

Cancer chemopreventive property of *Bidens pilosa* methanolic extract on two stage *in vivo* skin carcinogenesis model**S. PARIMALAKRISHNAN*, D. AKALANKA AND R. MANAVALAN***Department of Pharmacy, Annamalai University, Annamalai Nagar - 608002, India.*

The effect of the methanolic extract of whole plant of *B. pilosa* on drug metabolizing phase I and II enzymes, antioxidant enzymes, lactate dehydrogenase and lipid peroxidase, anticarcinogenic potential in dimethylbenzanthracene induced forestomach and tetradecanoyl acetate promoted skin papillomagenesis was studied in mice. The hepatic glutathione S-transferase and DT-diaphorase specific activities were elevated above basal level. *Bidens pilosa* methanolic extract was effective in elevating antioxidant enzyme response by increasing significantly the hepatic glutathione peroxidase, glutathione reductase, catalase and superoxide dismutase. Reduced glutathione measured as non protein sulphhydryl was found to be significantly increased in liver while in extrahepatic organs it was decreased. In the forestomach, kidney and lung, glutathione S-transferase and DT-diaphorase levels were significantly reduced. Chemopreventive response was calculated by the mean number of papillomas /mouse and percentage of tumor-bearing mice. There was a significant inhibition of tumor burden in both the tumor model systems studied.

Keywords: *Bidens pilosa*, chemopreventive, DT-diaphorase, glutathione S-transferase, lactate dehydrogenase.

INTRODUCTION

Bidens pilosa grows rarely in the south of India being known as "Otrancedi" and is frequently used in traditional medicine as a remedy to treat glandular sclerosis, wounds, cold and flu, acute or chronic hepatitis and urinary tract infections [1].

Chemoprevention is a means of cancer control by the use of natural or synthetic agents to interrupt the process of carcinogenesis and to prevent or delay tumor growth. Chemopreventive agents can be broadly classified as blocking and suppressing agents. The blocking agents prevent carcinogenic agents from reaching or reacting with critical target sites whereas suppressing agents prevent the evolution of neoplastic processes in the cells.

The inducibility of drug-metabolizing enzymes is one of the reliable biochemical markers to assess the chemopreventive potential of a test compound.

The most frequent skin cancers are melanomas, basal-cell cancers and squamous cell cancers.

For more than 50 years, the multistage model of carcinogenesis in mouse skin has provided a conceptual framework within which to study the carcinogenic process in tissues of epithelial origin. Stomach cancer is one of the most common forms of cancer in the world. Therefore, the modulatory influence of both the plant extracts on dimethylbenz(a)anthracene-induced skin and Tetradecanoyl acetate (TDA) promoted forestomach papillomagenesis at the per-initiation level was also evaluated.

Tetradecanoyl acetate is a well known tumor promoter, induces ornithine decarboxylase (ODC), which is a rate-limiting enzyme in polyamine biosynthesis and a tumor promoter marker in mouse skin. Topical application of TPA to mouse skin *in vivo* has been reported to cause transient induction of several genes, including metallothionein (MT), ODC, and early response genes, such as *c-fos* and *c-jun* [4]. Mechanistic studies on the effects of TDA

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have shown that it binds to and activates protein kinase C, which starts the protein kinase cascade. This finally leads to formation of activated protein 1 (AP-1), which induces expression of target genes [5].

Tetradecanoyl acetate is an irritant and inflammatory agent that has been used widely as a tumor promoter on the skin of mice previously initiated with 7, 12-dimethylbenz (a)anthracene (DMBA) or other polycyclic aromatic hydrocarbons. Topical application of 5–16 nmol of TPA alone to mouse skin twice a week for several months either has no tumorigenic effect by itself or results in only an occasional nonmalignant papilloma. Because of the potent effect of TPA in stimulating differentiation in myeloid leukemia cells at a very low concentration, the effects of TPA in mouse with papilloma were investigated in the present study.

