Antineoplastic potential of *Bryophyllum pinnatum lam.* on chemically induced hepatocarcinogenesis in rats

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ABSTRACT

Background: *Bryophyllum pinnatum* Lam. used in folk medicine in tropical Africa, tropical America, India, China and Australia contains a wide range of active compounds, well known for their haemostatic and wound-healing properties. **Objective:** The present study was designed to evaluate the effect of *Bryophyllum pinnatum* Lam. on N-diethylnitrosamine (DENA)-induced hepatic injury in rats. **Material and Methods:** The aerial part of *B. pinnatum* aqueous and ethanolic extract was prepared in doses of 250 mg/kg and 500 mg/kg. Hepatic injury was induced by DENA. Acute toxicity was also carried out. **Result:** Treatment with different doses of ethanolic extract of *B. Pinnatum* (250 mg/kg, p.o.) was not significantly able to treat the liver injury induced by DENA, but 500 mg/kg dose of ethanolic extract of *B. Pinnatum* protects the liver slightly. Treatment with different doses of aqueous extract of *B. Pinnatum* (250 and 500 mg/kg, p.o.) significantly ($P^* < 0.05$; $P^{**} < 0.01$ and $P^{***} < 0.001$) treated the liver injury induced by DENA. **Conclusion:** It may be inferred from the present study that the hepatoprotective activities of the aqueous extract of *B. Pinnatum* leaves in DENA-induced hepatotoxicity may involve its antioxidant or oxidative free radical scavenging activities by alleviating lipid peroxidation through scavenging of free radicals, or by enhancing the activity of antioxidants.

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INTRODUCTION

DENA (N-diethylnitrosamine) a hepatocarcinogen, is known to cause perturbations in the nuclear enzymes involved in DNA repair/replication and is normally used as a carcinogen to induce liver cancer in animal models.[1] Investigations have provided evidence that N-nitrosamines cause a wide range of tumors in all animal species and these compounds are considered to be effective health hazards to man.[2] Nitrosamines are a class of mutagenic, teratogenic and carcinogenic chemicals in the environment as by-products of various manufacturing, agricultural and natural processes.[3] DENA has been found in a variety of products that would result in human exposure, including mainstream and sidestream tobacco smoke, meat and whiskey.[4] Metabolism of certain therapeutic drugs is also reported to produce DENA. The International Agency for Research on Cancer (IARC) has classified DENA as a probable human carcinogen, despite the lack of epidemiologic data.[4] Administration of DENA to experimental animals has been shown to cause cancer in the liver and at lower incidences, in other organs as well.[5] It is also reported to be a hepatotoxic agent causing hepatocellular necrosis in experimental animals.[6] The formation of reactive oxygen species (ROS) is apparent during the metabolic biotransformation of DENA resulting in oxidative stress. Oxidative stress leads to carcinogenesis by several mechanisms including DNA, lipid and protein damage, change in intracellular signaling pathways and even changes in gene expression. Together, these oxidative modifications promote abnormal cell growth and carcinogenesis. Hence the model of DENA-induced hepatocellular carcinoma (HCC) is considered as one of the most accepted and widely used experimental models to study hepatocarcinogenesis.[7] Considering the above factors, it is likely that human exposure to DENA is inevitable. Hence, the development of an

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effective hepatoprotective agent against DENA-induced hepatotoxicity has become the need of the day.

The use of natural products with therapeutic properties is as ancient as human civilization and for a long time, mineral, plant and animal products were the main sources of drugs. Furthermore, throughout the development of human culture, the use of natural products had magical religious significance and different points of view regarding the concepts of health and disease existed within each culture. In recent years, there has been growing interest in alternative therapies and the therapeutic use of natural products, especially those derived from plants.[8,9]

