RESEARCH COMMUNICATION

Chemoprevention by *Butea Monosperma* of Hepatic Carcinogenesis and Oxidative Damage in Male Wistar Rats

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Abstract

In this communication, we document chemopreventive effects of *Butea monosperma* extract on hepatic carcinogenesis and on tumor promoter induced markers and oxidative stress in male Wistar rats. Treatment of male Wistar rats for five consecutive days with 2-AAF i.p. induced significant hepatic toxicity, oxidative stress and hyperproliferation. Pretreatment of *B.monosperma* extract (100 and 200 mg/kg body weight) prevented oxidative stress by restoring the levels of antioxidant enzymes and also prevented toxicity at both doses. The promotion parameters induced (ornithine decarboxylase activity and DNA synthesis) by 2-AAF administration in diet with partial hepatectomy (PH) were also significantly suppressed dose dependently by *B. monosperma*. Thereafter, we proceeded with studies on rat liver carcinogenesis. After fourteen days of DEN treatment, dietary administration of 2-AAF with PH resulted in a 100% incidence of tumors in the animals. However, *B.monosperma* caused reduction in the number of tumors/ rat and percentage of tumor bearing rats at the end of the study, as confirmed histologically. Thus, our data suggest that *B.monosperma* extract is a potent chemopreventive agent which suppresses 2-AAF-induced hepatic carcinogenesis and oxidative damage in Wistar rats. The protective activity of the plant might be due to the two major constituents (butrin and isobutrin).

Key Words: *Butea monosperma* - oxidative stress - chemoprevention - hepatocarcinogenesis - tumor promotion

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Introduction

Over the past decade, interest in evaluating oriental medicinal herbs and edible phytoproducts for use in cancer preventive strategies is encouraging and emerging as an acceptable approach for controlling the cancer incidence in many developing as well as developed countries (Kellof, 2000). Several experimental evidence for all common cancer sites have indicated that intake of fruits and vegetables and a number of other dietary items are associated with decreased cancer incidence (Morse and Stoner, 1996). These potential agents can either abolish or delay the development of cancer by interfering with one or more steps in the process of carcinogenesis such as preventing the activation of carcinogen, by increasing detoxification or by blocking the interaction of ultimate carcinogen with cellular macromolecules, or by suppressing the clonal expansion of neoplastic cells (Morse and Stoner, 1996; Tanaka, 1994). The potential of using medicinal herbs as cancer chemoprotective nutraceuticals and functional foods is promising.

Butea monosperma (Palash), traditionally employed intensively as folklore remedy for a wide spectrum of liver diseases in India. It has a wider array of uses than any other herb. The centuries old healing system, Ayurvedic medicine, has utilized flowers, bark, leaves, gum and even the seeds of *B.monosperma* to prepare herbal remedies (Wagner et al., 1986). Numerous scientific reports validate the traditional uses of *B.monosperma* in the maintenance of general health. Practically every part of *B.monosperma* (leaves, bark, fruit, flowers, oil, and gum) have been reported to be associated with various remedial properties such as, anti-diarrhoeal (Gunakkunru et al., 2005) and antiestrogenic effects (Shah et al., 1990), acceleration of cutaneous wound healing (Sumitra et al., 2005), anti-implantation and anti-ovulatory activity (Kamboj and Dhawan, 1982), and anthelminthic (Prashanth et al., 2001), nootropic (Gawale et al., 2001), bactericidal and fungicidal influence (Bandara et al.,1989). Recently we have reported the chemopreventive potential of *B.monosperma* on thioacetamide induced early tumor promotion related events in rat liver (Sehrawat et al., 2006). The important active principles of *B.monosperma* are butin, butein, butrin, isobutrin, palasitrin, coreopsin and isocoreopsin, chalcones, and aurones triterpene phenolics constituent (Gupta et al., 1970).

The inducibility of drug metabolizing enzyme, modulation of DNA synthesis and ornithine activity, which is the rate-limiting enzyme of polyamine biosynthesis are

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one of the reliable biochemical markers to assess the chemopreventive potential of a test compound (Banerjee and Rao, 1995). Therefore, in the present study, chemopreventive effect of B.monosperma extract was tested by evaluating these parameters in the liver of rats. Moreover, to further substantiate the enthusiastic biochemical findings, modulatory influence of *B.monosperma* extract on Solt-Farber induced hepatic carcinogenesis in male Wistar rats was investigated.

For the present study, we prepared the methanolic extract of the plant flowers that contains butrin and isobutrin as major active constituents (Figure. 1). The presence of butrin and isobutrin was identified by preparative TLC.

Materials and Methods

Chemicals

Reduced glutathione (GSH), oxidized glutathione (GSSG), glutathione reductase, γ-glutamyl p-nitroanilide, glycylglycine, 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), glucose-6-phosphate, Tween-20, 2,6,dichlorophenolindo-phenol, diethyl nitrosamines (DEN), 2-acetylaminofluorine (2-AAF) and thiobarbituric acid (TBA) were obtained from Sigma Chemical (St. Louis, MO, USA). Diacetylmonoxime, urea, picric acid, sodium tungstate, sodium hydroxide, trichloroacetic acid (TCA) and perchloric acid (PCA) were purchased from CDH, India. [14C] ornithine (sp.act. 56 m Ci mmol) and [3H] thymidine (sp.act. 82 Ci mmol) were purchased from Amersham Corporation (UK). All other chemicals and reagents were of the highest purity commercially available.

