Original Article

Chemopreventive Effect of *Bauhinia Purpurea* Against Chemically Induced Hepatocarcinogenesis via Amelioration of Oxidative Damage, Cell Proliferation and Induction of Apoptosis in Wistar Rats

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ABSTRACT

Objectives: In the present study we have evaluated the chemopreventive efficacy of *Bauhinia purpurea* against Diethylnitrosamine (DEN) initiated and 2 Acetylaminofluorine (2-AAF) promoted hepatocarcinogenesis in Wistar rats. **Materials and Methods:** Efficacy of *Bauhinia purpurea* against 2-AAF-induced hepatotoxicity was evaluated in terms of biochemical estimation of antioxidant enzyme activities (reduced hepatic GSH, glutathione peroxidase, glutathione reductase, catalase, and quinone reductase), histopathological changes and expressions of early tumor markers viz., ornithine decarboxylase activity (ODC) and proliferating cell nuclear antigen (PCNA) and also expressions of p53, Bax, Bcl-2, and caspase-3 were evaluated. **Results:** Oral pretreatment with *B. purpurea* significantly decreased the levels of serum toxicity markers, elevated antioxidant defense enzyme activities, suppressed the expression of ODC and PCNA and P53 along with the induction of apoptosis in the pretreatment groups. Tumor incidences are reduced by pretreatment of *B. purpurea.* Histopathological findings revealed that *B. purpurea*-pretreated groups showed marked recovery. **Conclusion:** The results support the protective effect of *B. purpurea* against chemically induced liver cancer and acts possibly by virtue of its antioxidant, antiproliferative, and apoptotic activities.

Key words: Apoptosis, *Bauhinia purpurea*, chemoprevention, hepatocarcinogenesis, oxidative stress

INTRODUCTION

Liver cancer is the sixth major cancer world wide and considered third leading cause of mortality with 598,000 patients every year.[1] Hepatocellular carcinoma (HCC), which is the major type, is the most common malignancy with a poor prognosis and inadequate treatment options, about 80% of patients die within a year of diagnosis.[2] HCC occurs in the epithelial cells of liver as a consequence of their malignant transformation. However, the exact molecular mechanism involved in cellular transformation and cancer cell development is still not clear.[3] Besides hepatitis C virus and alcohol consumption, oxidative stress is one of the most significant cause.[4] Oxidative stress is caused due to an imbalance between the production of reactive oxygen (ROS) species and the biological system ability to detoxify them.[5] It has been reported previously that in the process of carcinogenesis, increased oxidative stress plays a key role.[6] Liver is a target organ for detoxification of the xenobiotics. Liver injury can be caused due to the infections by viruses, protozoans, fungal toxins, and drug toxicity. It has been reported that *N*-nitrosamines produce a large number

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of tumors in animals. *N*-nitrosamines exposure may be caused through diet, by the use of tobacco products, agricultural chemicals, and cosmetics. DEN is one of the most important carcinogens of this class which induces tumors in the rats.[7] The dietary administration of 2-AAF with the involvement of the various *in vivo* proteins which are necessary for the progression of normal cell cycle, promote hepatocarcinogenesis. Tumors in rats are produced as a result of genotoxic metabolites of 2-AAF.[8] When cancer is diagnosed, therapists face a series of alarming challenges. Treatment usually constitutes the various combinations of surgery, radiation therapy and chemotherapy but despite these therapeutic options, cancer remains coupled with high mortality. Cancer can be prevented, suppressed, or reversed by the use of natural and some synthetic compounds.[9] Apoptosis is the result of an extremely complex cascade of cellular events that result in chromatin condensation, DNA fragmentation, cytoplasmic membrane blebbing, and cell shrinkage. Mitochondria play a vital role in apoptosis occurrence resulting from many chemotherapeutic compounds.[10] The generation of intracellular reactive oxygen species (ROS) plays a significant role in mitochondrial apoptosis occurrence through the disruption of redox.[11] ROS generation leads to the loss of mitochondrial membrane potential by activating mitochondrial permeability transition and induce apoptosis by releasing apoptotic proteins.[12] Due to the lack of efficient diagnostic tools for early detection and inadequate treatment options accessible to patients with advanced stages of HCC add to high mortality rate. Surgery is the treatment of choice for patients with well-preserved liver function. Liver transplantation provides a therapeutic option for early tumors, but this choice is of limited value due to the insufficient number of donors having predisposed factor.[13]

Chemoprevention is a new emerging strategy which reduced the hazard of cancer by the use of natural and synthetic agents. These compounds can delay, suppress or reverse the stages of carcinogenesis such as initiation, promotion, and progression.[14] A number of natural and synthetic compounds are known to possess chemopreventive potential.[15] Large amount of work has been done on a number of medicinal plants which have anticancer properties.[16]

B. purpurea is commonly known as Kachnar. In the indigenous system of Indian medicine it is used as a liver tonic and for the treatment of thyroid. It has anti-inflammatory, antipyretic, and hepatoprotective properties.[17] We examined its effects against 2-AAF-induced hepatotoxicity and also two stage hepatic carcinogenesis in Wistar rats. It was proved to be effective. We studied the expressions of ODC, PCNA, p53, Bax, Bcl-2, and caspase-3 which are known to be dysregulated in cancer cells and which may possibly be one of the targets of the chemopreventive action of *B. purpurea*.