B. pinnatum commonly known as life plant, air plant, love plant, miracle leaf, Canterbury bells is widely distributed in tropical Africa, America, Hawaii, India, China, Australia and Madagascar, and has been used in folk medicine.[10] In Europe, its use is limited almost exclusively to anthroposophic medicine.[11] The leaves are used as astringent, refrigerant, emollient, mucilaginous, hemostatic, vulnerary, depurative, constipating, anodyne, carminative, disinfectant and tonic. It is also useful in vitiated conditions like hematemesis, hemorrhoids, menorrhagia, cuts and wounds, discolorations of the skin, boils, sloughing ulcers, ophthalmia, burns, scalds, corn, diarrhea, dysentery, and vomiting.[12] A number of active compounds, including alkaloids, triterpenes, lipids,[13] flavonoids,[14] glycosides,[15] bufadienolides,[16] phenols[16] and organic acids[17] have been isolated. The leaves of this plant have been reported to possess anti-diabetic,[18] antihypertensive,[19] antimicrobial,[20] antifungal,[21] anti-inflammatory and analgesic[22] and anti-ulcer[23] activities. The plant is used for a variety of purposes in the Ayurvedic system of medicine. The root extracts are used as a laxative, as a diuretic, for liver troubles, for tuberculosis and for mental disorders. On the basis of its traditional uses to treat liver disease and as hepatoprotective, this study was conducted to explore the effect of the aqueous and ethanolic extracts of aerial parts of *B. pinnatum* on DENA-induced hepatic injury in rats.

MATERIALS AND METHODS

Animals

Wistar albino male rats of 150-200 g were used for the study. The inbred colonies of rats were obtained from Siddhartha Institute of Pharmacy, Dehradun (Uttarakhand). They were maintained in the animal house of Siddhartha Institute of Pharmacy, Dehradun (Uttarakhand) for experimental purpose. The animals were maintained under controlled conditions of temperature (23 ± 2°C), humidity (50 ± 5%) and 12-h light-dark cycles. The animals were randomized into experimental and control groups and housed individually in sanitized polypropylene cages containing sterile paddy husk as bedding. They had free access to standard pellets as basal diet and water *ad libitum*. All the studies conducted were approved by the Institutional Animal Ethical Committee (IAEC) of the Siddhartha Institute of Pharmacy, Dehradun (Uttarakhand).

Chemicals

Diethylnitrosamine (DENA) was purchased from Sigma Chemical, USA. All other chemicals used were of analytical grade and were purchased locally.

Collection of plant material

Aerial parts of *B. pinnatum* were collected in the month of September 2010 from the local region of Pauri, Garhwal (Uttarakhand, India) and identified by Dr. S. B. Singh, Scientist, NISCAIR, New Delhi. A voucher specimen (NISCAIR/RHMD/consult/-04-10-11/1573/236) was deposited in the herbarium of NISCAIR, India.

Preparation of the extract: Aerial parts of *B. pinnatum* were powdered; 150 g plant was soaked in cold ethanol (99.99%) for 48 h. The macerate was filtered and ethanol was evaporated in vacuum using a rotary evaporator. A final residue (13.90 g) was obtained after lyophilization. Before each pharmacological test, the lyophilized extract was freshly suspended in 1% Tween 80. For the aqueous extract, 1 L water was added to 150 g plant material, soaked for 48 h, and percolation was performed until the solvent became colorless. The extract was then concentrated in a vacuum to the desired volume (90% solvent out). It was dried completely and final residue (20.0 g) was obtained.

Acute (Oral) Toxicity Study (Fixed Dose Procedure)

Method

Acute toxicity studies for ethanolic and aqueous extracts of *B. pinnatum* were conducted as per Organisation for Economic Co-operation and Development (OECD) guidelines 420 using Albino Swiss mice. Each animal was administered ethanolic and aqueous extracts' solution of *B. pinnatum* by oral route. The test procedure minimizes the number of animals required to estimate the oral acute toxicity of a chemical and in addition estimation of LD_{50}, confidence intervals. The test also allows the observation of signs of toxicity and can also be used to identify chemicals that are likely to have low toxicity.

Principle of the fixed dose procedure

The fixed dose procedure is a method for assessing acute oral toxicity that involves the identification of a dose level that causes evidence of non-lethal toxicity (termed evident

toxicity) rather than a dose level that causes lethality. Evident toxicity is a term describing clear signs of toxicity following administration of a test substance, such that an increase to the next highest fixed dose would result in the development of severe toxic signs and probably mortality.

Procedure

As suggested, after acclimatization of animals for 4–5 days, the study was carried out as follows:

1. Healthy, young adult Albino Swiss mice (20–25 g), nulliparous and non-pregnant were used for this study. Food, but not water was withheld for 3–4 h and further 1–2 h post administration of sample under study.
2. Fixed dose levels of 5, 50, 500 mg/kg were initially chosen as dose levels that would be expected to allow the identification of the dose producing evident toxicity.
3. During the validation procedure, a fixed dose of 2000 mg/kg was added to provide more information on the substance of low acute toxicity.
4. Dosed one animal at the test dose by oral route.
5. Since this first test animal survived, four other animals were dosed (orally) on subsequent days, so that a total of five animals were tested.

