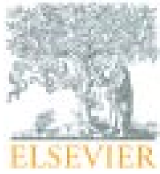

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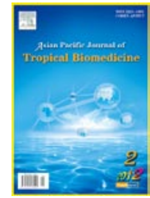

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In vivo antioxidant activity of bark extract of *Bixa orellana* L. against acetaminophen- induced oxidative stress

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ABSTRACT

Objective: To evaluate the *in vivo* activity of bark extract of *Bixa orellana* L. (*B. orellana)* against acetaminophen induced oxidative stress. **Methods:** In the present study, antioxidant activity of *B. orellana* was evaluated by using normal and acetaminophen induced oxidative stressed rats at the dose of 100 mg/kg and 200 mg/kg *p.o.* oraly daily for 20 days. The animal´s body weight was checked before and after treatment. Different biochemical parameters such as serum glutamate pyruvate transaminases, serum glutamate oxalo transaminases, alkaline phosphatase, total bilirubin, cholesterol, protein, lactate dehydrogenase, superoxide dismutase, catalase, ascorbic acid, lipid peroxide was performed. Histopathological analysis of the control and the hepatotoxicity induced rats were performed. **Results:** It was observed that the *B. orellana* bark extract showed significant protective activity against acetaminophen induced damage at 200 mg/kg dose level, while the 100 mg/kg dose showed moderate activity. **Conclusions:** From the result obtained in the present study suggest that *B. orellana* bark extract elicit protective activity through antioxidant activity on acetaminophen induced hepatic damage in rats.

1. Introduction

Liver disease is still a worldwide health problem. Unfortunately, conventional or synthetic drugs used in the treatment of liver diseases are inadequate and sometimes can have serious side effects [1]. Several hepatotoxic agents injure liver cells by inducing lipid peroxidation and oxidative stress. Some of the physiological factors also damage the liver in a drastic manner like alcohol consumption, malnutrition, anemia and availability of hepatotoxic drugs over the counter [2]. In spite of tremendous advances of modern medicine, there are no effective and reliable drugs available that can stimulate liver function[3] offer protection to the liver from damage or help to regenerate hepatic cells.

Acetaminophen, a widely used analgesic and antipyretic drug, becomes a hepatotoxin when taken in larger doses[4]. Under normal conditions, acetaminophen is primarily metabolized in the liver by glucuronidation and sulfation. A small proportion of the drug is metabolized by several of the cytochrome P-450 enzymes into the reactive intermediate N acetyl- p-benzoquinone imine (NAPQI), which is normally detoxified by glutathione (GSH) both non-enzymatically and enzymatically. Excess of NAPQI causes oxidative stress and binds covalently to liver proteins [5]. Acetaminophen poisoning in human briefly described about the hepatic necrosis, provoked a series of animal studies which demonstrate the acute centrilobular hepatic necrosis with collapse of the reticulum [6]. Hepatotoxicity induced by acetaminophen resembles various kinds of acute liver diseases with prominent elevations of liver marker enzymes and generation of reactive oxygen species (ROS). Oxidative stress caused by ROS plays an important role progression and/or perpetuation of a number of chronic diseases such as diabetes mellitus, cancer, atherosclerosis, arthritis, neurodegenerative diseases [7]. Oxidative stress caused by hepatotoxins plays a paramount role in the etiology of several diseases and antioxidant treatment might be a key element to combat such ailments.

Bixa orallana L. (*B. orallana*), belonging to the family Bixaceae, is used for the treatment of liver disorders [8]. It is a small handsome evergreen tree, leaves are cordate, acuminate, flowers are white or pink in terminal panicle,

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fruits reddish brown, and seeds are trigonous covered with a red pulp. The roots bark and seeds of *B. orellana* are antiperiodic, antipyretic and astringent. They are useful in intermittent fevers and gonorrhea. The pulp surrounding the seed is a mosquito repellent and is useful to treat dysentery. The decoction of the root is used for liver diseases. The whole plant is bitter, purgative, cures leprosy, biliousness, kidney disorders and vomiting.The methanol extract of *B. orellana* leaves showed neuropharmacological, anticonvulsant, analgesic and antidiarrhoeal activity [9]. Seeds and leaves of the annatto tree were used by the Aztecs to prepare remedies for a variety of illnesses such as tonsillitis, asthma, pleurisy, rectal disorders, headache, jaundice, sunstroke, and burns [10].