MATERIALS AND METHODS

Chemicals

Reduced glutathione (GSH), oxidized glutathione (GSSG), glutathione reductase, bovine serum albumin (BSA), 1,2, dithio-bis-nitrobenzoic acid (DTNB), 1, chloro-2,4, dinitrobenzene (CDNB), reduced nicotinamide adenine dinucleotide phosphate (NADPH), flavine adenine dinucleotide (FAD), Tween-20, 2,6, dichlorophenolindo-phenol (DCPIP), diethyl nitrosamines (DEN) and 2-Acetylaminofluorine (2-AAF) were obtained from Sigma Chemical (St. Louis, MO, USA). All other chemicals and reagents were of the highest purity and commercially available.

B. purpurea were collected from the herbal garden of Hamdard University, New Delhi, India. Freshly collected plant material was shade-dried and coarsely powdered in a grinder. The extraction procedure was followed as described by Didry *et al*. Briefly, 100-gm dried powdered parts of *B. purpurea* were extracted with methanol in a soxhlet for 72 hrs. Then by removing the solvent under reduced pressure in rotator evaporator (Buchi Rotavapour, Switzerland), the concentrated methanolic fraction obtained was stored at 4°C and was dissolved in distilled water to make the required doses.

Animals

Four to six-weeks old, male Wistar rats (130-150 g) were obtained from Central Animal House of Hamdard University, New Delhi, India. They were housed in a ventilated room at 25 ± 5°C under a 12-hr light/dark cycle. Acclimatization was for 1 week before the experiments and were given free access to standard laboratory feed (Hindustan Lever Ltd., Bombay, India) and water *ad libitum*. The study was approved by the Committee for the purpose of Control and Supervision of Experimental Animals (CPCSEA). Registration number and date of registration: (IAEC No: 173/CPCSEA 2000). CPCSEA guidelines were followed for animal handling and treatment.

Experimental protocols

To study the effect of pretreatment with *B. purpurea* against 2-AAF-induced hepatic oxidative stress, 30 rats were randomly divided into five groups of six rats each. Group I received only normal saline (2.5 mL/kg b.wt.) from day 1 to 11. Group II served as toxicant group and was given 2-AAF (0.02%) in powdered diet from day 7 to 11. Groups III and IV were pretreated with *B. purpurea* at a dose of 100 mg/kg b. wt. and 200 mg/kg b. wt.

orally from day 1 to 6. Thereafter, from day 7 to 11 the animals were pretreated with *B. purpurea* and fed with 2-AAF (0.02%) in powdered diet. Group V received only *B. purpurea* (200 mg/kg body weight) by gavage once daily for 11 days. Group V was used only to ensure that the higher dose does not produce any kind of hepatotoxicity if given alone. All animals were sacrificed by cervical dislocation on day 12 and liver samples were taken for various biochemical parameters and at the same time blood was taken out for the estimation of serum marker enzymes.

To study the effect of pretreatment with *B. purpurea* on 2-AAF-mediated expressions of ornithine decarboxylase (ODC) and proliferating nuclear cell antigen (PCNA) the groupings of animals were as described above. Group I received normal saline only (2.5 mL/kg b.wt.). Group II were administered 2-AAF (0.02%) in powdered diet for 14 days and subjected to partial hepatectomy (PH) on 7th day after 2-AAF diet initiation. Groups III and IV served as prevention groups and were administered *B. purpurea* at doses 100 and 200 mg/kg body weight, respectively, daily subsequent to 2-AAF administration in diet and subjected to PH on 7th day. All the animals were sacrificed on day 14th and livers were excised from each group for immunohistochemical studies.

For tumor inhibition studies, the experimental schedule of Solt and Farber was followed. Animals weighing 100-150g were randomized into five different groups (n = 20). Group I received only corn oil (2.5 mL/kg b. wt.) and was kept at normal basal diet. Group II served as toxicant group and was initiated by single i.p. dose of 200 mg/kg body weight of DEN in saline followed by 2-AAF (0.02% w/w in diet from day 14 for 8 weeks) and animals 'were subjected to partial hepatectomy on day 21. Groups III and IV served as experimental groups, in addition to carcinogen treatment as in Group II they received oral administration of *B. purpurea* at doses 100 and 200 mg/kg body weight, respectively, at alternate days for continuous 8 weeks along with 2-AAF in diet. At the end of eight weeks of initiation, half the number of animals from each group were left for progression until 22 weeks of initiation on basal diet for the tumor inhibition study. The remaining animals from each group were starved overnight and sacrificed after 8 weeks of initiation. Livers were excised and fixed quickly in 10% formalin for histopathological analysis and immunohistochemical studies. At the end of 24 weeks, all the animals were euthanized by light ether anesthesia. Their livers were quickly removed and processed for histopathological and immunohistochemical studies.