Observation

Animals were observed individually at least every 5 min once during the first 30 min after dosing, periodically at 2 h during the first 24 h (with special attention during the first four hours) and daily thereafter, for a total of 14 days.

An attempt was made to identify LD_{50} of ethanolic and aqueous extracts of *B. pinnatum*. Since no mortality was observed at 2000 mg/kg, it was thought that 2000 mg/kg was the cutoff dose. Therefore 1/8th and 1/4th doses (i.e. 250 mg/kg and 500 mg/kg) were selected for all further *in vivo* studies.

Experimental design

Albino wistar rats of either sex weighing between 150–200 g were divided into six groups of six animals each.

1. Group-I – Negative Control
2. Group-II– DENA (200 mg/kg, i.p.)
2. Group-III– DENA (200 mg/kg, i.p.) + Ethanolic extract of *B. Pinnatum* (250 mg/kg, p.o.)
3. Group-IV – DENA (200 mg/kg, i.p.) + Ethanolic extract of *B. Pinnatum* (500 mg/kg, p.o.)
4. Group-V – DENA (200 mg/kg, i.p.) + Aqueous extract of *B. Pinnatum* (250 mg/kg, p.o.)
5. Group-VI– DENA (200 mg/kg, i.p.) + Aqueous extract of *B. Pinnatum* (500 mg/kg, p.o.)

At the end of the experimental period, animals were subjected to ether anesthesia, blood was collected from retro-orbital plexus and serum was separated by centrifugation. Animals were sacrificed by cervical decapitation and the liver was excised, washed in ice-cold saline and blotted to dryness. A 1% homogenate of the

Table 1: Effect of aerial part of *B. pinnatum* on lipid profile in DENA induced hepatotoxicity in rats

Group	Mean ± SEM (mg/dl)		
	Cholesterol	Triglyceride	HDL
Group I	91.1 ± 2.56	74.79 ± 2.67	26.33 ± 0.70
Group II	163.9 ± 2.43[b]	152.6 ± 1.76[b]	20.53 ± 1.08[a]
Group III	171.0 ± 2.57	150.6 ± 1.22	19.67 ± 1.34
Group IV	159.7 ± 1.20	155.1 ± 2.19	19.75 ± 1.03
Group V	153.8 ± 1.90	142.0 ± 1.35[d]	21.80 ± 1.17
Group VI	128.3 ± 2.50[e]	119.8 ± 2.22[e]	25.12 ± 0.80[c]

Values are mean±SEM expressed as (n=6)
Whereas Group I as Negative control; Group II as DENA (200mg/kg); Group III as DENA (200mg/kg) + Ethanolic extract (250mg/kg); Group IV as DENA (200mg/kg) + Ethanolic extract (500 mg/kg); Group V as DENA (200mg/kg) + Aqueous extract (250 mg/kg); and Group VI as DENA (200mg/kg) + Aqueous extract (500 mg/kg). a)P*<0.05 as compared with Group I, b) P***<0.001 as compared with Group I, c) P*<0.05 as compared with Group II, d) P**<0.01 as compared with Group II, e) P***<0.001 as compared with Group II

Table 2: Effect of aerial part of *C. pinnatum* on antioxidant enzymes in DENA induced hepatotoxicity in rats

Group	Mean ± SEM (units/mg of proteins)		
	LPO	SOD	CAT
Group I	19.65 ± 0.62	2.35 ± 0.98	7.22 ± 0.10
Group II	26.24 ± 0.90[b]	0.92 ± 0.02[b]	3.48 ± 0.16[a]
Group III	25.57 ± 0.77	0.97 ± 0.16	3.36 ± 0.16
Group IV	24.02 ± 0.33	1.09 ± 0.27	3.80 ± 0.15
Group V	23.11 ± 0.88	1.26 ± 0.10[c]	4.11 ± 0.12[b]
Group VI	21.43 ± 0.88[c]	1.90 ± 0.03[d]	6.61 ± 0.73[d]