Animals

4-6 week old, male albino rats (130-150 g) of Wistar strain were obtained from Central Animal House of Hamdard University, New Delhi, India. They were housed in polypropylene cages in groups of six rats per cage in a room maintained at 25 ± 2 °C with a 12 h light/dark cycle. Acclimatization was for one week before the experiments and were given free access to standard laboratory feed (Hindustan Lever Ltd., Bombay, India) and water *ad libitum*.

Plant material

B. monosperma flowers were collected from the herbal garden of Hamdard University, New Delhi, India. Prof. M. Iqbal, Head, Department of Environmental Botany, Hamdard University, verified the identity of the plant. A voucher specimen has been deposited in the herbarium of Department of Medical Elementology and Toxicology. Freshly collected plant material was shade-dried and coarsely powdered in a grinder.

Preparation of extract

The extraction procedure was followed as described by

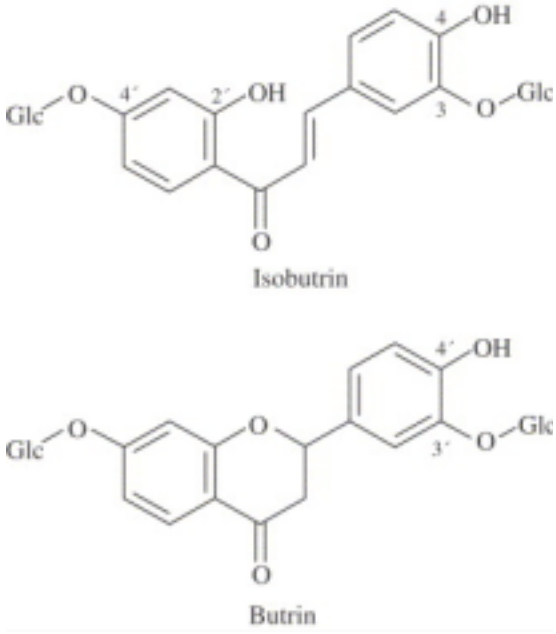

Figure 1. Structures of Isobutrin and Butrin

Wagner et al (1986). Briefly, 500g dried powdered parts of B.monosperma were extracted with methanol in a soxhlet for 20 hrs. Then by removing the solvent under reduced pressure in rotatory evaporator (Buchi Rotavapour, Switzerland), we obtained 145 g of orange powder. The concentrated methanolic fraction obtained was stored at 4°C and was dissolved in distilled water to make the required doses. For the screening of presence of antihepatotoxic compound isobutrin and butrin, 100 gm of the total methanol extract was partitioned three times between water and ethyl acetate. After ethyl acetate fraction removal, the remaining water phase was treated three times with n-butanol. The solvent was removed under reduced pressure to yield 2.7gm butanol fraction (yellow powder). To screen the presence of active principles, the butanol fraction was chromatographed on silica gel. Isobutrin gave an intense yellow spot in TLC (visible and UV fluorescent) while butrin did not show any fluorescence. For the quantitative assays of flavonoids, butrin and isobutrin was isolated by preparative TLC on silica gel (0.5mm, Merck) which give approximate yield 5mg of isobutrin and 4 mg butrin from 24 mg butanol fraction. This corresponds to 20-83 % isobutrin and 16.6 % butrin in the methanolic extract (Sehrawat et al., 2006).

Experimental Protocols

To study the effect of pretreatment with *B.monosperma* extract on 2-AAF-induced hepatic oxidative stress, thirty male Wistar rats were randomly allocated to five groups of six rats each. Group I received only saline injection intraperitoneally (0.85% NaCl) at a dose of 10 ml/kg body weight from days one to twelve. Group II served as treated control and was given i.p. injection of 2-AAF (50 mg/kg body wt) from days seven to eleven. Groups III and IV received pretreatment with *B.monosperma* extract at a dose of 100 mg/kg body weight and 200 mg/kg body weight respectively by gavage from days one to six. Thereafter, from

days seven to eleven the animals were pretreated with the plant extract and then administered 2-AAF i.p. Group V received only *B.monosperma* extract (200mg/kg body weight) by gavage once daily for 11 days. All animals were sacrificed by cervical dislocation after 24 h of last injection and processed for sub-cellular fractionation; their livers were quickly removed and perfused in ice-cold saline. Just before sacrifice, blood was collected in test tubes from retro-orbital sinus for the estimation of marker enzymes.

To study the effect of pretreatment with *B.monosperma* extract on 2-AAF mediated ODC activity, the groupings of animals were as described above. Each group contained six rats (6-8 week old). Group I served as saline control. Group II served as control and were administered 2-AAF (0.02%) in powdered diet for 14 days and subjected to partial hepatectomy (PH) on 7th day of 2-AAF diet initiation. Groups III and IV served as prevention groups and were administered *B.monosperma* extract at doses 100 and 200 mg/kg body wt. respectively daily subsequent to 2-AAF administration in diet and PH performed. Group V served as only plant treated group and received pretreatment with *B.monosperma* extract by gavage once daily for 14 days at a dose of 200 mg/kg body weight. All the animals were sacrificed on day 14th of 2-AAF administration in diet and liver were excised from each group and rinsed with ice cold saline and were processed for ODC activity.