A number of studies have shown that the plant extracts having antioxidant activity protect against acetaminophen induced hepatic damage by enhancing the activity of enzymatic antioxidants and inhibiting lipid peroxidation. Hence, an attempt was made to investigate the effects of bark extract of *B. orallana* L. against oxidative stress induced by acetaminophen hepatotoxicity.

2. Materials and methods

2. 1. Plant material and extraction

The bark of *B. orellana* was collected from Karpagam University and authenticated by the Botanical Survey of India (BSI), Coimbatore (Reference no: 625). The voucher specimen has been deposited in the laboratory for further references. After collection the bark of *B. orellana* were washed properly, shade dried at room temperature and powdered with electrical blunder. Thousand grams (1 000 g) of the powder was exhaustively extracted with 5 L of methanol [(MeOH 1:5 (w/v)] using soxhlet apparatus. The extract was then centrifuged (3 000 x g) thrice and the clear supernatants were combined. The combined supernatants were filtered over Whatman No.1 filter paper. The solvents were distilled off and evaporated to dryness by rotary evaporator (Buchi type, Switzerland) under reduced pressure at 45 ℃ (in vacuo) to leave the crude methanolic extract. The methanolic extract was stored in dark until further analysis.

2.2. Animals

Adult male wistar albino rats weighing about 150 – 180 g were procured from the Animal House of Karpagam University, and were used for experiments. The animals were grouped and housed in polyacrylic cages (cm) with six animals per cage. They were maintained at a constant temperature of 25 ℃ (12 h light/12h dark cycle) and were fed with commercial mouse diet (Gold Mohar rat feed, Hindustan Lever Ltd, Mumbai, India) and water ad libitum during the experiments. All animal procedures were performed in accordance with NIH guidelines, after getting the approval of the Institute's Animal Ethics Committee in Karpagam University, Coimbatore.

2.3. Acetaminophen induced hepatotoxicity

Animals were randomized and grouped into seven groups (I – V) of six animals per group. Animals of group I served as control and received only saline water for seven days. Group II rats were administrated with acetaminophen (300 mg/kg body weight) on the seventh day. Groups III and IV received a daily dose of plant extracts for 20 days (200 and 100 mg/kg body weight) and 2 mL of acetaminophen suspension (300 mg/kg body weight) 30 min after extract administration on the seventh day. Group V animals received reference drug (Silymarin) at a dose of 50 mg/kg on all the 20 days and 2 mL of acetaminophen suspension.

2.4. Preparation of serum and tissue samples

Animals were anesthetized with chloroform and sacrificed by cervical capitation on the seventh day after 48 hours of acetaminophen administration. Blood was obtained through cardiac puncture by means of hypodermal syringe and needle. The collected blood samples were placed in ice-cold micro centrifuge tubes. The blood was allowed to coagulate at 37 ℃ and centrifuged at 5 000 rpm for 10 min. The serum samples were collected and utilized for the respective assays. The liver and the kidney tissues were excised washed and homogenized in suitable buffer and centrifuged at 12 000 μg for 30 minutes at 4 ℃. The supernatant was collected and used for biochemical estimation of different antioxidant enzyme assays. The remaining liver tissues were used for the analysis of histopathological study.