Values are mean±SEM expressed as (n=6)
Whereas Group I as Negative control; Group II as DENA (200mg/kg); Group III as DENA (200mg/kg) + Ethanolic extract (250mg/kg); Group IV as DENA (200mg/kg) + Ethanolic extract (500 mg/kg); Group V as DENA (200mg/kg) + Aqueous extract (250 mg/kg); and Group VI as DENA (200mg/kg) + Aqueous extract (500 mg/kg). a)P***<0.001 as compared with Group I, b) P*<0.05 as compared with Group II, c) P**<0.01 as compared with Group II, d) P***<0.001 as compared with Group II

Table 3: Effect of aerial part of *B. pinnatum* on biochemical parameters in DENA induced hepatotoxicity in rats

Group	Mean ± SEM (IU/ml)		
	SGPT	SGOT	ALP
Group I	61.26 ± 0.86	86.77 ± 1.54	120.1 ± 1.59
Group II	79.42 ± 0.82[a]	143.5 ± 1.68[a]	171.9 ± 1.51[a]
Group III	82.04 ± 0.80	142.2 ± 1.10	165.7 ± 1.36
Group IV	75.59 ± 0.64[b]	138.8 ± 0.93	162.3 ± 1.68[c]
Group V	75.02 ± 0.86[c]	136.7 ± 0.72[b]	147.6 ± 1.98[d]
Group VI	65.41 ± 0.65[d]	98.65 ± 1.81[e]	128.5 ± 1.95[d]

Values are mean±SEM expressed as (n=6)
Whereas Group I as Negative control; Group II as DENA (200mg/kg); Group III as DENA (200mg/kg) + Ethanolic extract (250mg/kg); Group IV as DENA (200mg/kg) + Ethanolic extract (500 mg/kg); Group V as DENA (200mg/kg) + Aqueous extract (250 mg/kg); and Group VI as DENA (200mg/kg) + Aqueous extract (500 mg/kg). a) P***<0.001 as compared with Group I, b) P*<0.05 as compared with Group II, c) P**<0.01 as compared with Group II, d) P***<0.001 as compared with Group I

liver tissue sue was prepared in Tris–HCl buffer (0.1 M; pH 7.4), centrifuged and the clear supernatant used for further biochemical assays.

The activities of serum SGPT, SGOT and ALP were determined. Cholesterol, triglyceride and HDL were also determined in serum to assess the acute hepatic injuries by using standard enzyme kits.

For lipid peroxidation (LPO) liver homogenate was prepared in cold 50mM potassium phosphate buffer (pH 7.4), but for Superoxide dismutase (SOD) and Catalase (CAT) 1mM EDTA was added in it, using REMI homogenizer. The unbroken cells and debris were removed by centrifugation at 10,000 rpm for 15 min at 4°c using a REMI cooling centrifuge and the supernatant was used for the estimation of LPO, SOD and CAT by using standard enzyme kit.

Histopathology

The animals used in the curative study were sacrificed and liver tissue was examined grossly. A small portion of liver tissue of each animal was fixed in 10% neutral buffered formalin processed and embedded in paraffin wax to obtain 5–6 μm-thick hematoxylin and eosin-stained sections.[24]

Statistical analysis

All data were represented as mean ± SD. Significant difference between the mean values were statistically analyzed using one-way analysis of variance (ANOVA) using Prism 5 Graph pad software. The DENA alone treated group and the extract plus DENA treated groups were further analyzed by Tukey's test. *P* values less than 0.05 were considered as significant.

RESULTS

Acute toxicity studies for ethanolic and aqueous extracts of *B. Pinnatum* belonging to the family "Gentianaceae" were conducted as per OECD guidelines 420 using albino Swiss mice. Each animal was administered ethanolic and aqueous extracts by oral route. The animals were observed for any changes continuously for the first 2 h and up to 24 h for mortality. There was no mortality and noticeable behavioral changes observed in all the groups tested. The extracts were found to be safe up to 2000 mg/kg body weight.

An attempt was made to identify LD_{50} of ethanolic and aqueous extracts of aerial parts of *B. pinnatum*. Since no mortality was observed at 2000 mg/kg it was thought that 2000 mg/kg was the cutoff dose. Therefore, 1/8 and 1/4 doses i.e. 250 mg/kg and 500 mg/kg were selected for all further *in vivo* studies.