Experimental regimen for the quantitative analysis of [^{3}H]-thymidine was same as that for ODC activity except that animals were administered [^{3}H]-thymidine (25μCi/0.2 ml saline/100 g of animal) 2 h prior to killing for [^{3}H]-thymidine incorporation study.

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). The initial weights of rats were taken. Group I served as control was given 0.9% saline i.p. and kept at normal basal diet. Group II was given saline i.p. followed by 2-AAF (0.02% w/w in diet from day 14 for 6 weeks) and the animals were subjected to PH after one week of 2-AAF dietary administration. Group III served as treated control and was initiated by single i.p. dose of 200 mg/kg body wt. of DEN in saline followed by 2-AAF (0.02% w/w in diet from day 14 for 6 weeks) and animals subjected to PH on day 21. Groups IV and V served as experimental groups, in addition to carcinogen treatment as in group III they received oral administration of *B.monosperma* extract at doses 100 & 200mg/kg body wt. 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 eight weeks of initiation. Livers were excised and fixed quickly in 10% formalin for histopathological analysis. Animals in all the groups were observed for any apparent signs of toxicity as well as mortality.

Post-mitochondrial supernatant (PMS) and microsome preparation

Livers were removed quickly, cleaned free of extraneous material and immediately perfused with ice-cold saline (0.85% sodium chloride). The livers were homogenized in chilled phosphate buffer (0.1 M, pH 7.4) containing KCI (1.17%) using a Potter Elvehjen homogenizer. The homogenate was filtered through muslin cloth, and was centrifuged at 3000 rpm for 10 min at 4°C by Eltek Refrigerated Centrifuge (model RC 4100 D) to separate the nuclear debris. The aliquot so obtained was centrifuged at 12 000 rpm for 20 min at 4°C to obtain post-mitochondrial supernatant (PMS), which was used as a source of enzymes. A portion of the PMS was centrifuged for 60 min by ultracentrifuge (Beckman L7-55) at 34,000 rpm at 4 °C. The pellet was washed with phosphate buffer (0.1 M, pH 7.4) containing KCI (1.17%).

Biochemical determinations

a) Estimation of reduced glutathione

Reduced glutathione was determined by the method of Jollow et al. (1974). 1.0 ml sample of PMS was precipitated with 1.0 ml of sulfosalicylic acid (4%). The samples were kept at 4°C for 1 h and then centrifuged at 1200 _ g for 20 min at 4°C. The assay mixture contained 0.1 ml filtered aliquot, 2.7 ml phosphate buffer (0.1 M, pH 7.4) and 0.2 ml DTNB (100 mM) in a total volume of 3.0 ml. The yellow color developed was read immediately at 412 nm on a spectrophotometer (Milton Roy Model-21 D).

b) Estimation of lipid peroxidation

The assay for microsomal lipid peroxidation was done following the method of Wright et al. (1981) as modified by Khan et al. (2001). The reaction mixture in a total volume of 1.0 ml contained 0.58 ml phosphate buffer (0.1 M, pH 7.4), 0.2 ml microsomes, 0.2 ml ascorbic acid (100 mM), 0.02 ml ferric chloride (100 mM). The reaction mixture was incubated at 37°C in a shaking water bath for 1 h. The reaction was stopped by addition of 1.0 ml 10% trichloroacetic acid (TCA). Following addition of 1.0 ml 0.67% thiobarbituric acid (TBA), all the tubes were placed in boiling water-bath for 20 min and then shifted to crushed ice-bath before centrifuging at 2500g for 10 min. The amount of malondialdehyde formed in each of the samples was assessed by measuring optical density of the supernatant at 535 nm using spectrophotometer (Milton Roy 21 D) against a reagent blank. The results were expressed as nmol MDA formed/h/g tissue at 37 °C using molar extinction coefficient of 1.56 x 10^5/M/cm.

c) Assay for hydrogen peroxide

Hydrogen peroxide (H_2O_2) was assayed by H_2O_2-mediated horseradish peroxidase-dependent oxidation of phenol red by the method of Pick and Keisari (1981). 2.0 ml of microsomes was suspended in 1.0 ml of solution containing phenol red (0.28 nm), horseradish peroxidase (8.5

units), dextrose (5.5 nm) and phosphate buffer (0.05 M, pH 7.0) and was incubated at 37°C for 60 min. The reaction was stopped by the addition of 0.01 ml of NaOH (10 N) and then centrifuged at 800 g for 5 min. The absorbance of the supernatant was recorded at 610 nm against a reagent blank. The quantity produced was expressed as nmol H_2O_2/g tissue/ h based on the obtained standard curve of H_2O_2-oxidized phenol red.

d) Assay for glutathione-S-transferase activity

Glutathione-S-transferase activity was assayed by the method of Habig et al. (1974). The reaction mixture consisted of 1.475 ml phosphate buffer (0.1 M, pH 6.5), 0.2 ml reduced glutathione (1 mM), 0.025 ml CDNB (1 mM) and 0.3 ml PMS (10% w/v) in a total volume of 2.0 ml. The changes in the absorbance were recorded at 340 nm and enzymes activity was calculated as nmol CDNB conjugate formed/ min/mg protein using a molar extinction coefficient of 9.6 x 10^3/M/cm.

