Comparative hepatoprotective and antioxidant activity of *Berberis asiatica* stem bark and root

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Context
Berberis asiatica, a shrub with common name Kilmora in Kumaun, Assam, Madhya Pradesh, and Mount Abu of India, is used as a hepatoprotective, anti-inflammatory, antipyretic, analgesic, diuretic, antimicrobial, strong wound healer, and antirheumatic medicine.
Aim
The aim of this study was to evaluate *B. asiatica* stem bark and root extract for their antioxidant and hepatoprotective activity.
Materials and methods
The stem bark and root extracts were obtained with methanol for determining antioxidant activity and hydroalcoholic solvent for hepatoprotective activity using maceration process. The methanolic and hydroalcoholic extracts of *B. asiatica* stem bark and root were evaluated for in-vitro antioxidant activity using various methods, and the hepatoprotective activity was also assessed against paracetamol-induced hepatic damage in rats at the doses of 100 and 200 mg/kg.
Results
The half maximal inhibitory concentration value of root and stem bark extract for DPPH was found to be 102.31 and 120.7 μg/ml and for ABTS it was 32.24 and 26.724 μg/ml, respectively. There were significantly higher restorations of enzyme level on treatment with *B. asiatica* root extract (200 mg/kg) than with stem bark extract (200 mg/kg) observed.
Conclusion
The present study showed that both the stem bark and the root of *B. asiatica* possess antioxidant and hepatoprotective activity, although the root possesses significantly higher activity as well as higher antioxidant compounds – namely, total phenol, ascorbic acid, tannin, and flavonoid – compared with stem bark.

Keywords:
antioxidant, *Berberis asiatica*, free-radical scavenging activity, hepatoprotective, Kilmora

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Introduction

Free radicals can cause severe diseases such as diabetes, cancer, liver cirrhosis, atherosclerosis, etc. Antioxidant inhibits free radical formations and they have an essential role in liver protection. It is therefore vital to take antioxidants to support healthy liver function. They may prevent oxidative stress by scavenging free radicals, by inhibiting lipid peroxidation, and through other mechanisms, and thus help in averting free-radical-induced diseases [1]. *Berberis asiatica*, generally known as Kilmora (family Berberidaceae), is an evergreen spiky shrub, about 1.8–2.4 m in height, and it is commonly found in dry outer Himalaya from 600 to 2550 m height in Kumaun eastwards, Assam, Madhya Pradesh, and Mount Abu in India [2]. Its root as well as stem bark is a reputed drug in Ayurvedic system of medicine. It contains many alkaloids – namely, palmitine, berberine, columbamine, berbamine, jatrorrhizine, oxyacanthine, tetrahydropalmatine, and oxyberberine – and is conventionally used as a hepatoprotective, anti-inflammatory, antipyretic, analgesic, diuretic, antimicrobial, strong wound healer, antirheumatic, immunogogue, and antioxidant medicine [3,4]. Paracetamol is an effective and safe antipyretic and analgesic drug in therapeutic doses; however, overdose can induce severe liver injury [2]. The toxic mechanism of paracetamol includes the formation of reactive metabolite, primarily *N*-acetyl-*p*-benzoquinone imine, which is quickly conjugated by hepatic glutathione to yield mercapturic acid. Saturation of the conjugation pathway occurs due to overdose of paracetamol, which causes depletion of glutathione and increases the formation of toxic reactive metabolites. Because of depletion of glutathione, excess *N*-acetyl-*p*-benzoquinone imine binds to hepatic cell proteins and

DNA, which results in hepatic injury [3]. Oxidative damage to biomolecules (lipids, proteins, and DNA) occurs because of excessive production of free radicals, which eventually leads to many chronic diseases, including stroke, diabetes, cancer, myocardial infarction, cardiovascular diseases, rheumatoid arthritis, atherosclerosis, chronic inflammation, septic shock, postischemic perfusion injury, and other degenerative diseases in humans [5].