In the present study, the methanolic extract of entire plant of *Bidens pilosa* was used to evaluate these enzymes in the livers of mice, because the liver plays the pivotal role of carrying out various important reactions. Antioxidant enzymes are the counterparts of oxidative damage that protect the cell [3]. The levels of hepatic antioxidant defense enzymes, comprising superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), and glutathione reductase (GR) were therefore monitored. In addition to reduced glutathione (GSH), the intracellular antioxidant level was also evaluated. Lipid peroxidation and lactate dehydrogenase were measured in the microsome and cytosolic fractions, respectively, to evaluate free radical formation and cell-damage parameters. Furthermore the influence of *Bidens pilosa* methanolic extract (BPME) in increasing the detoxifying capabilities of extrahepatic organs such as the lung, kidney and forestomach was examined.

MATERIALS AND METHODS

Plant collection and preparation of modulator

The entire plant of *Bidens pilosa* (BP) was collected from Sengottai of Tamilnadu, India. The plant material was taxonomically identified and authenticated by the Department of Botany, Annamalai University,

Annamalainagar, India. The samples were cleaned, dried under shade, powdered by a mechanical grinder and stored in an airtight container.

Methanol was used as solvent in the extraction process. The methanol extract was prepared by using dried coarse material of the whole plant and methanol (yield 3.12%), in a Soxhlet apparatus. The methanol extract of *Bidens pilosa* (BPME) was suspended in 1% gum acacia and was administered to the mice at the doses of 200 and 400 mg/kg of body weight for each group except the control group in all the experiments.

Mice

Both the sexes of hairless 8 weeks old mice strain (25-30 g) were procured from the central animal house of the Department of Pharmacy, Annamalai University. They were kept in standard polypropylene cages maintained under standard room temperature (20±4 °C, relative humidity 60–70%) in a 12 h dark–light cycle. The mice were fed with standard pellet diet and water *ad libitum*. The experiments were conducted after getting the prior ethical approval. Three separate, independent experiments were carried out to demarcate specific aims of the work as mentioned earlier whereas experiment I was restricted to studying the inducibility of detoxifying parameters in hepatic and extrahepatic organs. Experiments II and III were aimed at evaluating the assumed efficacy of the modulator in chemoprevention in the mouse skin and forestomach tumorigenesis models.

Experiment I

The objective of experiment I was to investigate modulation of hepatic and extrahepatic carcinogen metabolising and antioxidant enzymes. Mice were grouped as follows:

Experimental design

Group I (n = 6): Mice were put on a normal diet and treated with 50 ml of an emulsion made of peanut oil and double-distilled water (ratio 4:1 ml) used as vehicle for feeding the modulator, by oral gavage daily for 15 days;

this group of mice served as a negative control.

Group II (n = 6): Mice were put on a normal diet and treated daily with 200 mg/kg body weight of lyophilized BPME extract, which was suspended in the control vehicle and was given to the mice (50 ml/mouse/day) by oral gavage for 15 days.

Group III (n = 6): Mice were put on a normal diet and treated daily with 400 mg/kg body weight of lyophilized BPME extract, which was suspended in the control vehicle and was given to the mice (50 ml/mouse/day) by oral gavage for 15 days.

Preparation of homogenates, cytosol and microsome fractions

Mice were sacrificed by cervical dislocation and the entire liver was perfused immediately with cold 0.9% NaCl and thereafter carefully removed, trimmed free of extraneous tissue and rinsed in chilled 0.15 M Tris–KCl buffer (0.15 M KCl + 10 mM Tris–HCl, pH 7.4). The liver was then blotted dry, weighed quickly, and homogenized in ice-cold 0.15 M Tris–KCl buffer (pH 7.4) to yield 10% (w/v) homogenate. An aliquot of this homogenate (0.5 ml) was used to assay reduced glutathione levels, while the remainder was centrifuged at 10,000 rpm for 20 min. The resultant supernatant was transferred into pre-cooled ultracentrifugation tubes and centrifuged at 105,000 x g for 60 min in a Beckman ultracentrifuge (Model-L870M). The supernatant (cytosol fraction), after discarding any floating lipid layer and appropriate dilutions were made and used in the assays of glutathione S-transferase, DT-diaphorase, lactate dehydrogenase, and antioxidant enzymes, whereas the pellet representing microsomes was suspended in homogenizing buffer and used for assaying cytochrome P450, cytochrome b5, cytochrome P450 reductase, cytochrome b5 reductase and lipid peroxidation.