e) Assay for glutathione peroxidase activity

Glutathione peroxidase activity was assayed by the method of Mohandas et al (1984). The reaction mixture consisted of 1.49 ml phosphate buffer (0.1 M, pH 7.4), 0.1 ml EDTA (1 mM), 0.1 ml sodium azide (1 mM), 0.05 ml glutathione reductase (1 IU ml/1), 0.05 ml GSH (1 mM), 0.1 ml NADPH (0.2 mM), 0.01 ml H_2O_2 (0.25 mM) and 0.1 ml 10% PMS in a total volume of 2 ml. The disappearance of NADPH at 340 nm was recorded at 25°C. Enzyme activity was calculated as nmol NADPH oxidized/min/mg protein using molar extinction coefficient of 6.22 x 10^3/M/cm.

f) Assay for glutathione reductase activity

Glutathione reductase activity was determined by method of Carlberg and Mannervik (1975). The reaction mixture consisted of 1.65 ml phosphate buffer (0.1 M, pH 7.6), 0.1 ml EDTA (0.5 mM), 0.05 ml oxidized glutathione (1 mM), 0.1 ml NADPH (0.1 mM) and 0.1 ml 10% PMS in a total volume of 2 ml. Enzyme activity was quantitated at 25°C by measuring disappearance of NADPH at 340 nm and was calculated as nmol NADPH oxidized/min/mg protein using molar extinction coefficient of 6.22 x 10^3/M/cm.

g) Assay for γ-glutamyl transpeptidase activity

This was determined by the method of Orlowski and Meister (1973) using γ-glutamyl p-nitroanilide as substrate. The reaction mixture in a total volume of 1.0 ml contained 0.2 ml 10% homogenate which was incubated with 0.8 ml substrate mixture (containing 4 mM γ-glutamyl p-nitroanilide, 40 mM glycylglycine and 11 mM $MgCl_2$ in 185 mM Tris-HCl buffer, pH 8.25) at 37 °C. Ten minutes after initiation of the reaction, 1.0 ml 25% TCA was added and mixed to terminate the reaction. The solution was centrifuged and the supernatant fraction read at 405 nm. Enzyme activity was calculated as nmol p-nitroaniline formed/min/mg protein using a molar extinction coefficient of 1.74 x 10^3/M/cm.

h) Assay for superoxide dismutase activity

Superoxide dismutase (SOD) activity was assayed by the method of Stevens et al (2000). The assay mixture consisted of 50mM, pH 10.4 glycine buffer, 20 mg/ml epinephrine solution, and cytosolic fraction (10% w/v) in a total volume of 1.0 ml. Enzyme activity was recorded at 480 nm and the activity was calculated as uM epinephrine oxidized /min/mg protein.

i) Assay for xanthine oxidase activity

The activity of xanthine oxidase was assayed by the method of Athar et al. (1996). The reaction mixture consisted of 0.2 ml PMS which was incubated for five minutes at 37°C with 0.8 ml phosphate buffer (0.1 M, pH 7.4). The reaction was started by adding 0.1 ml xanthine (9 mM) and kept at 37°C for 20 min. The reaction was terminated by the addition of 0.5 ml ice cold perchloric acid (10% v/v). After 10 min, 2.4 ml of distilled water was added and centrifuged at 4000 rpm for 10 min and μg uric acid formed/min/mg protein was recorded at 290 nm.

j) Assay for quinone reductase activity

The activity of quinone reductase was determined by the method of Benson et al (1980). The 3 ml reaction mixture consisted of 2.13 ml Tris-HCl buffer (25 mM, pH 7.4), 0.7 ml BSA, 0.1 ml FAD, 0.02 ml NADPH (0.1 mM) and 50 μL (10%) PMS. The reduction of dichlorophenolindophenol (DCPIP) was recorded calorimetrically at 600 nm and enzyme activity was calculated as nmol of DCPIP reduced/ min/mg protein using molar extinction coefficient of 2.1 x 10^4/M/cm.

k) Assay for lactate dehydrogenase (LDH) activity

LDH activity was estimated in serum by the method of Korenberg (1955). The assay mixture consisted of serum, 0.02 M NADH, 0.01 M sodium pyruvate, 0.1 M, pH 7.4-phosphate buffer and distilled water in a total volume of 3 ml. Enzyme activity was recorded at 340 nm and activity was calculated as nmol NADH oxidized/min/mg protein.

l) Assay for serum oxaloacetate and pyruvate transaminase activity (SGOT & SGPT)

SGOT and SGPT activity were determined by the method of Reitman and Frankel (1957). Each substrate (0.5 ml) (2mM α-ketoglutarate and either 200 mM · L-alanine or L-aspartate) was incubated for 5 min at 37°C in a water bath. Serum (0.1 ml) was then added and the volume was adjusted to 1.0 ml with 0.1 M, pH 7.4-phosphate buffer. The reaction mixture was incubated for exactly 30 and 60 min at 37°C for GPT and GOT, respectively. Then to the reaction mixture, 0.5 ml of 1mM DNPH was added, After another 30 min at room temperature, the color was developed by addition of 5.0 ml of NaOH (0.4 N) and the product read at 505 nm.