2.5. Estimation of biochemical parameters

Activities of serum hepato specific markers, serum glutamate oxaloacetic transaminase (SGOT), serum glutamate pyruvate transaminase (SGPT) [11] were estimated. The SGOT and SGPT activities were expressed as IU/L. The serum alkaline phosphatase (SALP) was assayed by the method of King[12] using di sodium phenyl phosphate as the substrate. The total bilirubin (TB) content of the serum was estimated by the method of Malloy and Evelyn[13]. Total cholesterol (TC) was estimated by the method of Parekh and Jung [14]. Total protein (TP) was estimated by following the method of Lowry OH *et al.* [15]. LDH was estimated using the method of King [16].

2.6. Assessment of enzymatic antioxidants

Super oxide dismutase (SOD) was estimated according to the method of Beauchamp Fridovich[17]. One unit of enzyme activity is defined as the enzyme reaction which

gave 50% inhibition of NBT reduction/minute and expressed as specific activity in units/g tissue. Catalase activity was determined according to the method described by Aeb[18]. One unit of Catalase activity is defined as the amount of enzyme required to decompose nano moles of H_2O_2/minute. The measurement of Glutathione peroxidase was conducted by modified methods from Flohe and Gunzler[19]. The absorbance of the reaction product was read at 412 nm and the enzyme activity was calculated as U/mg protein. Estimation of ascorbic acid (Vitamin C) was determined according to the method of Roe and Kuether[20]. The thiobarbituric acid reactive substances (TBARS) were measured by their reactivity with thiobarbituric acid (TBA) by following the method of Nichans and Samuelson[21]. Activity of lipid peroxidation was expressed as nano moles of MDA/mg protein.

2.7. Histopathological studies

A portion of the liver was cut into two to three pieces of approximately 6mm size and fixed in phosphate buffered 10% formaldehyde solution. After embedding in paraffin wax, thin sections of 5 μm thickness of liver tissue were cut and stained with haematoxylin – eosin. The thin sections of liver were made into permanent slides and examined under high-resolution microscope with photographic facility.

2.8. Statistical analysis

The data were expressed as mean ± deviation (SD). Statistical differences between the values were analyzed through one way analysis of variance (ANOVA) by SPSS version 11.5. Differences between the test and the control group were evaluated by least significant difference method at $P < 0.05$

3. Results

3.1. Changes in body weight

In acetaminophen induced group II rats the weight reduced significantly by 140 g from 157 g (Table 1). The rats pretreated with the *B. orellana* and those administered with acetaminophen the weight was gained significantly. The percentage of weight changed in group IV rats has found to be almost similar towards the control group I rats. The capability of *B. orellana* to protect body weight loss seems to be as a result of its ability to reduce hepatotoxicity.

3.2. Effect of B. orellana liver marker enzymes

As shown in Table 2 and 3, there was a significant increase in the levels of liver marker enzymes such as SGOT, SGPT, SALP, LDH, total cholesterol and bilirubin in acetaminophen intoxicated rats. A pretreatment with *B. orellana* (100 mg/kg and 200 mg/kg) significantly combated the liver toxicity by reducing the contents of the aforementioned marker enzymes. At a dose of 100 mg, the effect was only marginal where as at higher dose (200 mg/kg) the drug effectively prevented the acetaminophen induced liver damage. In our study malfunctioning of the liver was evidenced by the significant increase (P<0.05) in the level of unconjugated bilirubin in the serum of the group treated with only acetaminophen (group II) when compared with control (group I) and the other groups. Total protein level was significantly increased in acetaminophen intoxicant rat compared to normal. A significant decrease (P<0.01) in total protein was observed in *B. orellana* (Group III & Group IV) rats which were administered with *B. orellana*. Pretreatment with *B. orellana* (100, 200 mg/kg) reduced the elevated level of cholesterol which is shown table 2 respectively.

Table 1
Effect of methanolic bark extract of *B. orellana* (BME) on body weight of acetaminophen induced toxicity.