Extrahepatic organs

The lung, kidney and forestomach were carefully removed, along with the liver, trimmed free of extraneous tissue and rinsed in chilled 0.15 M Tris–KCl (pH 7.4). The lung

was cut into small pieces. The stomach was opened longitudinally.

The forestomach was separated from the glandular stomach and cleaned of all its contents by flushing with the buffer 5–6 times. The lung, kidney and forestomach were then blotted dry, weighed quickly and homogenized in ice-cold 0.15 M Tris–KCl buffer (pH 7.4) to yield a 10% (w/v) homogenate. A 0.5 ml aliquot of this homogenate was used to assay reduced glutathione. The rest of the homogenate was centrifuged at 15,000 x g for 30 min at 4 °C. The resulting supernatant obtained was used for assaying glutathione S-transferase and DT-diaphorase enzymes.

Assay method

Cytochrome P450 and cytochrome b5

Cytochrome P450 was determined using carbon monoxide difference spectra. Both cytochrome P450 and cytochrome b5 content were assayed in the microsomal suspension according to the method of Omura and Sato [6], using absorption coefficients of 91 and 185 cm² M⁻¹ m⁻¹, respectively.

Glutathione S-transferase

The cytosolic as well as supernatant of extrahepatic glutathione S-transferase activity was determined spectrophotometrically at 37°C according to the procedure of Habig *et al.* [7]. The reaction mixture (3 ml) contained 1.7 ml of 100 mM phosphate buffer (pH 6.5), 0.1 ml of 30 mM CDNB, and 0.1 ml of 30 mM of reduced glutathione. After pre-incubating the reaction mixture at 37 °C for 5 min, the reaction was started by the addition of 0.1 ml diluted cytosol and the absorbance was followed for 5 min at 340 nm. A reaction mixture without the enzyme was used as blank. The specific activity of glutathione S-transferase was expressed as mmoles of GSH–CDNB conjugate formed/min/mg of protein using an extinction coefficient of 9.6 mM⁻¹ cm⁻¹.

DT-diaphorase

DT-diaphorase activity was measured as described by Ernster *et al.* [8], with NADH as the electron donor and 2,6-dichlorophenol-

indophenol (DCPIP) as the electron acceptor at 600 nm. The activity was calculated using an extinction coefficient of $21 \text{ mM}^{-1}\text{cm}^{-1}$. One unit of enzyme activity has been defined as the amount of enzyme required to reduce one mole of DCPIP/min.

Reduced glutathione

Reduced glutathione was estimated as the total non-protein sulphhydryl group by the method described by Moron *et al.* [9]. Homogenates were immediately precipitated using 0.1 ml of 25 % trichloroacetic acid and the precipitate was removed following centrifugation. Free SH groups were assayed in a total volume of 3 ml by adding 2 ml of 0.6 mM DTNB prepared in 0.2 M sodium phosphate buffer (pH 8.0), to 0.1ml of the supernatant and absorbance was read at 412 nm using a Shimadzu UV-160 spectrometer. Reduced glutathione was used as a standard to calculate mmole-SH content/g tissue.