Post-mitochondrial supernatant preparation

Post-mitochondrial supernatant (PMS) was prepared as described by Naghma Khan *et al*.[18]

Measurement of liver toxicity markers serum aspartate aminotransferase and alanine aminotransferase

AST and ALT activity were determined by the method of Reitman and Frankel.[19]

Assay for lactate dehydrogenase activity

LDH activity has been estimated in serum by the method of Kornberg.[20]

Estimation of reduced glutathione

Reduced glutathione was assessed by the method of Jollow *et al*.[21]

Estimation of lipid peroxidation

The assay of lipid peroxidation was done according to the method of Wright *et al*.[22]

Assay for glutathione peroxidase activity

The activity of glutathione peroxidase was calculated by the method of Mohandas *et al*.[23]

Assay for glutathione reductase activity

The activity of glutathione reductase was measured by the method of Carlberg and Mannervik.[24]

Assay for catalase activity

Catalase activity was done by the method of Claiborne.[25]

Assay for quinone reductase activity

The activity of quinine reductase was measured by the method of Benson *et al*.[26]

Estimation of protein

The protein concentration in all samples was determined by the method of Lowry *et al*., using bovine serum albumin (BSA) as standard.[27]

Histopathological examination

After the rats were sacrificed, the livers were quickly removed and preserved in 10% neutral buffered formalin for histopathological processing. Sections were stained with hematoxyline and eosin before being observed under an Olympus microscope at ×40 magnification.

Immunohistochemistry

Liver sections on polylysine coated slides obtained were fixed in neutral buffered formalin, and embedded in paraffin. Following deparaffinization and rehydration, sections were irradiated in 0.1 mol/L sodium citrate buffer (pH 6.0)

in a microwave oven (medium low temperature) for 20 min and exposed to 3% H_2O_2 for 10 min to bleach endogenous per-oxidases, followed by rinsing three times in Tris buffer (pH 7.4) for 10 min and selectively incubated under humid conditions using an anti-ODC antibody (1:400; Thermo Fisher Scientific, USA), anti-PCNA antibody (1:200; Thermo Fisher Scientific, USA), anti-P53 antibody (1:200; Santacruz Biotechnology, Inc., USA), anti-Bax antibody (1:200; Santacruz Biotechnology, Inc., USA), anti-Bcl-2 antibody (1:200; Santacruz Biotechnology, Inc., USA) and anti-caspase-3 antibody (1:200; Santacruz Biotechnology, Inc) for overnight at 4°C. Next day, slides were washed three times in Tris buffer for 10 min each. The specificity of the antibodies was tested by omission of the primary antibodies and a positive control of rat tonsil tissue. After washing in Tris buffer (pH 7.4), tissues were visualized with DAB (3,3'-Diaminobenzidine) and counterstained with hematoxyline, mounted with DPX and cover slipped. Positive and negative controls were conducted in parallel with, ODC, PCNA-stained sections. Staining of sections with commercially available antibodies served as the positive control. Negative controls included staining tissue sections with omission of the primary antibody.

Statistical analysis

Differences between groups were analyzed using analysis of variance followed by Dunnet's multiple comparisons test. All data points are presented as the treatment groups mean ± S.E.

RESULTS

Pretreatment of *B. purpurea* modulates serum toxicity markers aspartate-aminotransferase, alanine-aminotransferase and lactate dehydrogenase

Protective effect of *B. purpurea* on serum AST, ALT and LDH level was observed. Significant protection ($P < 0.001$, $P < 0.01$, $P < 0.05$) in these serum marker enzymes was observed in the pre-treatment group and found to be effective in the reduction in level of these enzymes when compared to 2-AAF-treated group [Table 1].

Pretreatment of *B. purpurea* decreased malondialdehyde formation

MDA formation was measured to demonstrate the oxidative damage on LPO of 2-AAF induced liver injury in rats. A significant ($P < 0.001$) increase of the MDA formation was found in the 2-AAF-treated group when compared with control. It was found that pre-treatment with *B. purpurea* at both D1and D2 doses leads to the significant ($P < 0.05$ and $P < 0.001$, respectively) prevention of membrane damage when compared to 2-AAF-treated group. No significant difference was found in the MDA level between control and only D2 groups [Table 2].