m) Assay for ornithine decarboxylase activity

ODC activity was determined using 0.4 ml hepatic 105,000 g supernatant fraction per assay tube by measuring

release of $^{14}CO2$ from DL-[^{14}C] ornithine by the method of O'Brien et al. (1975). The livers were homogenized in Tris-HCl buffer (pH 7.5, 50 mM) containing EDTA (0.1 mM), pyridoxal phosphate (0.1 mM), PMSF (1.0 mM), 2-mercaptoethanol (1.0 mM), dithiothreitol (0.1 mM) and Tween-80 (0.1%) at 4°C. In brief, the reaction mixture contained 400 µl enzyme and 0.095 ml co-factor mixture containing pyridoxal phosphate (0.32 mM), EDTA (0.4 mM), dithiothreitol (4.0 mM), ornithine (0.4 mM), Brij 35 (0.02%) and [^{14}C]-ornithine (0.05 µCi) in a total volume of 0.495 ml. After adding buffer and co-factor mixture to blank and other test tubes, the tubes were closed immediately with a rubber stopper containing 0.2 ml ethanolamine and methoxyethanol mixture in the central well and kept in a water bath at 37°C. After 1hr of incubation, the enzyme activity was arrested by injecting 1.0 ml citric acid solution (2.0 M) along the sides of glass tubes and the incubation was continued for 1hr to ensure complete absorption of $^{14}CO_2$. Finally, the central well was transferred to a vial containing 2 ml ethanol and 10 ml toluene based scintillation fluid was added. Radioactivity was counted in a liquid scintillation counter (LKB Wallace-1410). ODC activity was expressed as pmol $^{14}CO_2$ released/h/mg protein.

n) Assay for hepatic DNA synthesis

The isolation of hepatic DNA and assessment of incorporation of [^3H]-thymidine into DNA were carried out by the method of Smart et al (1986). The rat livers were quickly removed and cleaned free of extraneous material and homogenate (10% w/v) was prepared in ice-cold water. The precipitate thus obtained was washed with cold TCA (5%) and incubated with cold PCA (10%) at 4 °C overnight. After this, incubation mixture was centrifuged and the precipitate was washed with cold PCA (5%). The precipitate was dissolved in warm PCA (10%), incubated in a boiling water bath for 30 min, and filtered through Whatman 50 paper. The filtrate was used for [^3H] counting in a liquid scintillation counter (LKB Wallace-1410) after adding scintillation fluid. The amount of DNA in filtrate was estimated by diphenylamine method of Giles and Myers (1965). The amount of [^3H] thymidine incorporated was expressed as dpm/µg DNA.

o) Estimation of protein

The protein concentration in all samples was determined by the method of Lowry et al (1951).

Statistical analysis

The level of significance between different groups was based on analysis of variance test (ANOVA) followed by the Dunnett's t-test.

Results

Effect of B.monosperma on glutathione metabolism and antioxidant enzymes

Table 1 shows the effect of pretreatment of rats with *B.monosperma* extract on 2-AAF mediated hepatic glutathione content, its metabolizing enzymes and antioxidant enzymes. Intraperitoneal administration of 2-AAF at dose (50 mg/kg body wt.) for five consecutive days caused significant depletion in the reduced glutathione content (77%), glutathione reductase activity (71%), glutathione peroxidase activity (35%) and quinone reductase (46%) and (46%) increase in glutathione S-transferase activities. However, pretreatment of animals with *B.monosperma* at 100 and 200 mg/kg body wt. partially recovered glutathione content, glutathione reductase activity, glutathione peroxidase activity, quinone reductase, and glutathione S-transferase levels to 9-48%, 26-38%, 5-23%, 5-11% and 9-21%, respectively as compared with 2-AAF-treated control groups.

Table 1. Effects of Prophylactic Treatment of Animals with *B.monosperma* on 2-AAF Induced Alteration in Hepatic Glutathione Content, Glutathione Metabolizing and Antioxidant Enzymes

Treatment group	GSH (nmol GSH/g tissue)	Glutathione reductase (nmol NADPH oxidized /min/mg protein)	Glutathione peroxidase (nmol NADPH oxidized /min/mg protein)	Glutathione -S transferase (nmol CDNB conjugate formed/ min/mg protein)	Quinone reductase (nmol dichloroin-dophenol reduced/ min/mg protein)
Saline-treated control	0.62±0.09	487.4±15.0	646.6±31.0	462.0±32.7	320.4±16.0
2-AAF (50mg /kg body wt.) alone	0.14±0.02$^{\#\#}$	138.7±11.1$^{\#\#}$	418.2±25.1$^{\#\#}$	674.0±28.4$^{\#\#}$	172.6±18.3$^{\#}$
B. monosperma (100 mg/kg body wt.) + 2-AAF (50mg /kg body wt.)	0.20±0.03****	268.1±18.9***	454.1±24.8^{NS}	630.4±25.6^{NS}	189.7±30.9^{NS}
B. monosperma (200 mg/kg body wt.) + 2-AAF (50mg /kg body wt.)	0.44±0.01***	324.8±13.8***	567.2±18.8**	574.7±15.2*	209.1±19.2****
B. monosperma (200 mg/kg body wt.)	0.62±0.04	489.6±21.0	666.4±13.2	450.7±18.1	344.1±15.8

Each value represents means ± S.E.; n=6. $^{\#}P< 0.01$ and $^{\#\#}P< 0.001$, compared to corresponding value for saline treated control
*P < 0.05, **P<0.01, ***P < 0.001, ****P < 0.1 and NS= not significant, compared with the corresponding value for treatment with 2-AAF

