the extracts in distilled water. On the 7th day, induction of liver damage was done 60 min after the last administration of test solution. Hepatic damage was done by oral administration of CCl_4 dissolved in olive oil (1:4 v/v) at a dose of 2 ml/kg body weight[12,13] except on the control Groups (I, IV, and V) which received only the vehicle. Food was withdrawn from the mice after CCl_4 administration, but they were given free access to water. Blood samples were drawn under mild anesthesia through cardiac puncture 24 h after the hepatotoxin administration. Anesthesia used was Zoletil 50, a solution of tiletamine hydrochloride and zolazepam hydrochloride. Animals were sacrificed through cervical dislocation.

Hepatoprotective Activity

Blood samples collected were centrifuged at 3000 g for 15 min at 4°C to obtain the serum. Serum samples were sent to the Clinical Laboratory of the Philippine General Hospital.

Table 1: Treatment groupings of the experimental subjects employed in the study

Groups	Treatment
I	Normal (untreated control)
II	Negative control (distilled water only+CCl_4)
III	Positive control (Vitamin E 200 IU/kg body weight+CCl_4)
IV	Control (500 ppm ethanolic leaf extract)
V	Control (500 ppm aqueous leaf extract)
VI	Ethanolic leaf extract given at 500 ppm+CCl_4
VII	Aqueous leaf extract given at 500 ppm+CCl_4

CCl_4: Carbon tetrachloride

Serum levels of AST and ALT were determined using an automated analyzer.

Histopathologic Examination

Immediately after the animals were sacrificed, the liver of each mouse was removed and placed in a container with 10% buffered formalin, subsequently sent to the Pathology Department of the Philippine General Hospital. Liver tissues were fixed using hematoxylin-eosin dye for microscopic examination.

Statistical Analysis

Serum enzyme levels, reported as means ± standard deviations, were compared using one-way analysis of variance with Bonferroni *post hoc* analysis at 5% level of significance using V12.0 Stata/SE statistical software.

RESULTS

Phytochemical Screening

There were different phytochemicals identified in the *B. orellana* L. leaf samples including alkaloids, anthraquinones, tannins, and sugars (Table 2).

Hepatoprotective Activity

Administration of ethanolic and aqueous extracts in mice without introducing CCl_4 was performed to assess if the extract alone can cause hepatic damage. Both the serum AST and ALT levels of the treated Groups (IV and V) are higher when compared with the normal group (Table 3), but these values do not significantly differ ($P > 0.05$). Moreover, the serum AST and ALT levels of Groups II, III, VI, and VII are significantly higher when compared with the normal Group I ($P < 0.00$) (Table 3). After CCl_4 administration, Group II showed a statistically significant difference in the serum AST and ALT levels when compared with Groups III, VI, and VII ($P < 0.00$) indicating more hepatic damage in the negative control Group II (Table 3). Finally, there is no significant difference in the hepatoprotective activities among Groups III, VI, and VII ($P > 0.05$).

Histopathologic Examination

Histopathology slides were viewed under a light microscope with liver section of normal group showing intact cells (Figure 1a), whereas the groups treated with aqueous and ethanolic extracts only showed cell inflammation but the cells remained intact (Figure 1b and c). Hepatocyte injury in treatment groups administered by CCl_4 is visible (Figure 1d-f). However, the damage is not as extensive when compared with Groups III, VI, and VII (Figure 1g-i).

Table 2: Phytochemicals detected in *Bixa orellana* L. leaf samples

Test	Result
Alkaloids	+
Flavonoids	-
Cardenolides	-
Anthraquinones	+
Tannins	+
Coumarins	-
Indoles	-
Sugars	+
Higher alcohols	-
Saponins	-

(-): Absent, (+): Present

Table 3: Serum enzyme levels across different experimental treatment groups

Group	Serum enzyme, IU/L (mean±SD)	
	AST	ALT
I (normal)	76.00±9.22[a]	24.80±3.63[a]
II (negative)	332.20±30.14	92.00±8.09
III (positive)	131.60±7.67[b]	45.00±5.24[b]
IV (ET)	93.00±4.47[a]	29.80±5.63[a]
V (AQ)	85.40±5.94[a]	29.40±4.39[a]
VI (EC)	155.40±11.06[b]	42.60±3.65[b]
VII (AC)	148.00±10.42[b]	38.60±4.62[b]

Means with the same superscript letters for a specific serum enzyme are not statistically different at 5% level of significance using Bonferroni test, AST: Aspartate aminotransferase, ALT: Alanine aminotransferase, SD: Standard deviation

DISCUSSION

B. orellana L. extracts have antiprotozoal, anthelmintic, platelet antiaggregant,[14,15] and anticlastogenic activity.[16] The root extracts have spasmolytic activity, whereas leaf and branch extracts were effective at neutralizing the effects of snake venoms.[17] Leaf and seed extracts displayed *in vitro* antimicrobial activity,[18] and the seed extracts exhibit chemopreventive and antioxidant activity.[19] In this study, both aqueous and ethanolic *B. orellana* L. leaf extracts have shown hepatoprotective effects on CCl_4-induced hepatic injury.