In the present study, *B. asiatica* stem bark and root extracts were evaluated for their antioxidant activity, and antioxidant compounds such as ascorbic acid, total phenols, tannins, and flavonoids were also determined along with their hepatoprotective activity. The aim of this study was to provide a cost-effective natural antioxidant material for pharmaceutical industry to develop a formulation with high impact of activity.

Materials and methods

Plant material

The stem bark and roots of *B. asiatica* were collected from herbal garden of Defence Institute of Bio-Energy Research (DIBER), field station, Pithoragarh, Uttarakhand, India, located in the Western Himalayan region. The plant was authenticated by Dr H.C. Pandey, Scientist D, Botanical Survey of India, Dehradun, and a herbarium specimen was deposited with reference number BSI/NRC/Tech (ident.)/2011-12/257.

Preparation of extract

The stem bark and roots were dried below 40°C in a drying chamber, powdered with a mechanical grinder, and extracted with methanol for determining antioxidant activity, and then a hydroalcoholic extract was prepared for determining hepatoprotective activity by means of maceration process followed by ultrasonication. The solvent was completely removed and dried extracts were stored in tightly closed containers until used.

Animals

Adult male Wistar rats were randomly assigned to the control and treatment groups in acrylic cages and were kept for 7 days before start of study to allow them to acclimatize under the prevailing laboratory conditions at a room temperature of 22±3°C, relative humidity of 50–70%, and 12 h light/dark cycle. Rat chow food and drinking water were given *ad libitum*. In the current study, all procedures were followed in accordance with the approved guidelines of Institutional Animal Ethics Committee, DIBER, field station, Pithoragarh, Uttarakhand, India (1306/c/09/CPCSEA).

Chemicals

Sodium carbonate, potassium permanganate, ferric chloride, hydrogen peroxide, methanol, and 2,6-dichlorophenolindophenol were purchased from E. Merck (Mumbai, India). 1,1-diphenyl-2-picryl-vhydrazyl (DPPH), 2,2′-azino-*bis*(3-ethylbenzo-thiazoline-6-sulfonicacid) (ABTS), butylated hydroxy toluene, aluminum chloride, trichloroacetic acid, potassium ferricyanide, catechol, ascorbic acid, quercetin, tannic acid, sulfuric acid, oxalic acid, and enzymatic kits for serological estimation were purchased from Sigma Aldrich Chemicals Pvt. Ltd, Bangalore, India. Silymarin was purchased from Microlab Pvt. Ltd (Baddi, Himachal Pradesh, India).

Free-radical scavenging activity (DPPH method)

Free-radical scavenging activity of methanolic extracts was determined by using the DPPH method [6]. The extract was prepared by adding various doses (40–200 µg) of methanolic extract (2 mg/ml) to 2 ml of methanolic solution of DPPH (0.1 mmol) of root and stem bark in volumetric flasks, and then the final volume was made up to 3 ml with methanol. After 40 min, the absorbance of mixture was measured at 517 nm of ultraviolet light. 0.1 mg/ml of ascorbic acid was used as standard in various doses (2–10 µg). The free-radical scavenging activity (%) and half maximal inhibitory concentration (IC_{50}) values of tested samples of *B. asiatica* stem bark and root were evaluated by comparing with control of methanol (1 ml) and DPPH solution (2 ml). For each sample, triplicate reading was taken and the values were averaged. Free-radical scavenging activity was determined by using the formula: free-radical scavenging activity=$[A_c-A_t/A_c-A_s]\times100$, where A_t is the absorbance of test, A_c is the absorbance of control, and A_s is the absorbance of standard.