Groups	Body weight (g)			
	Initial (g)	Final (g)	Difference in body weight (g)	% Weight change
Control	156.00±1.20	160.00±2.60	4.00±1.40	2.5
Acetaminophen (300mg/kg)	157.00±1.50	140.00±3.70	17.00±2.20	12.14
Acetaminophen+ BME (100mg/kg)	151.00±0.06	145.00±2.40	6.00±1.80	4.13
Acetaminophen + BME (200mg/kg)	179.00±2.50	170.00±3.00	4.00±0.50	2.35
Acetaminophen+ Silymarin (50mg/kg)	150.00±5.46	154.00±6.42	4.00±0.96	2.59

Values are expressed as mean ± SD for six animals in each group.

Table 2
Effect of *B. orellana* (BME) bark extract on LDH, total cholesterol, total protein and total bilirubin in acetaminophen induced hepatotoxicity in rats.

Groups	LDH(IU/L)	Total protein (mg/dL)	Total Cholesterol(mg/dL)	Total Bilurubin(mg/dL)
Control	108.94±2.06	9.06±0.54	49.55±3.04	0.83±0.9
Acetaminophen(300mg/kg)	148.50±0.51**	5.03±0.71**	82.75±2.79**	2.08±0.18**
Acetaminophen+ BME (100mg/kg)	130.18±0.86*	6.52±0.43*	71.16±1.88*	1.25±0.33*
Acetaminophen + BME (200mg/kg)	97.03±1.68*	7.15±0.50*	68.24±1.77*	0.95±0.10*
Acetaminophen+ Silymarin (50mg/kg)	107.59±0.50*	8.93±0.36*	46.30±2.10*	0.82±0.21*

Each value in the table was obtained by calculating the average of all animals (n=6), ± standard deviation. *P<0.01 indicate significantly values compared with control group, **P<0.05 significantly different values from Acetaminophen group.

Table 3
Effect of *B. orellana* (BME) bark extract on SGOT, SGPT and SALP in acetaminophen induced hepatotoxicity in rats.

Groups	Dose (mg/kg)	SGOT(U/L)	SGPT(U/L)	SALP (U/L)
Control	–	218.00±7.00	187.56±5.30	220.43±6.14
Acetaminophen	300	314.60±2.10**	211.90±3.70**	378.10±5.04**
Acetaminophen+BME	100	258.66±1.50*	151.33±2.50*	287.06±5.40*
Acetaminophen + BME	200	241.16±1.90*	178.0±2.90*	203.90±7.40*
Acetaminophen+ Silymarin	50	217.10±3.50*	186.43±0.50*	220.36±5.20*

Each value in the table was obtained by calculating the average of all animals (n=6), ± standard deviation. *P<0.01 indicate significantly values compared with control group, ** P<0.05 significantly different values from Acetaminophen group.

Table 4
Effect of *B. orellana* (BME) bark extract on GSH-Px, SOD, catalase and vitamin C in liver.

Groups	GSH-Px(U/mg)	SOD(U/mg)	Catalase(U/mg)	Vitamin C (U/mg)
Control	190.16±3.71	144.36±4.15	78.61±1.11	37.05±1.46
Acetaminophen(300 mg/kg)	72.22±0.82**	55.01±4.22**	23.36±2.27**	11.31±4.64**
Acetaminophen+ BME (100 mg/kg)	142.73±1.75*	71.74±2.92*	44.41±4.00*	16.29±0.63*
Acetaminophen + BME (200 mg/kg)	176.34±2.92*	93.83±3.25*	63.80±4.78*	19.62±0.82*
Acetaminophen+ Silymarin (50 mg/kg)	192.29±3.39*	140.06±4.37*	71.94±3.23*	36.05±4.79*

Each value in the table was obtained by calculating the average of all animals (n=6), ± standard deviation. *P<0.01 indicate significantly values compared with control group, ** P<0.05 significantly different values from Acetaminophen group.

