Effect of *Bryophyllum pinnatum* Lam. On N-diethylnitrosamine Induced Hepatic Injury in Rats

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ABSTRACT

This study was designed to evaluate the effect of *Bryophyllum pinnatum* Lam. on n-diethylnitrosamine (DENA) induced hepatic injury in rats. The aerial part of *B. pinnatum* aqueous and ethanolic extract was prepared in the doses of 250 and 500 mg kg^{-1}. Hepatic injury was induced by DENA. Acute toxicity was also carried out. Treatment with different doses of ethanolic extract of *B. pinnatum* (250 mg kg^{-1}, p.o.) not significantly able to treat the liver injury induced by DENA, but 500 mg kg^{-1} dose of ethanolic extract of *B. pinnatum* slightly protect the liver. Treatment with different doses of aqueous extract of *B. pinnatum* (250 and 500 mg kg^{-1}, p.o.) significantly ($p < 0.05$; $p < 0.01$ and $p < 0.001$) treat the liver injury induced by DENA. 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.

Key words: *Bryophyllum pinnatum*, n-diethylnitrosamine, histopathology, rats, aqueous extract, hepatotoxicity

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]. N-nitrosamines have been proved by too many investigators that causes a wide range of tumors in animals and considered to be effective health hazards to man[2]. Nitrosamines, the by-products of various manufacturing, agricultural and natural processes falls under the category of mutagenic, teratogenic and carcinogenic chemicals in the environment[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]. It has been reported by the researchers that biotransformation of certain drugs also lead 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]. It is well studied and established that administration of DENA to experimental animals cause hapatocellular carcinoma[5] and reported to be selective hepatotoxic[6]. Further DENA-induced HCC considered as one among the most accepted and widely used animal model. 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 and these oxidative modifications promotes carcinogenesis[7]. Considering the above factors, it is likely that human exposure to DENA is inevitable. Hence, the development of an effective hepatoprotective agent against DENA induced hepatotoxicity has become the need of the day.

Natural products for therapeutic properties are being utilized from an ancient time and for decades, mineral, plant and animal products were used to be the main sources of drugs. Moreover, during the development of human civilization, natural products had magical religious significance and different perspectives regarding the concepts of health and disease existed within each culture. Recently, investigators showed a growing interest in alternative therapies and use of natural products, especially derived from plants[8,9].

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B. pinnatum known as life plant, air plant, love plant, miracle leaf, Canterbury bells is widely distributed and had been used in folk medicine from ancient times in different civilization[10]. In Europe, its use is limited almost exclusively to anthroposophic medicine[11]. Traditionally the leaves are known to possess the biological activities like, astringent, refrigerant, emollient, mucilaginous, haemostatic, vulnerary, depurative, constipating, anodyne, carminative, disinfectant and tonic. It is also useful in vitiated conditions haematemesis, haemorrrhoids, menorrhagia, cuts and wounds, discolorations of the skin, boils, sloughing ulcers, ophthalmia, burns, scalds, corn, diarrhea, dysentery, 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 it's traditionally uses to treat the liver disease and as hepatoprotective activity, this study was conducted to explore the effect of 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 ($23 \pm 2°C$), humidity ($50 \pm 5\%$) 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 assessed to standard pellets as basal diet and water *ad libitum*. All the studies conducted were approved by the Institutional Animal Ethical Committee (IAEC) of 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. Imran Kazmi, Assistant Professor, Siddhartha Institute of Pharmacy, Dehradun. A voucher specimen (Ref. SIP/IAEC.CLEAR/85/2009-10) was deposited in the herbarium of Siddhartha Institue of Pharmacy.

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 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 method for assessing acute oral toxicity that involve the identification of a dose level that cause evidence of non-lethal toxicity (termed evident toxicity) rather than a dose level that cause lethality. Evident toxicity is a term describing clear signs of toxicity following administration of 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, study was carried out as follows:

- Healthy, young adult albino swiss mice (20-25 g), nulliporous and non pregnant were used for this

study. Food, but not water was with held for 3-4 h and further 1-2 h post administration of sample under study
- Fixed dose level of 5, 50 and 500 mg kg^{-1} were initially chosen as dose level that would be expected to allow the identification of dose producing evident toxicity
- During the validation procedure, a fixed dose of 2000 mg kg^{-1} was added to provide more information on substance of low acute toxicity
- Dosed one animal at the test dose by oral route
- 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 first 30 min after dosing, periodically at 2 hrs during the first 24 h (with special attention during the first four hour) 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^{-1}, it was thought that 2000 mg kg^{-1} was the cut off dose. Therefore 1/8th and 1/4th dose (i.e. 250 and 500 mg kg^{-1}) 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:

Group 1: Negative control
Group 2: DENA (200 mg kg^{-1}, i.p.)
Group 3: DENA (200 mg kg^{-1}, i.p.) +Ethanolic extract of *B. pinnatum* (250 mg kg^{-1}, p.o.)
Group 4: DENA (200 mg kg^{-1}, i.p.) +Ethanolic extract of *B. pinnatum* (500 mg kg^{-1}, p.o.)
Group 5: DENA (200 mg kg^{-1}, i.p.) +Aqueous extract of *B. pinnatum* (250 mg kg^{-1}, p.o.)
Group 6: DENA (200 mg kg^{-1}, i.p.) +Aqueous extract of *B. pinnatum* (500 mg kg^{-1}, p.o.)

At the end of experimental period, animals were subjected to ether anaesthesia, 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 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) 1 mM 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 Graphpad 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 and up to 24 h for mortality. There were no mortality and noticeable behavioral changes in all the groups tested. The extracts were found to be safe up to 2000 mg kg^{-1} 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^{-1}. It was thought that 2000 mg kg^{-1} was the cut off dose. Therefore, 1/8 and 1/4 dose i.e., 250 and 500 mg kg^{-1}. were selected for all further *in vivo* studies.

Administration of DENA (200 mg kg^{-1}, i.p.) to rats caused significant liver damage, as evidenced by the altered serum biochemical parameters. Administration of DENA significantly (p<0.001) increased the cholesterol level (163.9 mg dL^{-1}) as

compare to the normal control (91.07 mg dL^{-1}). The level of triglyceride significantly (p < 0.001) increased up to 152.6 mg dL^{-1} as compare to normal control (74.79 mg dL^{-1}). LPO level also significantly (p < 0.001) increased up to 26.24 units mg^{-1} of proteins as compare to normal control (19.65 units mg^{-1} of proteins). SGPT significantly (p < 0.001) increased up to 79.42 IU mL^1 as compare to normal control (61.26 IU mL^{-1}). SGOT significantly (p < 0.001) increased up to 143.5 IU mL^{-1} as compare to normal control (86.77 IU mL^{-1}). Further, ALP also significantly (p < 0.001) increased up to 171.9 IU mL^{-1} as compare to normal control (120.1 IU mL^{-1}). It also significantly (p < 0.05, p < 0.001) decreased HDL (26.33 to 20.54 mg dL^{-1}), SOD level from 2.35 units mg^{-1} of proteins to 0.92 units mg^{-1} of proteins and CAT level 7.22 units mg^{-1} of proteins to 3.48 units mg^{-1} of proteins.

Treatment with different doses of ethanolic extract of *B. pinnatum* (250 and 500 mg kg^{-1}, p.o.) not significantly reversed these parameters. The level of cholesterol, triglyceride, HDL, LPO, SOD, CAT, SGPT, SGOT and ALP after treatment with 250 mg kg^{-1} ethanolic extract dose was 171.0, 150.6, 19.67, 25.57, 0.97, 3.36, 82.04, 142.2 and 165.7, respectively. The 500 mg kg^{-1} dose of ethanolic extract significantly (p < 0.05 and p < 0.01) increased SGPT and ALP level. The level of cholesterol, triglyceride, HDL, LPO, SOD, CAT, SGPT, SGOT and ALP after treatment with 500 mg kg^{-1} ethanolic extract dose was 159.7, 155.1, 19.75, 24.02, 1.09, 3.80, 75.59, 138.8 and 162.3, respectively. Treatment with different doses of aqueous extract of *B. pinnatum* (250 and 500 mg kg^{-1}, p.o.) significantly (p < 0.05, p < 0.01 and p < 0.001) reversed these parameters dose dependently. The level of cholesterol, triglyceride, HDL, LPO, SOD, CAT, SGPT, SGOT and ALP after treatment with 250 mg kg^{-1} aqueous extract dose was 152.8, 142.0, 21.80, 23.11, 1.26, 4.11, 75.02, 136.7 and 146.6, respectively. The level of cholesterol, triglyceride, HDL, LPO, SOD, CAT, SGPT, SGOT and ALP after treatment with 500 mg kg^{-1} aqueous extract dose was 128.3, 119.8, 25.12, 21.43, 1.90, 6.61, 65.41, 98.65 and 128.5, respectively (Table 1-3).

The histopathological studies also supported the protective properties of aerial parts of *B. pinnatum*. The 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^{-1} not shown any protective effect and ethanolic extract at 500 mg kg^{-1} dose shown minor decrease in necrotic zone. The photomicrographs of the liver sections were given in Fig. 1.