Oyaizu method for determining reductive ability

The Oyaizu method [7] was used for determining the reducing ability of both extracts. Various doses (400–20 000 µg) of methanolic extract (4 mg/ml) of stem bark and root were dissolved in 1 ml of distilled water and then mixed with 2.5 ml of potassium ferricyanide [$K_3Fe(CN)_6$] (1%) and 2.5 ml of phosphate buffer (0.2 mol/l; pH 6.6). The mixture was then incubated at a temperature of 50°C for a duration of 20 min, followed by the addition of 2.5 ml of trichloroacetic acid (10%). The mixture was then centrifuged at 3000 rpm for 10 min, and from the upper layer 2.5 ml of solution was taken out and mixed with 2.5 ml of distilled water and 0.5 ml of $FeCl_3$ (0.1%). Absorbance of the solution was then measured at 700 nm. Butylated hydroxy toluene (1 mg/ml) was

used in various doses of 250–1250 μg as a reference standard. All analyses were performed in triplicate and the results were averaged.

ABTS method

For ABTS assay, some modifications were made to the method described by Aranao *et al.* [8]. In this method, 2.6 mmol of potassium persulfate solution and 7.4 mmol of ABTS solution were used as stock solution. For preparing working solution, equal quantities of both stock solutions were mixed and then allowed to react for 12 h at room temperature in dark. Dilution was carried out by mixing 60 ml of methanol in 1 ml of working solution to obtain an absorbance of 1.1±0.02 U at 734 nm using a spectrophotometer. Methanolic extract (2 mg/ml) in various doses of 20–60 μg were allowed to react with 2 ml of ABTS solution, and using methanol the final volume in each test tube was made up to 2.5 ml. Thereafter, absorbance was measured at 734 nm using a spectrophotometer. Trolox was used for preparing standard curve.

Determination of total phenols

The concentrations of total phenolic content in various extracts were determined using the Folin–Ciocalteu method [9]. After weighing, 1 g of the sample was grinded with 10 times the volume of 80% ethanol, the mixture was centrifuged at a speed of 10 000 rpm for 20 min. Finally, re-extraction of residue was carried out with five times the volume of 80% ethanol by means of centrifugation and the supernatant was pooled and dried by means of evaporation. The residue was dissolved in 5 ml of distilled water. Test tubes were taken and different aliquots (10–50 μl), followed by 0.5 ml Folin–Ciocalteu reagent, were pipetted out into each test tubes and mixed thoroughly. A volume of 2 ml of sodium carbonate solution (20%) was added after three minutes and mixed. For 1 min, the test tubes were kept in boiling water and cooled. Thereafter, the absorbance was measured at 650 nm. Standard curve was prepared by using different concentrations of 0–5 μg catechol. Standard calibration curve was prepared for measuring the concentration of the total phenolic content equivalent to catechol.

Determination of total tannins

The Folin–Denis method was used for determining total tannin in both extracts [10]. In this method, 0.5 g powdered extract was boiled for 30 min with 75 ml of double-distilled water, cooled, and centrifuged at 2000 rpm for 20 min. Using double-distilled water, the volume of supernatant liquid was made up to 100 ml in a volumetric flask (100 ml). Thereafter, 10 ml of sodium carbonate solution (35%), 75 ml of water, and 5 ml of Folin–Denis reagent was taken in 100 ml volumetric flask, followed by addition of 1 ml of extract solution. Thereafter, the mixture was diluted up to 100 ml with water, shaken well, and the absorbance was taken at 700 nm after 30 min. A blank solution was prepared with water instead of the extract. For preparing a standard graph, 0–625 μg of tannic acid was used. Total tannin content of the extract was determined by plotting standard graph.