Pretreatment of *B. purpurea* increased the hepatic antioxidant enzymes level

Tables 2 and 3 shows the effect of pretreatment of rats with *B. purpurea* on 2-AAF-mediated hepatic glutathione content, its metabolizing enzymes and antioxidant enzymes. 2-AAF caused significant depletion in the reduced glutathione content ($P < 0.001$), glutathione reductase activity ($P < 0.001$), glutathione peroxidase activity ($P < 0.001$) quinone reductase ($P < 0.01$), and xanthine oxidase activity ($P < 0.001$) when compared with the control. However, pretreatment of animals with *B. purpurea* at 100 and 200 mg/kg body weight was found to be significantly effective in restoring the activity of these enzymes at both doses of *B. purpurea* ($P < 0.001$, $P < 0.01$, $P < 0.05$) when compared with 2-AAF-treated group. We have observed that there is no significant difference in the

Table 1: Results of pre-treatment of *B. purpurea* on serum enzymes (AST, ALT and LDH)

Treatment regimen per group	AST (IU/L)	ALT (IU/L)	LDH (n mol NADH oxidised/ min/mg protein)
Group I (control)	67.47±4.5	74.06±3.05	259.21±29.73
Group II (2-AAF only)	174.89±12.7***	125.66±13.04***	616.93±35.25***
Group III (D1+2-AAF)	128.49±2.37###	102.73±4.46###	380.88±11.59#
Group IV (D2+2-AAF)	99.02±0.49###	86.01±18.5###	296.24±40.7###
Group V (D2 Only)	72.07±1.45	79.51±1.02	269.79±5.79

Results represent mean±SE of six animals per Group. 2-AAF treatment leads to significant elevation in the serum marker enzymes in Group II as compared to Group I (***P<0.001). Pretreatment of *B. purpurea* restored activity of these enzymes in the Groups III and IV significantly as compared to 2-AAF treated Group II (###P<0.001, #P<0.05). *B. purpurea*; D1=100 mg/kg b. wt. D2=200 mg/kg b. wt

Table 2: Results of pre-treatment of *B. purpurea* on serum on LPO, GSH and XO against 2-AAF administration in liver of rat

Treatment regimen per group	LPO (nmol MDA formed/ hr/g tissue)	GSH (n mol CDNB conjugate formed /g tissue)	XO (µg uric acid formed/ min/mg protein)
Group I (control)	1.05±0.08	0.55±0.003	0.268±0.005
Group II (2-AAF only)	3.41±0.10***	0.148±0.01***	0.77±0.03***
Group III (D1+2-AAF)	2.63±0.19#	0.284±0.01##	0.44±0.06#
Group IV (D2+2-AAF)	1.74±0.02###	0.461±0.02###	0.31±0.02###
Group V (D2 Only)	1.26±0.01	0.56±0.002	0.28±0.004

Results represent mean±SE of six animals per Group. 2-AAF treatment leads to significant elevation in the MDA, GSH and XO levels in Group II as compared to Group I (***P<0.001). Pretreatment with *B. purpurea* restored activity of these enzymes in the Groups III and IV significantly as compared to 2-AAF treated Group II (###P<0.001, ##P<0.01, #P<0.05,). *B. purpurea*; D1=100 mg/kg b. wt. D2=200 mg/kg b. wt

activity of the antioxidant enzymes between control and only *B. purpurea*-treated groups.

Effect of *B. purpurea* on expression of ODC and PCNA

In Figures 1 and 2 Immunohistochemical evaluation showed more intense expressions of ODC and PCNA in rats subjected to 2-AAF alone when compared with control group. The immunohistochemical studies revealed that low and much diffused staining of hepatocytes was observed after treatment with both the doses of *B. purpurea*.

Table 3: Results of pretreatment of *B. purpurea* on antioxidant enzymes like quinone reductase, glutathione reductase, glutathione peroxidase against 2-AAF administration in liver of rat

Treatment regimen per group	Quinone reductase (n mol dichloroindophenol reduced/min/mg protein)	Glutathione reductase (n mol NADPH oxidized/min/ mg protein)	Glutathione peroxidase (n mol NADPH oxidized/min/mg protein)
Group I (control)	337.97±5.83	337.97±5.83	559.62±29.24
Group II (2-AAF only)	149.50±5.88**	149.50±5.88***	170.47±16.05***
Group III (D1+2-AAF)	242.39±9.02$^{\#}$	242.39±9.02$^{\#}$	399.50±22.99$^{\#}$
Group IV (D2+2-AAF)	337.01±25.32$^{\#\#}$	337.01±25.32$^{\#\#\#}$	556.80±51.13$^{\#}$
Group V (D2 Only)	338.01±1.91	338.7±1.91	558.35±45.45

Results represent mean±SE of six animals per Group. 2-AAF treatment leads to significant depletion in the activities of antioxidant enzymes in Group II as compared to Group 1 (***P<0.001, (**P<0.01). Pretreatment of *B. purpurea* restored activity of these enzymes in the Groups III and IV significantly as compared to 2-AAF treated Group II ($^{\#\#\#}P$<0.001), ($^{\#\#}P$<0.01) and ($^{\#}P$<0.05). *B. purpurea*; D1=100 mg/kg b. wt. D2=200 mg/kg b. wt