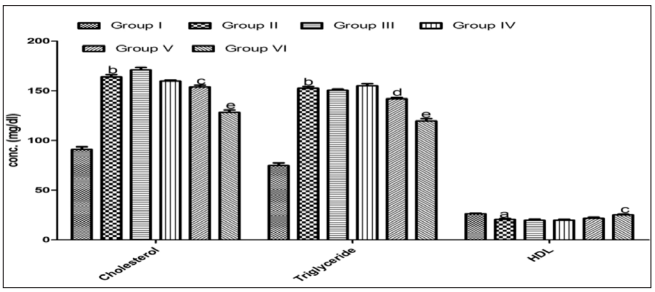

Figure 1: Effect of aerial part of *B. pinnatum* on lipid profile in DENA-induced hepatotoxicity in rats. Whereas Group I as Negative control; Group II as DENA (200mg/kg); Group III as DENA (200mg/kg) + Ethanolic extract (250mg/kg); Group IV as DENA (200mg/kg) + Ethanolic extract (500 mg/kg); Group V as DENA (200mg/kg) + Aqueous extract (250 mg/kg); and Group VI as DENA (200mg/kg) + Aqueous extract (500 mg/kg). a) *P**<0.05 as compared with Group I, b) *P****<0.001 as compared with Group I, c) *P**<0.05 as compared with Group II, d) *P***<0.01 as compared with Group II, e) *P****<0.001 as compared with Group II.

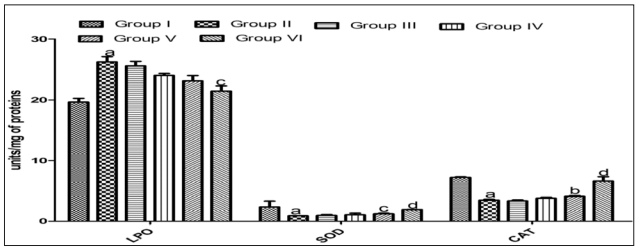

Figure 2: Effect of aerial part of *B. pinnatum* on antioxidant enzymes in DENA-induced hepatotoxicity in rats. Whereas Group I as Negative control; Group II as DENA (200mg/kg); Group III as DENA (200mg/kg) + Ethanolic extract (250mg/kg); Group IV as DENA (200mg/kg) + Ethanolic extract (500 mg/kg); Group V as DENA (200mg/kg) + Aqueous extract (250 mg/kg); and Group VI as DENA (200mg/kg) + Aqueous extract (500 mg/kg). a) *P****<0.001 as compared with Group I, b) *P**<0.05 as compared with Group II, c) *P***<0.01 as compared with Group II, d) *P****<0.001 as compared with Group II

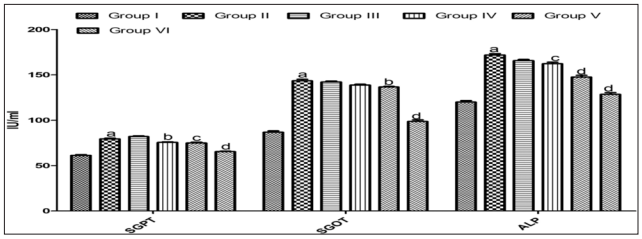

Figure 3: Effect of aerial part of *B. pinnatum* on biochemical parameters in DENA-induced hepatotoxicity in rats. Whereas Group I as Negative control; Group II as DENA (200mg/kg); Group III as DENA (200mg/kg) + Ethanolic extract (250mg/kg); Group IV as DENA (200mg/kg) + Ethanolic extract (500 mg/kg); Group V as DENA (200mg/kg) + Aqueous extract (250 mg/kg); and Group VI as DENA (200mg/kg) + Aqueous extract (500 mg/kg). a) *P****<0.001 as compared with Group I, b) *P**<0.05 as compared with Group II, c) *P***<0.01 as compared with Group II, d) *P****<0.001 as compared with Group II

Administration of DENA (200 mg/kg, i.p.) to rats caused significant liver damage, as evidenced by the altered serum biochemical parameters. DENA significantly (*P**<0.05) increased cholesterol, triglyceride, LOP, SGPT, SGOT and