Determination of ascorbic acid content

Total ascorbic acid content in plant extract was determined by using the 2,6-dichlorophenolindophenol method [11]. In this method, 1 g of dried powdered sample was taken and extracted with 4% oxalic acid. Thereafter, the volume of extract was made up to 20 ml, followed by centrifugation at 10 000 rpm for 10 min, and then 5 ml of supernatant liquid was transferred to a conical flask and 10 ml of 4% oxalic acid was added. Titration was carried out to attain a pink end point against standard dye solution (2,6-dichlorophenolindophenol). For blank solution (without adding extract) the same procedure was repeated. A volume of 5 ml of ascorbic acid of 100 ppm was used as standard. Ascorbic acid content was determined by using the following formula:

$$\text{Ascorbic acid (mg/100 g)} = \frac{\text{Wt of sample} \times \text{titer vol. against test} \times \text{vol. made} \times 100\ \text{ml}}{\text{Titer vol. against ref.} \times 5\ \text{ml} \times \text{wt of sample}} \times 100.$$

Determination of flavonoid content

For determination of flavonoid content, the aluminum chloride method was modified from the procedure given by Woisky and Salatino [10]. Quercetin was used for making calibration curves. Different dilutions of 25, 50, and 100 μg/ml of sample were prepared by dissolving 10 mg of quercitin in 80% ethanol. Thereafter, 1.5 ml of ethanol (95%), 0.1 ml of aluminum chloride (10%), 0.1 ml of potassium acetate (1 mol/l), and 2.8 ml of distilled water were added in 0.5 ml of diluted standard solution. The mixture was then incubated for 30 min at room temperature and the absorbance of reaction mixture was determined using an ultraviolet spectrophotometer at 415 nm. Similarly, solution of stem bark and root (100 ppm) was reacted separately with aluminum trichloride and used for the determination of flavonoid content as described above [12].

Hepatoprotective activity

The hepatoprotective activity of stem bark and root extract was determined by using paracetamol-induced

hepatotoxicity rat model. Thirty-five rats were divided into seven groups of five rats each. Group 1 was assigned as the normal control and group 2 served as paracetamol-treated control. All groups were treated once with paracetamol (3000 mg/kg, orally) excluding normal control. After 48 h of paracetamol administration, group 3 received silymarin (100 mg/kg), groups 4 and 5 received 100 and 200 mg/kg of *B. asiatica* root extract, respectively, and groups 6 and 7 received 100 and 200 mg/kg of *B. asiatica* stem bark extract, respectively, orally, once daily for 21 consecutive days. Sixteen hours after administration of last dose of drugs, the animals were anesthesized with chloroform, and using a fine glass capillary blood was collected from the retinobulbar venous plexus. Blood samples were clotted at room temperature. For separation of serum, centrifugation was carried out at 2500 rpm for 15 min and used for the estimation of the biochemical markers of liver damage – namely, alanine aminotransferase (SGPT), aspartate aminotransferase (SGOT), direct bilirubin, total bilirubin, alkaline phosphatase, cholesterol, total protein, and albumin.

After collection of blood samples, the rat was killed and the abdomen was cut open and the liver was dissected out. The liver was then preserved in 10% formalin solution and then processed for histopathological investigations.

Statistical analysis

The data are presented as mean±SD. Data obtained from biochemical studies of rat serum were determined with one-way analysis of variance followed by post-hoc analysis of significance with Dunnett's multiple comparison test. Two-way analysis of variance followed by post-hoc Bonferroni's multiple comparison tests was performed for analyzing the data obtained from antioxidant activities. Differences were considered significant at *P* value less than 0.05.

Results and discussion

Antioxidant activity

The antioxidant properties of *B. asiatica* stem bark and root have been evaluated by measuring their reducing ability, DPPH, and ABTS free-radical scavenging ability and by determining total phenol, total tannin, flavonoid, and ascorbic acid contents using methanolic extract of stem bark and root of *B. asiatica,* which were found significant in a dose-dependent manner.