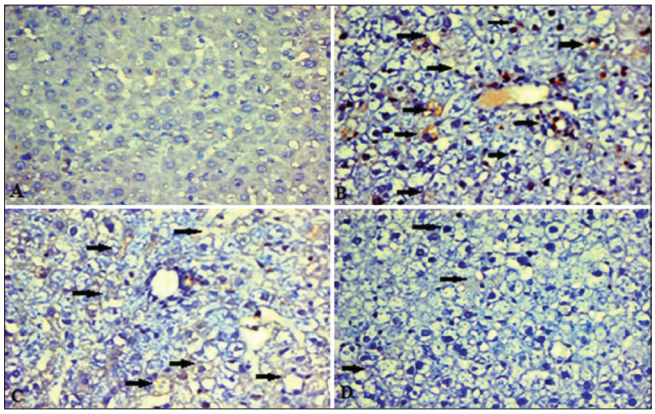

Figure 1: (a) ODC protein expression in control group showing negligible staining of hepatocytes (b) ODC protein expression in 2-AAF treated group showing intense and positive stained hepatocytes as shown by the arrows (c) ODC immunostaining of liver treated with low dose of *B. purpurea* showing weak and diffuse staining of hepatocytes as shown by arrows (d) ODC immunostaining of liver treated with high dose of *B. purpurea* showing negligible staining of hepatocytes as shown by arrows

Histopathology

In the control group a normal histological appearance was seen and the liver architecture was maintained. In II group i.e., DEN + 2-AAF + PH, development of tumor nodules formed of neoplastic liver cells was observed. The residual normal liver tissue was seen as a thin strip at the edge of the nodule and the edge of the tumor nodule showed infiltration by lymphocytes. The tumor cells have large hyperchromatic nuclei with prominent nucleoli. There was a significant lymphocytic infiltration around the portal triad area of the liver lobules. Oral administration of *B. purpurea* at doses 100 and 200 mg/kg body weight significantly caused regression of tumor formation dose dependently as shown by decreased nodule and carcinoma formation in Figure 3.

Effect of *B. purpurea* on expressions of p53, Bax, Bcl-2 and caspase-3

Immunohistochemical expression of p53 protein in rat liver tissue is shown in Figure 4.

Immunohistochemical expression of Bax protein in rat liver tissue is shown in Figure 5.

Immunohistochemical expression of Bcl-2 protein in rat liver tissue is shown in Figure 6.

Immunohistochemical expression of caspase-3 protein in rat liver tissue is shown in Figure 7.

Tumor incidences

Table 4 shows incidence of tumors in control and experimental animals. The data gives a summary of the

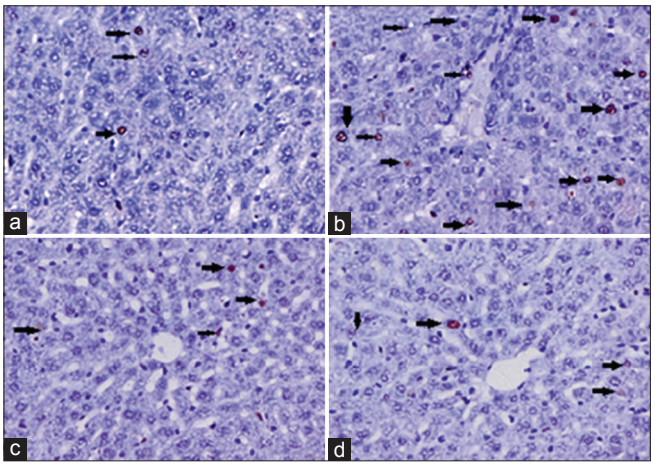

Figure 2: (a) PCNA protein expression in control group, (b) PCNA protein expression in 2-AAF-treated group showing intense and positive-stained hepatocytes as shown by arrows (c) PCNA immunostaining of liver treated with low dose of *B. purpurea* showing less staining around central vein as shown by arrows (d) PCNA expression in higher dose of *B. purpurea*-treated hepatic tissue showing very weak PCNA staining as shown by arrows