Glutathione reductase

Glutathione reductase was determined by the procedure as described by Carberg and Mannervik [10]. The reaction mixture (final volume 1 ml) contained 0.2 M sodium phosphate buffer (pH 7.0), 2 mM EDTA, 1 mM oxidised glutathione (GSSG) and 0.2 mM NADPH. The reaction was started by adding 25 μl of cytosol and the enzyme activity was measured indirectly by monitoring the oxidation of NADPH following decrease in OD/minute for a minimum of 3 min at 340 nm. One unit enzyme activity has been defined as nmoles of NADPH consumed/minute/mg of protein based on an extinction coefficient of $6.22 \text{ mM}^{-1}\text{cm}^{-1}$. [10]

Glutathione peroxidase

Glutathione peroxidase activity was measured by the coupled assay method as described by Paglia and Valentine [11]. Briefly, 1 ml of the reaction mixture contained 50 mM sodium phosphate buffer (pH 7.0) containing 1 mM EDTA, 0.24 Uml^{-1} yeast glutathione reductase, 0.3 mM glutathione (reduced), 0.2 mM NADPH, 1.5 mM H_2O_2 and cytosol sample. The reaction was initiated by adding NADPH and its oxidation was monitored at 340 nm by observing the decrease in OD/min

for 3min. One unit of enzyme activity has been defined as nmoles of NADPH consumed/min/mg of protein based on an extinction coefficient of $6.22 \text{ mM}^{-1}\text{cm}^{-1}$.

Catalase

Catalase was estimated at 240 nm by monitoring the disappearance of H_2O_2 as described by Aebi [12]. The reaction mixture (1 ml) contained 0.02 ml of suitably diluted cytosol in phosphate buffer (50 mM, pH 7.0) and 0.1 ml of 30 mM H_2O_2 in phosphate buffer. Catalase enzyme activity has been expressed as moles of H_2O_2 reduced/min/mg of protein.

Superoxide dismutase

Superoxide dismutase was assayed utilizing the technique of Marklund and Marklund [13], which involves inhibition of pyrogallol autoxidation at pH 8.0. A single unit of enzyme was defined as the quantity of superoxide dismutase required to produce 50 % inhibition of autoxidation.

Lipid peroxidation

Lipid peroxidation (LP) in the microsomes was estimated spectrophotometrically by thiobarbituric acid reactive substances (TBARS) method, as described by Varshney and Kale [14], and is expressed in terms of malondialdehyde (MDA) formed/mg of protein. In brief, 0.4 ml of microsomal sample was mixed with 1.6 ml of 0.15 M Tris-KCl buffer to which 0.5 ml of 30 % TCA was added. Then 0.5 ml of 52 mM TBA was added and placed in a water bath for 45 min at 80°C , cooled in ice and centrifuged at room temperature for 10 min at 3000 rpm. The absorbance of the clear supernatant was measured against blank of distilled water at 538.1 nm in spectrophotometer (Hitachi U-2000).

Lactate dehydrogenase

Lactate dehydrogenase (LDH) was assayed by measuring the rate of oxidation of NADH at 340 nm, according to the method of Bergmeyer and Bernt [15]. The reaction mixture contained 50 mM potassium phosphate buffer (pH 7.5), 0.5 mM sodium

pyruvate, 0.1 mM NADH and the required amount of cytosolic fraction in a final volume of 1 ml. The reaction was started at 25 °C by addition of NADH and the rate of oxidation of NADH was measured at 340 nm. The enzyme activity was calculated using an extinction coefficient of 6.22 mM⁻¹cm⁻¹. One unit of enzyme activity is defined as that which causes the oxidation of one mole of NADH/min.

Protein determination

Protein was determined following the method of Lowry *et al.* [16] using bovine serum albumin (BSA) as a standard at 660 nm.

Experiment II

Experiment II studied the effect of BPME on DMBA-induced skin papillomagenesis:

Preparation of chemicals and modulator

DMBA was dissolved in acetone at a concentration of 0.05 mg in 0.01 ml acetone. 200 and 400 mg/kg body weight of BPM extract was prepared in the control vehicle.

Experimental design

The DMBA-induced skin papillomagenesis was studied in nu/nu mice as described by Elegbede *et al.* [17] with some modifications. The hairs on the dorsum (2 cm diameter) of the mice were clipped off 3 days before the application of the chemicals, and mice in the resting phase of hair growth cycle were selected for the experiment. The mice were assorted into the following groups:

Group I (n = 6): Mice were treated for 21 days through oral gavage route with the emulsion of peanut oil-double distilled water (4:1), used as vehicle for feeding the modulator. On the 14th day, a single dose of DMBA (0.05 mg/0.1 ml acetone) was applied on the shaven area. Two weeks after the carcinogen application, 0.1 ml of 1% croton oil in acetone was applied twice a week until termination of the experiment. This group of mice served as positive control group.