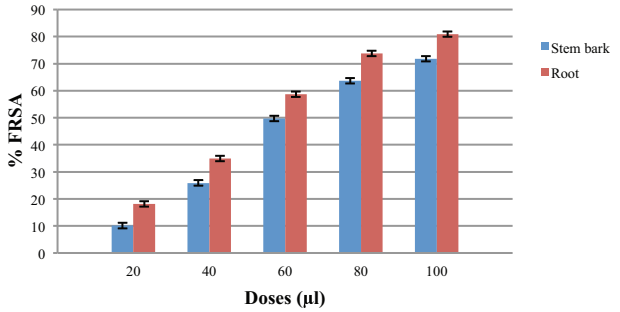
DPPH free-radical scavenging activity

The methanolic extract of stem bark and root of *B. asiatica* exhibited very good antioxidant activity in a dose-dependent manner. However, the root of *B. asiatica* at different doses exhibited significantly higher (10%) antioxidant activity as compared with stem bark (Fig. 1). The IC_{50} value of root and stem bark extract was found to be 102.31 and 120.7 µg/ml, respectively, as compared with standard drug ascorbic acid (4.981 µg/ml).

ABTS free-radical scavenging activity

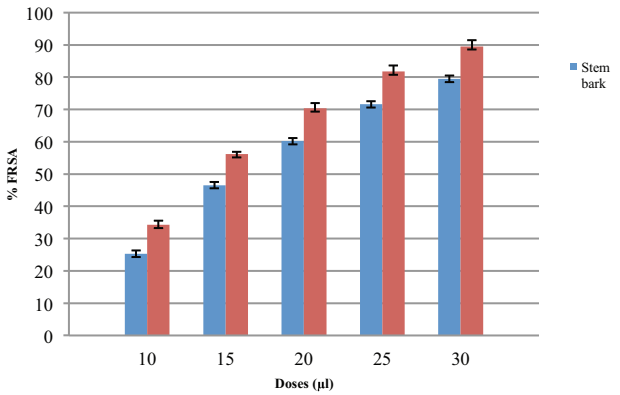
The stem bark and the root of *B. asiatica* showed very good antioxidant activity in a dose-dependent manner. As compared with stem bark methanolic extract, root extract at different doses showed significant (9% more) antioxidant activity (Fig. 2). The IC_{50} value of stem bark and root extract was found to be 32.24 and 26.724 µg/ml, respectively. For standard drug trolox it was found to be 3.258 µg/ml. Scavenging of proton radical is a vital characteristic of antioxidants. Maximum absorbance of ABTS (a protonated radical) is obtained at 734 nm and this absorbance decreases with the scavenging of the proton radicals [13].

Figure 1



1,1-Diphenyl-2-picryl-hydrazyl (DPPH) free-radical scavenging ability of *Berberis asiatica* stem bark and root. FRSA, free-radical scavenging activity

Figure 2



Free-radical scavenging ability of *Berberis asiatica* stem bark and root. FRSA, free-radical scavenging activity

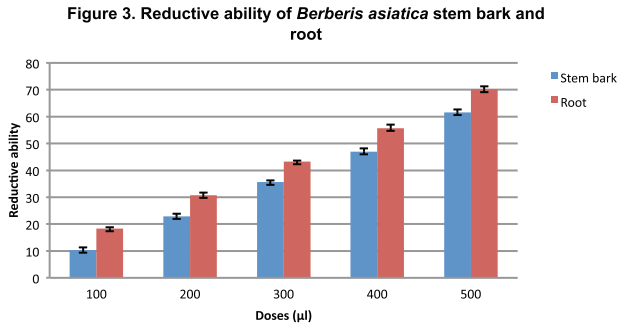
Reductive ability

The methanolic extract of *B. asiatica* root at different doses exhibited significant reductive ability (9% higher) as compared with stem bark. The absorbance of solution increased with the increase in concentration of plant extract. It indicates the increase in the reducing power of extracts or an increase in the concentration of the hydrogen donating group (Fig. 3). Potential antioxidant activity of the plant extract may serve as an indicator of its reducing capacity [14].

Total phenols

The total phenolic content of methanolic extract of *B. asiatica* stem bark and root was observed to determine the presence of antioxidant constituent as they are extremely vital plant constituents due to the scavenging ability of their hydroxyl groups [6]. Total phenol in the present study was found higher in *B. asiatica* root (59.07 mg/100 g) than in the stem bark (52.2 mg/100 g) (Table 1).