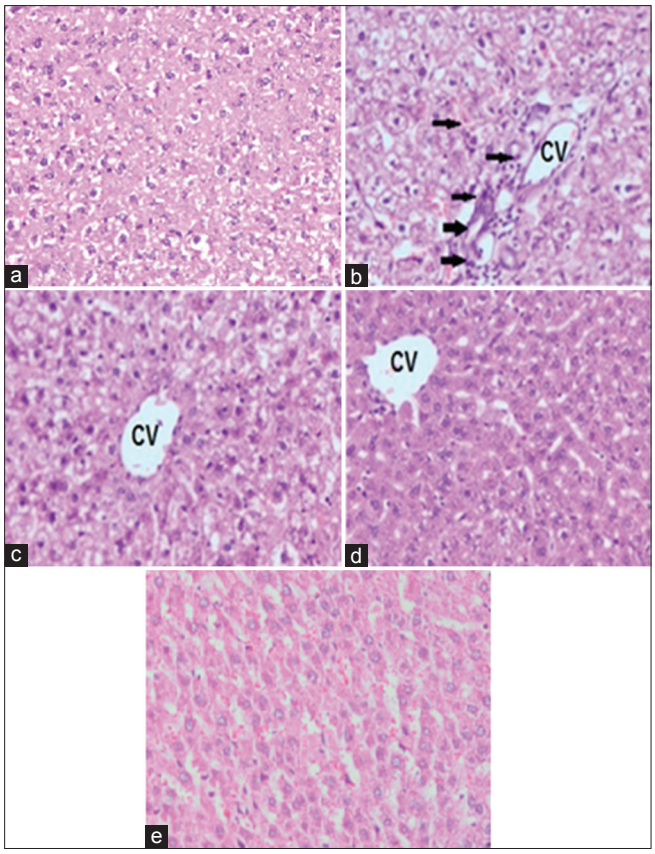

Figure 3: (a) Showing normal histology of untreated rat liver. (b) DEN+ 2-AAF+ PH group shows liver tumors with dark eosinophilic staining. The tumor cells have large hyperchromatic nuclei with prominent nucleoli as shown by arrows (c and d) Oral administration of *B. purpurea* at doses 100 and 200 mg/kg body weight caused regression of tumor formation dose dependently as shown by decrease in hyperchromatic nuclei (e) Only higher dose of *B. purpurea* shows almost similar morphology as control group

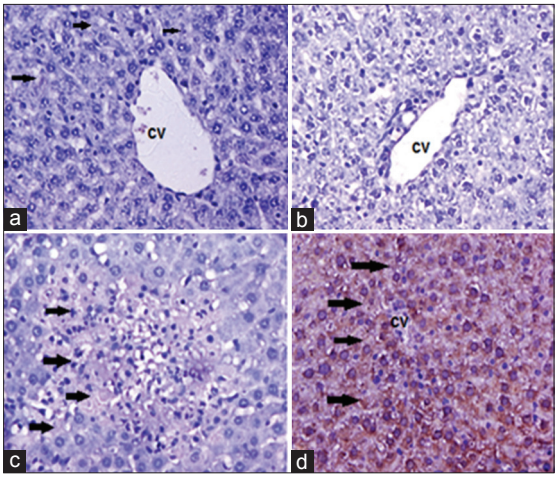

Figure 5: (a) Bax protein expression in control group showing negligible staining. (b) Bax protein expression in DEN + 2-AAF + PH-treated group showing weak and diffused staining as shown by the arrows. (c) Bax immunostaining of liver treated with low dose of *B. purpurea* showing intense staining of hepatocytes as shown by arrows. (d) More intense staining of Bax protein at high dose of *B. purpurea*-treated rat hepatic tissue as shown by arrows

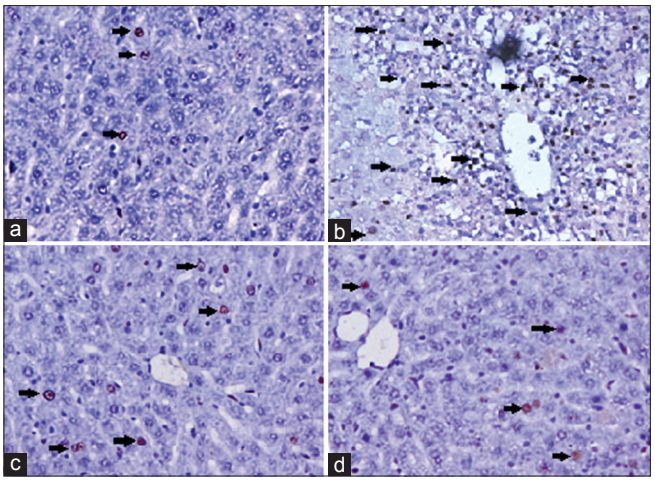

Figure 4: (a) p53 protein expression in control group, (b) p53 protein expression in DEN + 2-AAF + PH-treated group showing intense and positive-stained hepatocytes near central vein as shown by arrows. (c) p53 immunostaining of liver treated with low dose of *B. purpurea* showing fewer stainingof hepatocytes around central vein as shown by arrows. (d) p53 expression in higher dose of *B. purpurea*-treated hepatic tissue showing very weak p53 staining as shown by arrows

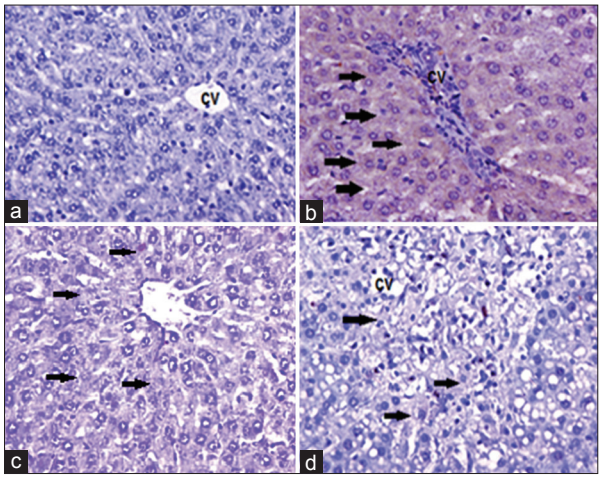

Figure 6: (a) Bcl-2 protein expression in control group almost showing negligible staining (b) Bcl-2 protein expression in DEN + 2-AAF + PH-treated group showing intense staining as shown by the arrows (c) Bcl-2 immunostaining of liver treated with low dose of *B. purpurea* showing less intense staining of hepatocytes as shown by arrows (d) Less intense staining of Bcl-2 protein expression at high dose of *B. purpurea*-treated rat hepatic tissue as shown by arrows