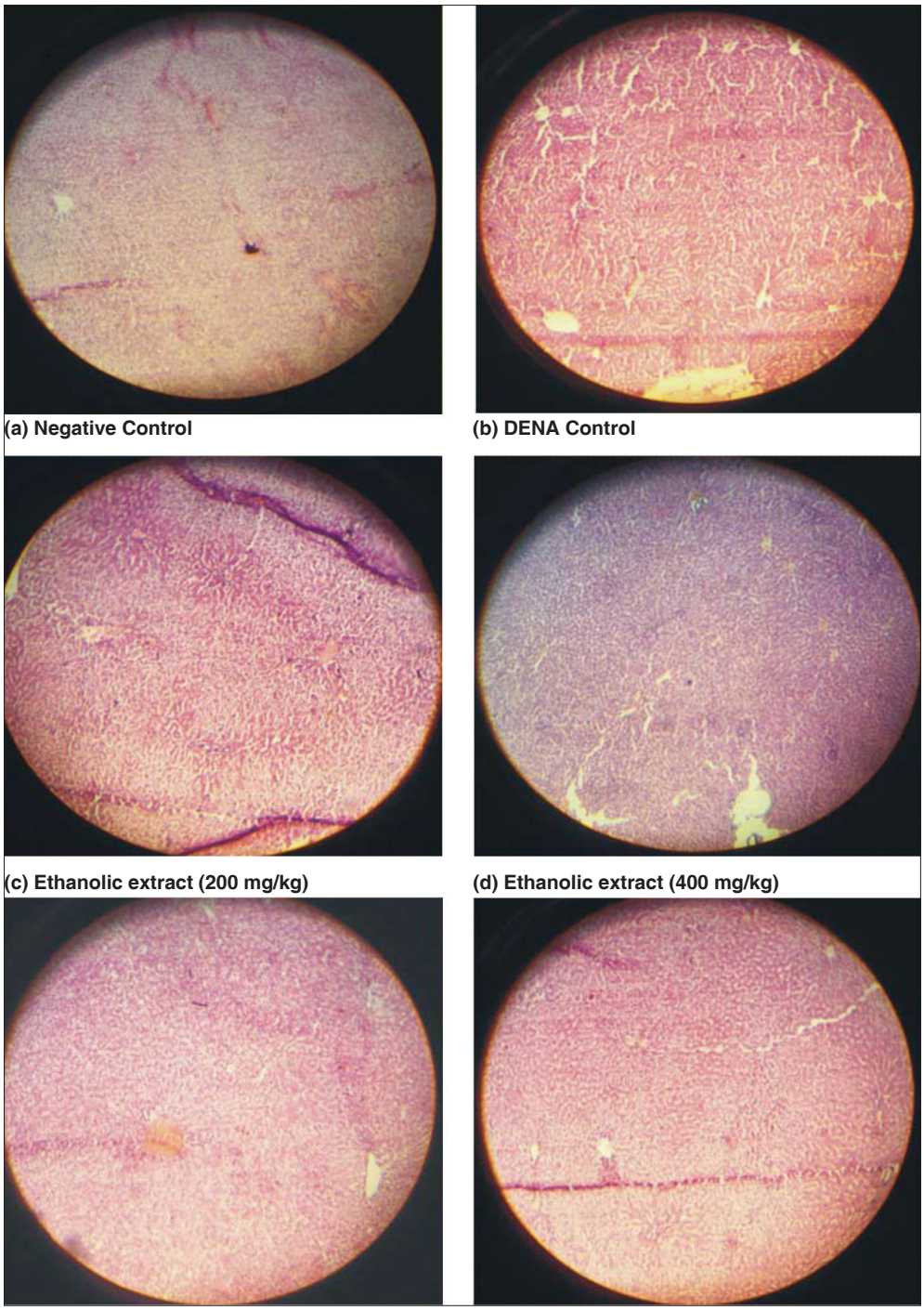

Figure 4: Histopathological architecture of the rat liver in DENA-induced hepatotoxicity (a) Section of the liver tissue of control rats showing normal histology; (b) Section of the liver tissue of rats treated with DENA showing necrosis & Fatty vacuole; (c) Section of the liver tissue of ethanolic extract of *B. pinnatum* (250 mg/kg) -treated rat showing necrosis, inflammation and fatty vacuole; (d) Section of the liver tissue of ethanolic extract of *B. pinnatum* (500 mg/kg) -treated rat showing necrosis and fatty vacuole; (e) Section of the liver tissue of aqueous extract of *B. pinnatum* (250 mg/kg) - treated rat showing normal arrangements of hepatocytes around the central vein; (f) Section of the liver tissue of aqueous extract of *B. pinnatum* (500 mg/kg)-treated rat showing normal arrangements of hepatocytes around the central vein, absence of necrosis

ALP. It also decreased HDL, SOD and CAT significantly ($P^{*}<0.05$ and $P^{***}<0.001$). Treatment with different doses of ethanolic extract of *B. Pinnatum* (250 and 500 mg/kg, p.o.) did not significantly reverse these parameters, but 500 mg/kg dose of ethanolic extract significantly ($P^{*}<0.05$ and $P^{**}<0.01$) increased SGPT and ALP level, whereas treatment with different doses of aqueous extract of *B. Pinnatum* (250 and 500 mg/kg, p.o.) significantly ($P^{*}<0.05$,

*P***<0.01 and *P****<0.001) reversed these parameters dose-dependently [Tables 1-3 and Figures 1-3].