CCl_4 is produced industrially by the chlorination of methane, propane, ethane, propene, or carbon disulfide and is soluble in alcohol and acetone and miscible with benzene, ether, and chloroform and has been used as a grain fumigant, rodenticide and solvent for oils, fats, rubber cements, and resins.[20] This was introduced in dry-cleaning because of the high cost of the earlier petroleum solvents and was used as a domestic spot remover. It was discontinued because of

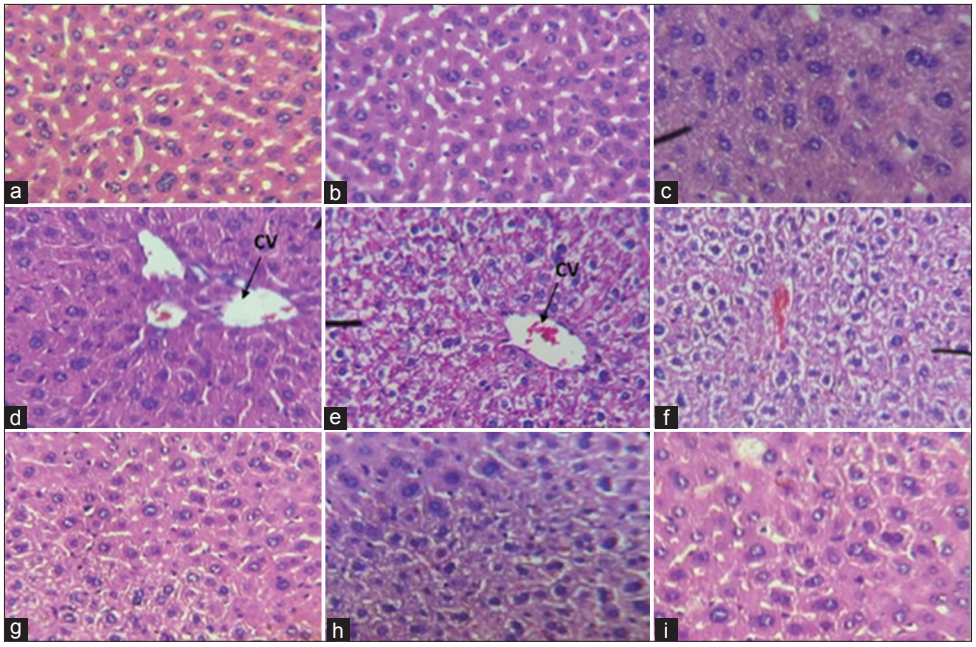

Figure 1: Liver sections viewed under light microscope with ×400: (a) Normal untreated control, (b) aqueous extract only, (c) ethanolic extract only, (d-f) negative control group with carbon tetrachloride (CCl_4), (g) Vitamin E with CCl_4, (h) ethanolic extract with CCl_4, (i) aqueous plus with CCl_4. CV: Central vein

its toxicity and corrosiveness.[1] As such, many studies have been conducted to study the toxicology of CCl_4. The primary target organs of CCl_4 toxicity are the liver and kidney,[20] and when CCl_4 is metabolically activated, free radical reactions are initiated leading to oxidative stress and the peroxidation of cellular lipids[21] hypothesized in the development of CCl_4-induced liver toxicity.[22] The action of CCl_4 is due to its free-radical transformation into a trichloromethyl radical which binds to macromolecules or attack polyenoic fatty acid in hepatocyte membrane.[23,24] An increase in serum AST and ALT is associated to damaged hepatocytes.[1] Hepatocyte damage occurred after CCl_4 administration since significant increase in AST and ALT levels were noted particularly in Groups II, III, VI, and VII. However, this damage is reduced in the positive control Group III, ethanolic extract treated Group VI, and aqueous extract treated Group VII.

Liver damage reflects abnormal liver enzyme levels,[25] an increase in serum concentrations of AST and ALT. AST is diffusely represented in the heart, skeletal muscle, kidneys, brain, and red blood cells, whereas ALT has low concentrations in skeletal muscle and kidney[26] suggesting ALT's specificity for liver damage. In the liver, ALT is localized solely in the cellular cytoplasm, whereas AST is both cytosolic and mitochondrial.[27] The hepatocyte damage resulted from the collapse of the previously existing collagen framework of the cells.[20] In this study, vacuole formation and lesions were observed after CCl_4 treatment. For Groups III, VI, and VII, there was reduced lesion in the liver tissue. Between Groups VI and VII, the aqueous extract treated group has less vacuole formation and more intact cells than the ethanolic extract treated group.

The aqueous and ethanolic *B. orellana* L. leaf extracts have comparable hepatoprotective activity when compared to Vitamin E (α-tocopherol). Vitamin E is an antioxidant that is soluble in non-polar organic solvent which protects fatty acid chains from degradation through oxidation. It reacts more rapidly to oxygen than triacylglycerols. This prevents biological membranes from reacting with oxidants which cause damage to membrane structures.[21,28,29]

CONCLUSION

Alkaloids, anthraquinones, tannins, and sugars were identified in *B. orellana* L. leaf extract. Preliminary toxicity study revealed that aqueous and ethanolic *B. orellana* L. leaf extract has no lethal or toxic effect to mice up to a dose of 2000 ppm. Oral pre-treatment with aqueous and ethanolic leaf extracts at a dose of 500 mg/kg body weight showed no hepatotoxic damage before CCl_4 induction. Biological markers (AST and ALT) in the aqueous and ethanolic extract groups were significantly lower compared to the negative control group with histopathologic analysis showing less inflammatory lesion and vacuole formation in the liver tissues. However, it is recommended that different treatment dosages of the extracts be further tested to establish a better range of hepatoprotection. Moreover, crude aqueous and ethanolic extracts should be fractionated, and fractions likewise tested further for hepatoprotection potential. Phytochemical screening of the active fractions should also be done to determine which secondary metabolites are responsible for the hepatoprotective effect.

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