Table 4: Summary of tumor data on the effect of *B. purpurea* on DEN-initiated and 2–AAF promoted liver tumors

Treatment Groups	No. of animals treated	No. of animals studied for histopathology	No. of animals with liver cell tumors	Incidence of liver cell tumors (%)
Group I	20	20	0	0
Group II	20	14	11	78
Group III	20	16	9	56.2
Group IV	20	15	6	40

Group I – Control Group; Group II – DEN + 2-AAF + PH Group; Group III; DEN + 2-AAF + PH + *B.purpurea* (100 mg/kg b. wt.); Group IV – DEN + 2-AAF + PH + *B.purpurea* (200 mg/kg b. wt); DEN- Diethylnitrosamine; 2-AAF - 2-Acetylaminofluorine

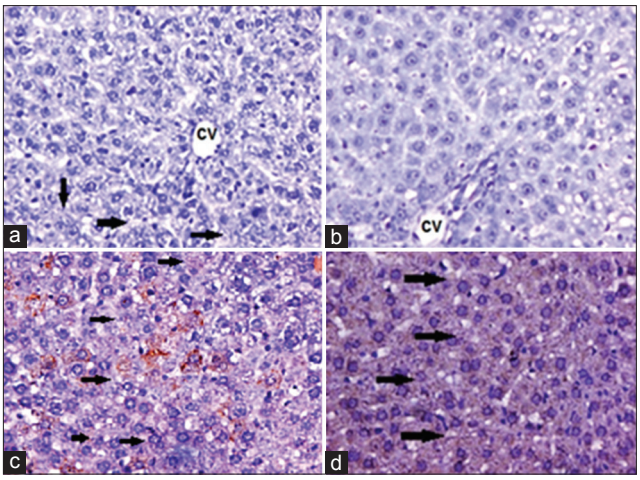

Figure 7: (a) Caspase-3 protein expression in control group showing weak staining of hepatocytes (b) Caspase-3 protein expression in DEN + 2-AAF + PH-treated group showing negligible staining of hepatocytes (c) Caspase-3 immunostaining of liver treated with low dose of *B. purpurea* showing moderate staining of hepatocytes as shown by arrows (d) Intense stainingof hepatocytes was observed at high dose of *B. purpurea*-treated rat hepatic tissue as shown by arrows

percentage incidence of liver cell tumors in treatment groups. Group I did not show any incidence of liver tumors. However, treatment with2-AAF of DEN-initiated animals enhanced the development of liver tumors in 78% of the animals studied. In comparison, tumor incidence in the group of animals cotreated with *B. purpurea*, at a lower dose (100 mg/kg body weight) was 56% whereas in the group receiving the higher dose of *B. purpurea* (200 mg/kg body weight) the tumor incidence was reduced to almost 40%.

DISCUSSION

Since past few years, due to the exposure of genotoxic and carcinogenic compounds the risk of malignancy has increased world wide. This has bought an awareness to prevent the harmful effect of these chemical compounds. This has lead to the development of a number of preventive compounds. These compounds significantly decrease tumor incidences, delay tumor onset and also have minimal long-term toxicity. Any natural or synthetic agents, which exhibits any, or when they are combine together act as a cancer-chemopreventive agent.[28] Several dietary compounds are recommended as a effective cancer chemopreventive agents due to their safety and less toxicity.[29] As a result various new classes of chemical compounds are evaluated in clinical trials as cancer preventive compounds for a number of malignancies. Further lot of scientific efforts are currently focused on exploration of safe and effective phytochemicals for the management of liver cancer. The aim of this study is that *B. purpurea* inhibits 2-AAF-dependent HCC, hyperproliferative response induces apoptosis and reduces oxidative stress. In this study, two-stage hepatocarcinogenesis was accompanied by single i.p. administration of DEN followed by 2-AAF in diet and PH in male Wistar rats. Partial hepatectomy used as a mitogenic stimuli to increased hepatic cellular carcinoma (HCC) development through the induction of hyperplasia.[30] We have observed a marked reversal of pathological manifestation which gives evidence of modulatory effect of *B. purpurea* on the development of tumor. In DEN and 2-AAF-treated group well-developed tumors with neoplastic development was seen. With the pretreatment of the *B. purpurea* to the rats the incidence of tumor was decreased. These results were further confirmed by studying the antitumor promotion activity of *B. purpurea* extract against the toxicity of 2-AAF in rat liver by studying the oxidative stress and hyperproliferation markers. The organism has antioxidative defense system, including the non-enzymatic mainly GSH and enzymatic antioxidant defenses including GR, GPx, and CAT which shield the cellular macromolecules against the damage cause by oxidative stress by detoxification of carcinogens either by destruction of their reactive centers or by conjugating them with endogenous ligands to facilitate their excretion.[31] *B. purpurea* extract ameliorated 2-AAF-induced inhibition of the activities of antioxidant enzymes viz., glutathione peroxidase, glutathione reductase, quinone reductase, and xanthine oxidase. Lipid peroxidation plays a significant role in the process of carcinogenesis and can lead to the production of several toxic products, like malondialdehyde (MDA) and 4-hydroxynonenal which can attack DNA, thus inducing mutagenecity and carcinogenicity.[32] It has been evaluated that there is an increase in lipid peroxidation during 2-AAF-induced liver toxicity (Jeyabal PV *et al.*, 2005).[33] In line with this finding there was a significant increase in the level of lipid peroxidation in the liver of rats treated with 2-AAF. However, groups pretreated with *B. purpurea* showed a significant reduction in lipid peroxidation when compared to animals treated with 2-AAF alone. The observed reduction in the level of lipid perioxidation in *B. purpurea*-treated animals was presumably due to its ability to scavenge the hydroxyl and peroxyl radicals. Any structural damage in the liver is reflected by an enhancement in the level of serum transaminases because these are cytoplasmic in location and after cellular damage they are released into circulation.[34] *B. purpurea* used in the present study has dose-dependent protection and maintain the structural integrity of hepatic cells. This was evident from the significant reduction in serum AST, ALT, and LDH activities.