Table 5
Effect of *B.orellana* (BME) bark extract on GSH-Px, SOD, catalase and vitamin C in kidney.

Groups	GSH-Px (μm/mg)	SOD (U/mg)	Catalase (U/mg)	Vitamin C (U/mg)
Control	172.57±5.06	143.65±2.64	83.95±0.72	30.50±0.63
Acetaminophen(300 mg/kg)	56.10±0.82**	26.34±2.58**	31.55±1.71**	12.49±2.27**
Acetaminophen+ BME (100 mg/kg)	98.43 ±2.92*	52.51±2.04*	44.40±1.94*	16.89±2.08*
Acetaminophen + BME (200 mg/kg)	116.23±0.64*	75.81±0.93*	75.23±1.73*	22.82±2.28*
Acetaminophen+ Silymarin(50 mg/kg)	170.08±9.47*	134.03±1.24*	80.37±3.55*	32.12±2.64*

Each value in the table was obtained by calculating the average of all animals (n=6), ± standard deviation. *P<0.01 indicate significantly values compared with control group, ** P<0.05 significantly different values from Acetaminophen group.

Table 6
Effect of *B. orellana* (BME) bark extract on lipid peroxidation in different organs of acetaminophen induced hepatotoxicity in rats.

Groups	Liver (nmol/mg)	Kidney (nmol/mg)	Brain (nmol/mg)
Control	0.47±1.27	0.074±5.52	0.32±4.18
Acetaminophen 300 mg/kg	1.85±0.56**	0.49±0.75**	0.95±0.85**
Acetaminophen+ BME (100 mg/kg)	0.81±0.72*	0.35±0.86*	0.84±0.35*
Acetaminophen + BME (200 mg/kg)	0.52±1.53*	0.15±2.13*	0.65±2.16*
Acetaminophen+ Silymarin (50 mg/kg)	0.80±3.33*	0.07±3.75*	0.32±1.05*

Each value in the table was obtained by calculating the average of all animals (n=6), ± standard deviation. *P<0.01 indicate significantly values compared with control group, ** P<0.05 significantly different values from Acetaminophen group.

3.3. Effect of B. orellana on antioxidant enzymes

Table 4 and 5 demonstrates the effects of *B. orellana* on the activities of enzymatic antioxidants CAT, glutathione peroxidase, SOD and vitamin C in liver and kidney of control and experimental groups.

The SOD activity of the tested organs, liver and kidney significantly decreased in acetaminophen treated group. Administration of *B. orellana* extract proved significantly better in restoring the altered activity of SOD, and increased the activity in a dose dependent manner in both organs. The catalase and the Glutathione peroxidase activity of tissue homogenate of both the organs of acetaminophen treated rats (31.55 & 56.10 U/mg tissue) was significantly (P< 0.05) decreased than that of normal rats (83.95 & 172.57 U/mg tissue). In the *B. orellana* and silymarin treated rats the catalase activity was significantly (P< 0.01) higher than acetaminophen treated rats and approximately similar towards the control group.

Acetaminophen intoxication led to severe oxidative stress in the rats as could be seen from the dramatic increase in the lipid peroxide level of the acetaminophen treated group (P< 0.05) compared with the control group. The *B. orellana* extracts significantly reduced the elevated lipid per oxide levels at the 100-200 mg/kg dose levels (Table 6). Apparently, the group pretreated with *B. orellana* at 200 mg/kg dose seemed to be more effective than the other.

3.4. Histopathology studies

Histopathological studies of the liver of control animals showed normal hepatocytes. Animals in group II (acetaminophen treated) demonstrate severe hepatotoxicity

as evident by showing perivenular hepatocytes and bridging fibrosis (Figure 1). The groups of administration of various doses of *B. orellana* pretreated with acetaminophen also showed regenerating hepatocytes (group III and IV). The animals exhibited mild fatty changes and mild perivenular congestions. The animals with group V shows almost normal hepatocytes and regeneration of hepatocytes. The histological changes associated with the hepatoprotective activity in two prescriptions of *B. orellana* supported the estimation of the biochemical results.