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

| Group | Conc. (mg kg^{-1}) | | |
	Cholesterol	Triglyceride	HDL
1	91.07 ± 2.56	74.79 ± 2.67	6.33 ± 0.70
2	463.09 ± 2.43^b	152.06 ± 1.76^b	20.54 ± 1.08^a
3	171.00 ± 2.57	150.06 ± 1.22	19.67 ± 1.34
4	159.07 ± 1.20	155.01 ± 2.19	19.75 ± 1.03
5	153.08 ± 1.90^c	142.00 ± 1.35^d	21.80 ± 1.17
6	128.03 ± 2.50^e	119.08 ± 2.22^e	25.12 ± 0.80^e

Values are Mean ± SEM (n = 6), ^{a}p < 0.05 as compared with group 1, ^{b}p < 0.001 as compared with group 1, ^{c}p < 0.05 as compared with group 2, ^{d}p < 0.01 as compared with group 2 and ^{e}p < 0.001 as compared with group 2

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

| Group | Conc. (mg kg^{-1}) | | |
	LPO	SOD	CAT
1	19.65 ± 0.62	2.35 ± 0.98	7.22 ± 0.10
2	26.24 ± 0.98^a	0.92 ± 0.02^a	3.48 ± 0.16^a
3	25.57 ± 0.77	0.97 ± 0.16	3.36 ± 0.16
4	24.02 ± 0.33	1.09 ± 0.27	3.80 ± 0.15
5	23.11 ± 0.88	1.26 ± 0.10^c	4.11 ± 0.12^b
6	21.43 ± 0.88^c	1.90 ± 0.03^d	6.61 ± 0.73^d

Values are Mean ± SEM (n = 6), ^{a}p < 0.001 as compared with group 1, ^{b}p < 0.05 as compared with group 2, ^{c}p < 0.01 as compared with group 2 and ^{d}p < 0.001 as compared with group 2

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

| Group | Conc. (mg kg^{-1}) | | |
	SGPT	SGOT	ALP
1	61.26 ± 0.86	86.77 ± 1.56	120.1 ± 1.59
2	79.42 ± 0.82^a	143.05 ± 1.68^a	171.9 ± 1.51^a
3	82.04 ± 0.80	142.02 ± 1.10	165.7 ± 1.36
4	75.59 ± 0.64^b	136.07 ± 0.72^b	147.6 ± 1.98^d
5	75.02 ± 0.86^c	136.07 ± 0.72^b	147.6 ± 1.98^d
6	65.41 ± 0.65^d	98.65 ± 1.81^d	128.5 ± 1.95^d

Values are Mean ± SEM (n = 6), ^{a}p < 0.001 as compared with group 1, ^{b}p < 0.05 as compared with group 2, ^{c}p < 0.01 as compared with group 2 and ^{d}p < 0.001 as compared with group 1