Table 2. Effect of Prophylactic Treatment of Animals with *B.monosperma* on 2-AAF Mediated Alterations in Hepatic Lipid Peroxidation, Hydrogen Peroxide Content, Xanthine Oxidase and Superoxide Dismutase Enzyme

Treatment groups	Lipid peroxidation (nmol malondialdehyde formed / hr / gm tissue)	Hydrogen peroxide content (nmol H_2O_2/g formed)	Superoxide dismutase (uM epinephrine oxidized /g tissue) /min/mg protein)	Xanthine oxidase (ug uric acid formed/mg protein
Saline-treated control	0.90±0.07	997.7±26.2	150.5±23.5	0.307±0.030
2-AAF (50mg /kg body wt.) alone	3.59±0.03$^{\#\#\#}$	2939 ±50.1$^{\#\#\#}$	58.1±19.3$^{\#}$	0.675±0.045$^{\#\#}$
B. monosperma (100 mg/kg body wt.) + 2-AAF (50mg /kg body wt.)	2.67±0.07**	1971 ±25.6**	75.1±14.1^{NS}	0.593±0.012***
B. monosperma (200 mg/kg body wt.) + 2-AAF (50mg /kg body wt.)	2.08±0.15**	1896 ±43.2**	119.7±21.6***	0.466±0.018*
B. monosperma (200 mg/kg body wt.)	0.89±0.07	924.8±28.0	158.4±18.1	0.296±0.024

Each value represents means _ S.E.; n=6. $^{\#}$P< 0.05, $^{\#\#}$P< 0.01, $^{\#\#\#}$P< 0.001, compared to corresponding value for saline treated control.
*P<0.01, **P < 0.001 and ***P < 0.1, compared with the corresponding value for treatment with 2-AAF.

Table 3. Effect of Prophylactic Treatment of Animals with *B.monosperma* on TAA-mediated Alteration in Activities of Serum Oxaloacetate and Pyruvate Transaminases (SGOT & SGPT), Lactate Dehydrogenase (LDH) and γ-Glutamyl-transpeptidase (GGT) Enzymes

Treatment groups	SGOT (IU/ litre)	SGPT (IU/ litre)	LDH1	GGT2
Saline-treated control	78.3 ± 14.8	65.9 ± 11.1	432.3 ± 22.8	543.2 ± 18.8
2-AAF (50mg /kg) alone	276.0 ± 13.7$^{\#}$	187.8 ± 31.5$^{\#}$	741.4 ± 38.3$^{\#}$	874.4 ± 17.5$^{\#}$
B. monosperma (100 mg/kg) + 2-AAF (50mg /kg)	212.1 ± 14.5**	175.0 ± 21.1**	656.8 ± 16.2*	766.6± 19.7**
B. monosperma (200 mg/kg) + 2-AAF (50mg /kg)	161.9 ± 11.0**	72.5 ± 30.1**	454.6 ± 21.1**	668.6 ± 11.9**
B. monosperma (200 mg/kg)	78.7 ± 17.5	62.5 ± 21.1	430.2 ± 20.5	541.1 ± 6.5

1nmol NADH oxidized/min/mg protein; 2 nmol p-nitroaniline formed/min/mg protein; Values are means ± S.E., n=6.
$^{\#}$P < 0.001, as compared to the saline treated control. *P < 0.05, **P < 0.01 as compared with the 2- AAF treated group.

Effect of B.monosperma extract on xanthine oxidase, lipid peroxidation, hydrogen peroxide content and superoxide dismutase

Table 2 shows the effects of *B.monosperma* against 2-AAF mediated decrease in superoxide dismutase and induction in lipid peroxidation, hydrogen peroxide formation and xanthine oxidase activity. 2-AAF alone resulted in the down regulation of superoxide dismutase activity (61%), parallel to these changes; it increased the level of lipid peroxidation (75%), hydrogen peroxide content (195%) and xanthine oxidase activity (120%). The activity of superoxide dismutase was significantly recovered (41%) on *B.monosperma* pretreatment especially at the higher dose. The percentage of recovery in case of xanthine oxidase was 12-31%. *B.monosperma* caused suppression in the formation of MDA (26-42%) and H_2O_2 (33-35%) in a dose dependent manner.

Effects of B.monosperma extract on hepatic toxicity markers

Table 3 shows the effect of pretreatment of animals with *B.monosperma* on 2-AAF induced hepatotoxicity. 2-AAF treatment leads to the enhancement in serum oxaloacetate and pyruvate transaminases (252%, 185% respectively), lactate dehydrogenase (61%) and γ-glutamyl transpeptidase activity (71%), as compared with saline treated control animals. Pretreatment with *B.monosperma* resulted in the 7-23% and 39-61% at lower (100 mg/kg body wt.) and higher (200 mg/kg body wt) doses respectively.

Effect of B.monosperma extract on percentage incidence of liver tumors

Effect of prophylactic treatment of *B. monosperma* on body weight, liver weights and relative weights of rats during long-term hepatic carcinogenic studies are shown in Table 4. DEN initiated and 2-AAF promoted rats showed a significant increase in body weight, liver weight and liver-to- body weight ratio. *B. monosperma* supplementation caused a reduction in the body and liver weight and liver-to- body weight ratio. Also, the number of rats with tumors decreased on pretreatment with *B. monosperma* extract.