Figure 3



Reductive ability of *Berberis asiatica* stem bark and root

Table 1 Quantities of phytochemical constituents with antioxidant property in *Berberis asiatica* stem bark and root

Constituent names	*Berberis asiatica* stem bark	*Berberis asiatica* root
Phenols (mg/100 g)	52.240±0.119	59.076±0.219
Tannins (%)	0.706±0.234	1.101±0.177
Ascorbic acid (mg/100 g)	22.221±2.749	28.571±4.760
Flavonoids (%)	1.735±0.396	1.576±0.197

Flavonoid content

It has been reported that the flavonoid constituents of the plant possess antioxidant properties and was found to be useful in the treatment of liver damage and possess an ideal structure for the scavenging of free radicals, which makes them important antioxidant agents [1,15–17]. In the present study, root powder of *B. asiatica* was found to exhibit less flavonoid content (1.57%) as compared with stem bark (1.73%) (Table 1).

Tannin content

In the present study, higher tannin content was recorded in the root, whereas they were moderately low (0.4%) in stem bark (Table 1). Many higher plants contain tannins as one of their major phytoconstituents. Tannins have been known since long time as the astringent, having the capacity to combine with tissue proteins and precipitate them [18]. They are antioxidants often characterized by reducing power and scavenging activities [19,20]

Ascorbic acid content

Vitamin C performs several biochemical and physiological actions as it acts as a reducing agent due to its electron-donating nature. It is a powerful antioxidant with water-soluble nature. It donates two electrons, to form a double bond between the second and third carbons of 6-carbon molecule [1]. The 2,6-dichlorophenolindophenol method was used for determining ascorbic acid, and

Table 2 Effect of hydroalcoholic extract of *Berberis asiatica* stem bark and root on paracetamol-induced hepatotoxicity

Serological parameters	Groups						
	Normal control	PC control (3 g/kg)	PC+silymarin (100 mg/kg)	PC+BSB (100 mg/kg)	PC+BSB (200 mg/kg)	PC+BR (100 mg/kg)	PC+BR (200 mg/kg)
SGPT	3.80±5.12	108.4±6.48#	53.4±4.33*	69.2±4.60*	66.4±4.77*	66.2±4.76*	62.8±3.96*
SGOT	52.2±5.80	120.8±4.65#	62.2±4.91*	77.4±5.07*	72.2±4.76*	74.8±5.63*	68.2±5.06*
ALKP	111.8 ±4.65	298.2±4.91#	119.2±4.60*	140.2±4.60*	133.4±3.64*	132.4±4.44*	127.4±3.64*
TPR	4.93±0.03	2.19±0.34#	4.73±0.06*	4.22±0.07*	4.340±0.05*	4.30±0.07*	4.42±0.06*
CHL	102.4 ±4.77	178.8±4.43#	109.4±3.84*	6.2±5.40*	130.8±4.60*	127.8±5.54*	122.2±5.63*
TBIL	0.99±0.02	2.24±0.18#	1.47±0.01*	1.82±0.07*	1.718±0.05*	1.73±0.02*	1.64±0.04*
DBIL	0.23±0.03	0.71±0.02#	0.37±0.04*	0.42±0.02*	0.414±0.03*	0.41±.053*	0.40±0.04*
ALB	3.91±0.41	1.20±0.44#	3.88±0.38*	3.65±0.38*	3.72±0.58*	3.61±0.40*	3.51±0.22*