4. Discussion

Chemicals that cause liver injury are called hepatotoxins. Around 900 drugs have been found to cause liver damage. Acetaminophen is one among to produce acute liver damage if over doses have been consumed [22]. It is mainly metabolized in the liver to glucuronide and sulphate conjugates that are subsequently excreted. The hepatotoxicity of acetaminophen has been attributed to the formation of a highly reactive metabolite N-acetyl-P-benzoquinoneimine (NAPQI) by the hepatic cytochrome P-450 which in turn disrupts the structure and function of lipid and protein macromolecules in the membrane [23].

The decrease in body weight in acetaminophen induced rats shows that the loss or degradation of structural proteins which is due to liver damage and structural proteins are known to contribute to the body weight. The toxic effect of acetaminophen was controlled in the animals treated with methanol extract (100 mg/kg and 200 mg/kg) of *B. orellana* by restoring the levels of liver function. The rise in serum level of SGOT, SGPT and SALP has been attributed to the damaged structural integrity of the liver. Hence, the *B. orellana* mediated reduction in level of SGOT, SGPT and ALP towards the respective normal values which indicates the stabilization of plasma membrane as well as repair of hepatic tissue damage caused by acetaminophen intoxication. Increased activity of alkaline phosphatase, which℃curs due to de novo synthesis by liver cells, is a reliable marker of hepatobiliary dysfunction due to damage[24]. Our results indicate that methanolic extract of *B. orellana* could increase the synthesis of protein in liver after damage inflicted by acetaminophen.

The increase in plasma LDH activity may be due to hepatocellular necrosis leading to leakage of the enzyme to the blood stream[2]. Thus when acetaminophen leads to the release of these enzymes into plasma as result of autolysis breakdown or cellular necrosis, the plants infusion supplement impart protection against carcinogenic chemical induced oxidative injury that may result in development of liver damage.

Presumably a decrease in CAT activity could be attributed to cross linking and inactivation of the enzyme protein in the lipid per oxidase and leads to oxidative stress. The activity was restored to normal after treatment with *B. orellana* methanolic extracts evidently shows the antioxidant properties of the extracts against oxygen free radicals. Moreover our findings showed a decrease in Glutathione peroxidase activity in acetaminophen intoxicated control rats. It is an enzyme that catalyzes the reduction of hydroperoxides, including hydrogen peroxides, and functions to protect the cell from peroxidative damage [26].

An increase in peroxidase activity was observed in control acetaminophen plus plant extracts Group III rats, when compared with Group II rat's decreased peroxidase activity. Peroxidase estimation is based on periodide formation. Periodide can be spectrophotometrically determined, and this is directly proportional to the peroxidase concentration in the reaction mixture containing approximate amounts of H2O2 and enzyme. It plays an important role in pro-oxidant/antioxidant balance. Free radicals induced lipid peroxidation is belived to be one of the major causes of cell membrane damage leading to a pathological condition including autocatalytic process of cell death [27]. The increase in LPO level in all the 3 organs suggests enhanced lipid peroxidation leading to tissue damage and failure of antioxidant defense mechanism to prevent formation of excessive free radicals [28]. The diminished LPO activity after treatment with the BME may be attributed to the antioxidant activity of the plant by scavenging the free radical generated due to the metabolic transformation in the organs.

The results of the present study indicated that the methanolic extracts of *B. orellana* bark possess hepatoprotective property and this may in part be explained by the presence of important antioxidative factors. There is an alarming increase in the incidence of drug-related liver damage and the use of acetaminophen is widespread. Therefore, further studies along these lines would be worthwhile. However, detailed toxicity studies and investigations on possible contraindications that might result from the use of the plant should be carried out.

Conflict of interest statement

We declare that we have no conflict of interest.

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