ODC activity is extensively used as biochemical marker to assess the tumor-promoting potential of a compound.[16] In our study 2-AAF administration led to a marked increase in the ODC expression in the liver, which was significantly and dose dependently ameliorated by pretreatment with *B. purpurea* which suggested the antipromoting effect of *B. purpurea*. One of the important characteristics of carcinogenesis is dysregulation of the cell proliferation.

PCNA is a functional marker for the evaluation of tumor cell proliferation and progression.[35] The number of PCNA-positive cells increased significantly in DEN + 2-AAF + PH-treated group but *B. purpurea* suppressed the proliferation of liver cells dose dependently. Thus, we can suggest that PCNA expression was downregulated by *B. purpurea* pretreatment which inhibited the proliferation induced by 2-AAF administration. Apoptosis or programmed cell death is highly organized process which is necessary to eliminate damaged or abnormal cells. Induction and inhibition of apoptosis both are related to either physiological stimulus or exposure to stress-inducing agents. Apoptosis is a novel target for cancer chemoprevention which removes the cells undergoing neoplastic transformation, in conditions where other defense mechanisms are unsuccessful to obstruct the carcinogenesis process.[36] Initiation, progression and maintenance of tumor usually involve variation in programmed cell death. It has been evaluated that dysregulation of apoptosis is a main root for the formation of HCC.[37] Various studies showed that induction of apoptosis is essential for chemoprevention of cancer by natural compounds.[38] Thus, we evaluated the changes of apoptotic-related factors in protein expression levels. p53 is a transcription factor and a tumor-suppressor protein and helps in regulation of transcription of genes involved in cell cycle, DNA repair and apoptosis.[39] The present study showed that the DEN + 2-AAF + PH group has more p53 immunopositive staining as compared to the control group, while pretreatment with *B. purpurea* attenuated the p53 expression. The antiapoptotic Bcl-2 family proteins neutralize proapoptotic proteins and play a crucial role in determining the capability of a cancer cell to go through apoptosis.[40] Bax usually present in the cytosol, translocates to mitochondria to help in the progress of apoptosis, but its activity is counteracted by antiapoptotic proteins like Bcl-2.[41] Several cancer cells avoid apoptosis with upregulation of the expressions of Bcl-2.[42] In the present study, *B. purpurea* upregulated the expression of Bax, and downregulated the expression of Bcl-2 when compared with DEN + 2-AAF + PH group. In carrying out apoptosis activation of caspases plays a significant role. In the two major pathways, death receptor and mitochondrial pathways caspase-3 activation is common.[43] In the present study, DEN + 2-AAF group showed inhibition in the expression of caspase-3, but *B. purpurea* activated the expression of caspase-3. We demonstrated that *B. purpurea*-induced apoptosis by downregulation of Bcl-2, upregulation of Bax and caspase-3. The exact mechanism of the action of *B. purpurea* extract has not been fully elucidated. Its chemopreventive effect is suggested because of the presence of its active constituents viz., kaempferol, quercetin, and isorhamnetin. In the present study, it can be concluded that the mechanism of action of *B. purpurea* extract is induction of various antioxidant and phase II enzymes, scavenging reactive oxygen species, reduction in the levels of tumor promoter marker, decrease in the percentage incidence of tumors and downregulation of Bcl-2, upregulation of Bax and caspases-3 in two-stage hepatocarcinogenesis. Thus, our data suggest that *B. purpurea* extract inhibits 2-AAF-induced hepatic carcinogenesis by delaying tumor development and reducing the incidence of tumors and oxidative damage in Wistar rats.

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