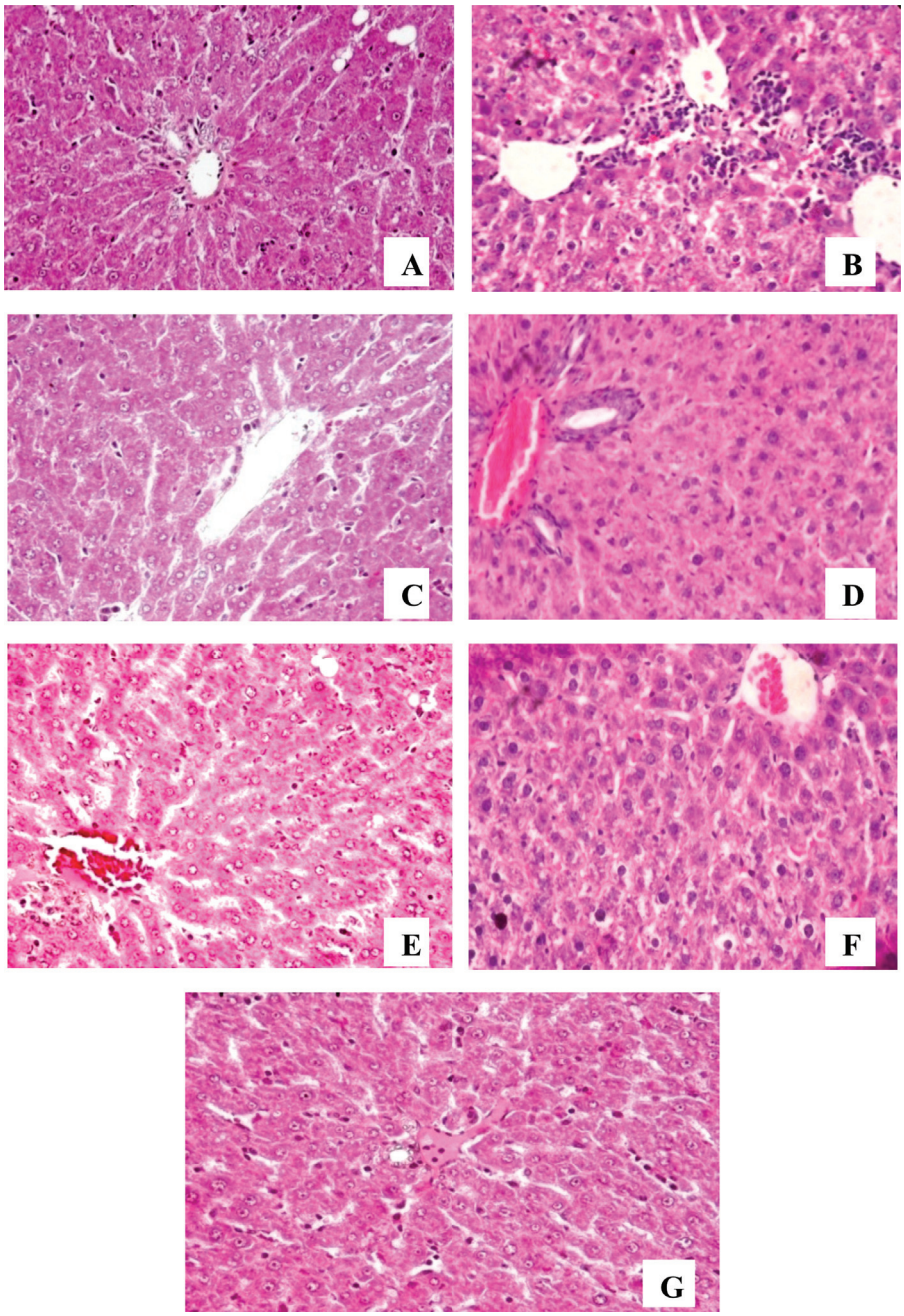
Values are expressed as mean±SD. Number of animals in each group=5. ALB, albumin; ALKP, alkaline phosphatase; BR, *Berberis asiatica* root; BSB, *Berberis asiatica* stem bark; CHL, cholesterol; DBIL, direct bilirubin; SGOT, aspartate aminotransferase; SGPT, alanine aminotransferase; TBIL, total bilirubin; TPR, total protein. #$P<0.01$, when compared with the normal control group. *$P<0.01$, when compared with the paracetamol-treated group.

higher ascorbic acid content was found in root powder (28.57 mg/100 g) of *B. asiatica* in comparison with stem bark (22.22 mg/100 g) (Table 1).