DISCUSSION

In this present study it was noted that a significant increase in the levels of triglycerides and cholesterol in the group 2 rats clearly indicate the hyperlipidaemic conditions caused by exposure to DENA[25]. These parameters were brought back to the normal levels in the group 5 and 6 rats indicate the beneficial effects of administration of aqueous extract of *B. pinnatum* during DENA-induced hepatocarcinogenesis in rats[26]. 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
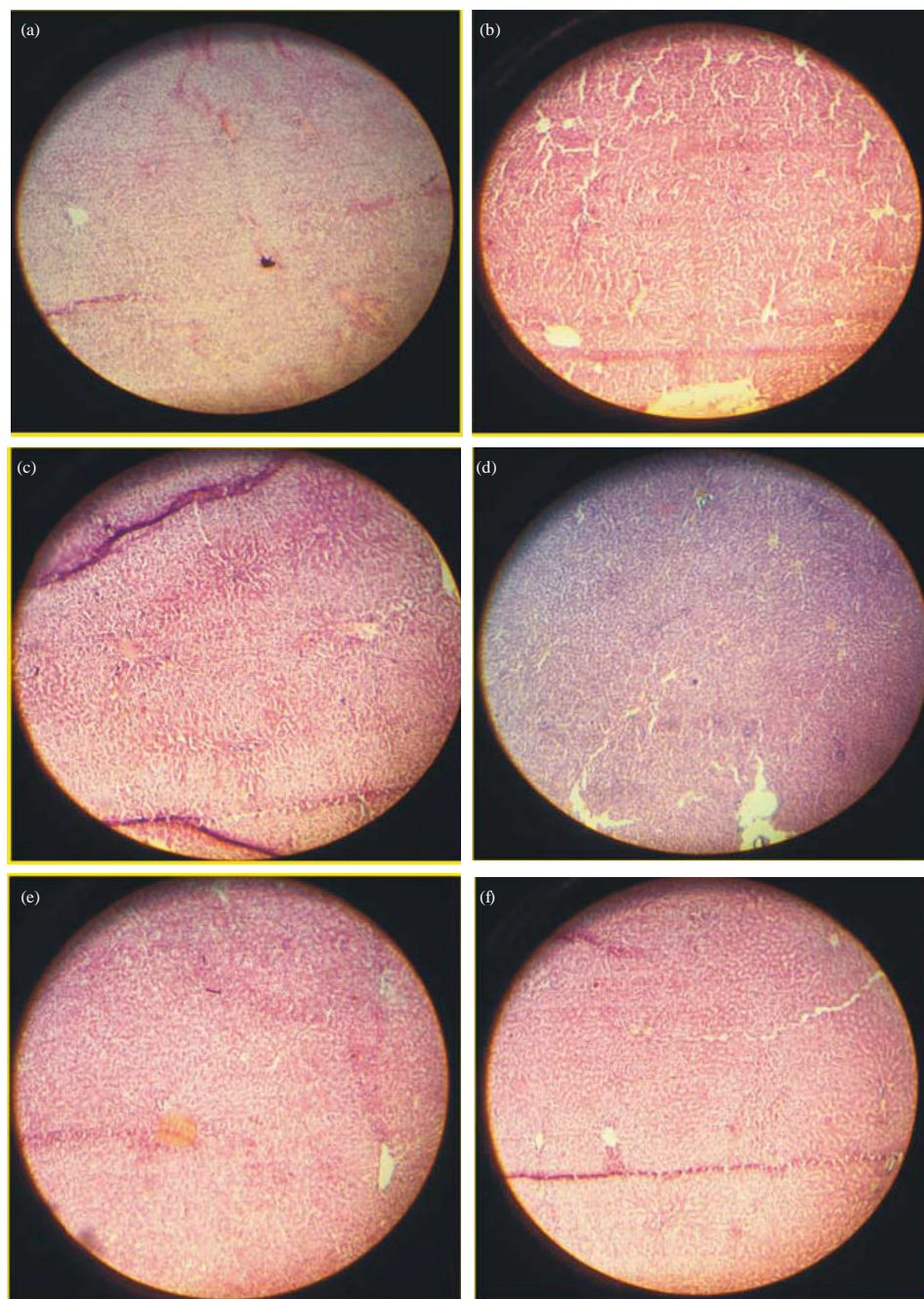
Fig. 1(a-f): Histopathological architecture of the rat liver tissue in DENA induced hepatotoxicity (a) Negative control showing normal histology, (b) DENA constrol showing necrosis and fatty vacuole, (c) *B. pinnatum* (ethanolic extract 250 mg kg^{-1})-treated showing necrosis, inflammation and fatty vacuole, (d) *B. pinnatum* (ethanolic extract 500 mg kg^{-1})-treated showing necrosis and fatty vacuole, (e) *B. pinnatum* (aqueous extract 250 mg kg^{-1})-treated showing normal arrangements of hepatocytes around the central vein and (f) *B. pinnatum* (aqueous extract 500 mg kg^{-1})-treated showing normal arrangements of hepatocytes around the central vein, absence of necrosis

transferase, lipoprotein lipase and hepatic triglyceride lipase, but the clear mechanism of action for the observed hypolipidaemic effects is not well understood at this stage of study[27]. DENA is a major environmental hepatocarcinogen[28,29]. Since liver is the main site of DENA metabolism, the production of ROS in the liver may be responsible for its carcinogenic effects[30]. DENA is well known to generate free radicals, disturbing the antioxidant status and ultimately leading to oxidative stress and carcinogenesis[31]. In group 2, DENA (200 mg kg^{-1}) treated rats were shown an decrease in SOD along with catalase[32]. The group II rats also showed increase in liver lipid peroxidation, which indicate a cellular damage caused by free radicals[33]. In group 5 and group 6, 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 extreme 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[34]. 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 high concentration in liver. Due to hepatocyte necrosis or abnormal membrane permeability, these enzymes are released from the cells and their levels in the blood increases[35]. In the present study, the activities of these enzymes were found to increase in the group 2, 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 aqueous extract of B. pinnatum against DENA induced hepatic damage. The effect was more pronounced with 400 mg kg^{-1} 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 contents of xanthones present in the extract which could have reduced the accumulation of toxic DENA derived metabolites[36]. Histopathological examination of the liver section of the group 2, DENA treated rats showed intense centrilobular necrosis and vacuolization. The rats treated with aqueous extract of B. pinnatum extracts along with DENA showed sign of protection against these toxicants to considerable extent as evident from 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 free radical scavenging properties found to be present in the Aerial parts of B. pinnatum.

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