The histopathological studies also supported the protective properties of aerial parts of *B. pinnatum*. Areas of necrosis and ballooning degeneration of hepatocytes were observed in the DENA control group. The group of animals pre-treated with aqueous extract of aerial parts of *B. pinnatum* showed a dose-dependent marked protective effect with decreased necrotic zones and hepatocellular degeneration whereas ethanolic extract at dose of 250 mg/kg did not show any protective effect and ethanolic extract at 500 mg/kg dose showed minor decrease in necrotic zone. The photomicrographs of the liver sections were given in Figure 4.

DISCUSSION

In the present study a significant increase in the levels of triglycerides and cholesterol in the Group II rats clearly indicated the hyperlipidemic conditions caused by exposure to DENA. These parameters were brought back to the normal levels in the Group V and Group VI rats, which indicates the beneficial effects of the administration of the aqueous extract of *B. Pinnatum* during DENA-induced hepatocarcinogenesis in rats. It is believed that it may be due to the antioxidant and antiperoxidative effects coupled with an ability to correct the abnormalities in lipid and lipoprotein metabolism through an increase in the activities of few lipid metabolizing enzymes, viz. lecithin cholesterol acyl transferase, lipoprotein lipase and hepatic triglyceride lipase, but the clear mechanism of action for the observed hypolipidemic effects is not well understood at this stage of study. DENA is a major environmental hepatocarcinogen. Since the liver is the main site of DENA metabolism, the production of ROS in the liver may be responsible for its carcinogenic effects.[25] DENA is well known to generate free radicals, disturbing the antioxidant status and ultimately leading to oxidative stress and carcinogenesis.[26] In Group II, DENA (200 mg/kg)-treated rats showed a decrease in SOD along with catalase. The Group II rats also showed an increase in liver lipid peroxidation, which indicated cellular damage caused by free radicals. In Group V and Group VI, aqueous extract of *B. Pinnatum*-treated rats in the present study showed an extremely significant rise in SOD along with catalase. The extract also showed extremely significant decrease in liver lipid peroxidation, which signifies the antioxidant activity on liver of the treated animals. ALT is more selectively a liver paranchymal enzyme than AST. ALT is a sensitive indicator of acute liver damage and elevation of this enzyme in non-hepatic diseases is unusual. Normally, AST and ALP are present in a high concentration in the liver. Due to hepatocyte necrosis or abnormal membrane permeability, these enzymes are released from the cells and their levels in the blood increase. In the present study, the activities of these enzymes were found to increase in Group II, DENA-treated rats, and were significantly reduced in groups of aqueous extract of *B. Pinnatum*-administered rats as compared to that of hepatotoxic rats. This confirms the protective effect of the aqueous extract of *B. Pinnatum* against DENA-induced hepatic damage. The effect was more pronounced with 400 mg/kg extract. A possible mechanism of the *B. Pinnatum* extract as hepatoprotective may be due to its anti-oxidant effect. This might be due to the higher content of xanthones present in the extract which could have reduced the accumulation of toxic DENA-derived metabolites. Histopathological examination of the liver section of Group II, DENA-treated rats showed intense centrilobular necrosis and vacuolization. The rats treated with the aqueous extract of *B. Pinnatum* along with DENA showed signs of protection against these toxicants to a considerable extent, which was confirmed by the formation of normal hepatic cards and absence of necrosis and vacuoles, dose dependently respectively.

CONCLUSION

It may be inferred from the present study that the hepatoprotective activities of the aqueous extract of *B. Pinnatum* leaves in DENA-induced hepatotoxicity may involve its antioxidant or oxidative free radical scavenging activities by alleviating lipid peroxidation through scavenging of free radicals, or by enhancing the activity of antioxidants. The mechanism of action is yet to be investigated but may be due to the antioxidant effects of xanthones and the free radical scavenging properties found to be present in the aerial parts of *B. pinnatum*.

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