Hepatoprotective activity

The hepatoprotective activity of hydroalcoholic extract of *B. asiatica* stem bark and root was evaluated using the paracetamol-induced toxicity model. Its results are given in Table 2. For assessment of paracetamol-induced liver toxicity, the levels of serum enzymes such as SGPT and SGOT were estimated, as these enzymes are released into circulation due to necrosis and membrane damage, and therefore increased levels of these serum enzymes serve as an indicator of loss in cell membrane integrity of liver [17]. Conversely, alkaline phosphate and bilirubin level in serum is related to the function of hepatic cell [21]. The liver is a large organ responsible for metabolism, detoxification, and protein synthesis. Drug-induced hepatotoxicity is one of the major causes of human mortality all over the world [22]. In the present

Figure 4



Photomicrograph of rat liver (×400): (a) the control group, (b) the paracetamol (3000 mg/kg)-treated group, (c) the paracetamol+silymarin (100 mg/kg)-treated group, (d) the paracetamol+root extract (100 mg/kg)-treated group, (e) the paracetamol+root extract (200 mg/kg)-treated group, (f) the paracetamol+stem bark extract (100 mg/kg)-treated group, and (g) the paracetamol+stem bark extract (200 mg/kg)-treated group

study, administration of paracetamol produced a marked elevation in the serum levels of SGOT, SGPT, direct bilirubin, total bilirubin, alkaline phosphatase, and cholesterol, whereas reduction in serum levels of total protein and albumin was observed when compared with that of the control group. After treatment with *B. asiatica* stem bark extract (200 mg/kg) and root extract (200 mg/kg), restoration of enzyme levels was observed. There were significantly higher restorations of enzyme level on treatment with *B. asiatica* root extract (200 mg/kg) compared with stem bark extract (200 mg/kg). Although restoration of enzyme level was also observed at low doses (100 mg/kg), it was less as compared with 200 mg/kg dose. The decrease in the levels of SGOT and SGPT toward the normal value in serum indicates repair of hepatic tissue damage caused by paracetamol, whereas improvement in the secretary mechanism of the hepatic cells is indicated by significant control of bilirubin and alkaline phosphate levels. In the histopathological examination, the liver of control group rats showed normal hepatic cells and normal hepatocytes, whereas in the paracetamol (3000 mg/kg)-treated group congestion of the central vein at few places, necrosis of hepatocytes around the central veins, and degeneration of hepatocytes in many of the hepatic lobes were observed. In the *B. asiatica* stem bark extract (100 mg/kg)-treated group, slight congestion of large and small blood vessels, a slight increase in sinusoidal space, degenerated hepatocytes having vacuoles in the cytoplasm, minor degeneration of hepatocytes with swelling of hepatocytes particularly around the central vein leading to occlusion of sinusoids, at places were observed, whereas 200 mg/kg dose of *B. asiatica* stem bark extract showed slight congestion and no sign of necrosis. *B. asiatica* root extract 100 mg/kg showed congestion of large and small blood vessels and mild degeneration of hepatocytes, whereas 200 mg/kg *B. asiatica* root extract showed minimal inflammatory condition with near-normal architecture. The silymarin-treated group showed almost normalization of congestion of the central vein, necrosis, and degeneration of hepatocytes (Fig. 4).

Conclusion

It may be concluded from the present study that both the stem bark and the root of *B. asiatica* possess antioxidant and hepatoprotective activity, although *B. asiatica* root possess a significantly higher activity as well as higher antioxidant compounds – namely, total phenol, ascorbic acid, tannin, and flavonoid as compared with stem bark. Higher antioxidant and hepatoprotective activity of the root compared with the stem bark may be due to the higher antioxidant compounds in root compared with stem bark.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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