Effect of B.monosperma extract on induction of hepatic ODC activity

2-AAF administration in the diet for 14 days with PH

Table 4. Effects of Prophylactic Treatment with *B.monosperma* on Body, Liver and Relative Weights

Treatment groups	Effective no of rats	No. of rats with tumors (%)	Body weight	Liver weight (g)			Liver- to body wt ratio (x 10 –2)
				Initial (g)	Final (g)	Increase (g)	
Saline-treated control	20/20	0	146.4 ± 3.5	571.7 ± 22.6	425.3 ± 24.0	14.2 ± 1.5	2.5 ± 0.3
DEN/2-AAF	14/20	14 (100)	146.6 ± 4.1	857.0 ± 28.6	810.4 ± 28.9$^{\#}$	41.9 ± 2.5$^{\#}$	4.8 ± 0.4
2-AAF	17/20	3 (17.6)	129.5 ± 5.1	587.4 ± 20.8	457.9 ± 20.5^{NS}	22.0± 6.7^{NS}	3.7 ± 0.1
DEN/2-AAF + *B. monosperma* (100 mg/kg)	16/20	14 (87.5)	143.3 ± 6.8	796.7 ± 20.8	653. 4 ±20.1*	36.1± 2.1**	4.5 ± 0.5
DEN/2-AAF +*B. monosperma* (200 mg/kg)	18/20	12 (66.6)	148.6 ± 3.9	692.2 ± 18.7	543.6 ± 19.2***	29.8 ± 1.3*	4.3 ± 0.4

Each value represents means ± S.E.; n=6. $^{\#}P< 0.01$, compared to corresponding value for saline treated control
*P < 0.05, **P<0.01, ***P < 0.001, and NS= not significant, compared with the corresponding value for treatment with DEN/2-AAF

augmented a 276% increase in hepatic ODC activity when compared to saline treated control. The pretreatment of rats with *B.monosperma* extract at a dose of 100 mg/kg body weight caused inhibition in the elevation of ODC activity by 14 % and at a dose of 200 mg/kg body weight by 28 %, when compared to the 2-AAF + PH-treated group.

Effects of B.monosperma extract on hepatic DNA synthesis

2-AAF administrations in the diet for 14 days with PH caused a 266% increase in rate of [^{3}H] thymidine incorporation when compared to saline treated control. *B.monosperma* extract pretreatment (100 and 200 mg/kg body weight) caused reduction in the enhancement of DNA synthesis by 13-35 %, when compared to the 2-AAF + PH-treated group.

Effect of B.monosperma extract on histopathological alterations in rat liver

B.monosperma was tested for its chemopreventive effect in the hepatocarcinogenesis assay, induced by DEN as initiator and 2-AAF as a promoter. In the control group a normal histological appearance was seen and the hepatic architecture was maintained. In DEN + 2-AAF + PH group development of tumor nodules formed of neoplastic hepatocytes were 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 shows infiltration by lymphocytes. The tumor cells have large hyperchromatic nuclei with prominent nucleoli. In group 2-AAF + PH any development of tumor nodules in the liver parenchyma was not seen. There was however a significant degree of lymphocytic infiltration around the portal triad area of the hepatic lobules. Oral supplements of *B.monosperma* extract at doses 100 & 200 mg/kg body wt significantly caused regression of tumor formation as evidenced by decreased nodule and carcinoma formation in dose dependent manner. No differences in liver tissue architecture were observed in the DEN + 2-AAF plus *B.monosperma* groups when compared with control group. At the dose of 100 mg/kg body wt. small foci of tumor nodule development were seen with no fatty changes in the liver parenchyma. While at 200 mg/kg body wt. almost a normal histological appearance with no tumor development or fatty change in any area was observed.

Effects of B.monosperma extract on tumor formation

Livers of rats with DEN+ 2-AAF + PH showed carcinoma formation and cirrhotic conditions. Oral supplemention with *B.monosperma* extract at doses 100 & 200 mg/kg body wt. significantly caused reduction in tumor formation (Table 4).

Discussion

Many plants conveniently available in India are used in traditional folklore medicine have been accepted as main source of drug discovery and development for cancer. The chemical composition and medicinal uses of *B.monosperma* extract have been reported widely. This plant has been reported to contain butin, butein and butrin, isobutrin, palasitrin, coreipsin and isocoreipsin, chalcones, and aurones. In the present study, the methanolic extract of *B.monosperma* extract was found to possess butrin and isobutrin as its major active constituents. Butrin and isobutrin has been reported to act as strong antihepatotoxic agents. We have recently reported that *B.monosperma* flower extract having butrin and isobutrin as main component that inhibits early liver tumor promotion markers in rats (Sehrawat et al., 2006). Since the active constituents of *B.monosperma* extract have known protective effects in other studies, so the observed chemopreventive activity of *B.monosperma* extract in our study may be suggested due to the presence of these compounds.

The major advantage of the Solt -Farber model used in this study to investigate the effect of *B.monosperma* extract is that cancer development by this model passes through multiple stages just like human cancer does (Enzmann et al., 1998) and it has emerged as useful tool in field of chemoprevention for evaluating the chemoprotective effect of various agents on the promotion stage of hepatocarcinogenesis (Lee et al., 2005, Sultana et al., 2005). In the present study, two-stage carcinogenesis was accompanied by single i.p. administration of DEN followed by 2-AAF in diet and PH in male Wistar rats. Partial hepatectomy (PH) enhanced hepatic cellular carcinoma (HCC) development by inducing hyperplasia (Park and Suh, 1999). The marked reversal of pathological manifestation gives ample evidence of modulating effect of *B.monosperma*

on tumor development. Commonly observed in DEN and 2-AAF treated group were well developed tumors with neoplastic development. With the pretreatment of the *B.monosperma* extract to the rats, on the contrary, not only the incidence of tumor was less, reversal of the pathological sequences with small foci of tumor nodule development was also seen.

These results were further confirmed by testing the anti-tumor promotion ability of a *B.monosperma* extract against the toxicity of 2-AAF in rat liver by studying the oxidative stress and hyperproliferation markers. Oxidative stress is implicated in all the stages of the development of cancer as well as in the genesis of other diseases. Antioxidant defense enzymes comprising of superoxide dismutase (SOD), glutathione peroxidase (GPx) and glutathione reductase (GR); and reduced glutathione (GSH) protect the cellular macromolecules against oxidative damage by detoxifying carcinogens either by destroying their reactive centers or by conjugating them with endogenous ligands facilitating their excretion (Banerjee and Rao, 1995). 2-acetylaminofluorene (AAF) is metabolized in rat liver to give genotoxic metabolites, and is able to initiate rat liver tumors. One of the possible mechanisms responsible for tumor promotion by 2-AAF is the microsomal generation of reactive oxygen by redox cycling of suitable 2-AAF metabolites (Hillesheim et al., 1995). Dietary administration of 2-AAF promotes hepatocarcinogenesis, by interfering with the in vivo regulation of several proteins essential for a normal cell cycle progression. Loss of cellular proliferation control causes the loss of lipid peroxidation in preneoplastic hepatocellular membranes (Benedetti et al., 1984). *B.monosperma* extract ameliorated 2-AAF-induced inhibition of the activities of antioxidant enzymes, viz., glutathione peroxidase, glutathione reductase and phase-II metabolising enzymes such as glutathione-S-transferase and quinone reductase. *B.monosperma* extract has established antioxidant properties that might have counteracted the oxidant effects of 2-AAF. The present study shows induction of hepatic glutathione-S-transferase and quinone reductase activity following *B.monosperma* extract treatment. Many chemopreventive agents are known to degrade electrophilic metabolites by induction of glutathione-S-transferase and quinone reductase activities. Quinone reductase is a major enzyme of xenobiotic metabolism that protects cells against mutagenicity and carcinogenicity resulting from free radicals and toxic oxygen metabolites by carrying out obligatory two-electron reductions. Induction of quinone reductase activity has been reported to have correlation with the prevention of cancer (De Flora and Ramel, 1988).

There was also dose-dependent decrease in the 2-AAF mediated susceptibility of hepatic microsomal membrane for iron-ascorbate induced lipid peroxidation through decreased production of free radicals as shown by ameliorated malendialdehyde levels. The decreased level of reduced glutathione following 2-AAF administration due to decreased reduction of oxidized glutathione and increased activity of xanthine oxidase cause accumulation of peroxides thereby leading to oxidative stress. There was a decrease in the activities of xanthine oxidase, H_2O_2 and an increase in hepatic glutathione content and superoxide dismutase.

Damage to the structural integrity of liver is reflected by an increase in the level of serum transaminases because these are cytoplasmic in location and are released into circulation after cellular damage (Recknagel et al., 1991). *B.monosperma* extract used in the present study seems to offer dose dependent protection and maintain the structural integrity of hepatic cells. This was evident from the significant reduction in serum GOT, GPT, LDH and GGT activities.It has been shown that 2-AAF + PH induces ornithine decarboxylase (ODC) activity and rate of DNA synthesis by several folds in rat liver (Olson and Russell, 1980). ODC activity and [^{3}H]- thymidine incorporation are used extensively as a biochemical marker to evaluate the tumor promoting potential of an agent. Various inhibitors of ODC induction and enhanced DNA synthesis have been shown to suppress tumor promotion. The prophylactic treatment of plant markedly suppressed 2-AAF + PH induced ODC activity and [^{3}H] thymidine incorporation into regenerating hepatic DNA. As observed in the present study, *B.monosperma* extract dose-dependently inhibited the induction of ODC activity and [^{3}H] thymidine incorporation suggesting its antihyperproliferative potential.

The exact mechanism of the action of *B.monosperma* extract has not been fully elucidated. However, its chemopreventive effect is suggested due to the presence of its active constituents, viz., butrin and isobutrin in addition with other minor constituents. In the present study, it can be concluded that the mechanism of action of *B.monosperma* extract is (1) induction of various antioxidant and phase II enzymes, (2) scavenging reactive oxygen species, (3) sharp reduction in the levels of tumor promoter markers and (4) decrease in the percentage incidence of tumors in two-stage hepatic carcinogenesis. Thus, our data suggest that *B.monosperma* extract is a potent chemopreventive agent and inhibits 2-AAF-induced hepatic carcinogenesis by delaying tumor formation and reducing the frequency of tumors and oxidative damage in Wistar rats.

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