Group II (n = 6): All mice within this group were treated through oral gavage route with

BPME suspended in the control vehicle at dose level of 200 mg/kg of body weight for 21 days. On the 14th day DMBA was topically applied to these mice in the shaven area (after a gap of minimum 6 h after treatment with the modulator) followed by croton oil treatment as given to Group I mice.

Group III (n = 6): All mice within this group were treated through oral gavage route with BPME suspended in the control vehicle at dose level of 400 mg/kg of body weight for 21 days. On the 14th day DMBA was topically applied to these mice in the shaven area (after a gap of minimum 6 h after treatment with the modulator) followed by croton oil treatment as given to Group I mice.

Mice were weighed initially, then weekly and finally at autopsy. Papillomas appearing in the shaven area were recorded at weekly intervals and papillomas >1 mm in diameter were included in data analysis only if they persisted for 2 weeks or more. Mice were sacrificed 20 weeks after commencement of the treatments.

Experiment III

Effect of BPM extract on TPA promoted mouse fore-stomach papillomagenesis was studied as described by Kundoor *et al.* [18].

Preparation of chemicals and modulator

Initiation of skin carcinogenesis was accomplished by a single application of DMBA (200 nmol /100 µl acetone) on the back of nu/nu mice. For promotion, TPA (5 nmol/100 µl acetone) was applied twice a week from 1 week after the initiation. Concentrations of 200 and 400 mg/kg body weight of both extracts were prepared in the control vehicle.

Experimental design

The mice were assorted into the following groups:

Group I (n = 6): After one week mice were treated with topical administration of acetone, used as vehicle modulator. Two weeks after initiation of vehicle treatment, each mouse received 5 nmol of TPA twice per week for 20

weeks. This group of mice served as positive control group.

Group II (n = 6): Mice were treated with 200 mg/kg body weight of BPM extract starting 2 weeks before, during and after the carcinogen treatment (5 nmol of TPA twice per week for 20 weeks) as given to Group I mice.

Group III (n = 6): Mice were treated with 400 mg/kg body weight of BPM extract starting 2 weeks before, during and after the carcinogen treatment as given to Group I mice.

Statistical Analysis

After calculating the mean and standard deviation, the Mann-Whitney Rank Sum test was performed to obtain the significant difference among groups. A value of $P < 0.05$ was considered as significant.

RESULTS

Hepatic studies: Body and organ weight and general observations

Body weight and relative liver weight at the termination of experiment have been summarized in Table 1. There was no significant difference in either the mean body weight gain profile or terminal in mice treated with the two different doses of the BPM extracts was compared to control. Further, there was no alteration in the liver-somatic index, in the microsomal and cytosolic protein values between the control and modulator treated mice. The oral administration of the *Bidens pilosa* methanolic extract did not cause any apparent clinical signs such as survivability or any gross visible changes attributable to toxicity in the liver, lung, and kidney of mice.

Lactate dehydrogenase

The activity of LDH was diminished by 0.76 folds ($P < 0.001$) following in lower dose of the BPM extract and at higher dose the activity was further diminished to 0.86 ($P < 0.005$) folds, as compared to that of control group.

Lipid peroxidation

Lipid peroxidation (LP), measured as formation of MDA, was inhibited at lower dose of BPM extract to 0.8 folds, as compared to the control group and at higher dose showed significant inhibition at both dose levels with of the BPM extract treatment to 0.73 fold, as compared to that of control group.

Phase I enzymes

The major phase I enzymes, cytochrome P450 and cytochrome b5 work as an important cofactor component of mono-oxygenase enzymatic activity. Cytochrome P450 revealed no significant alterations from its basal constitutive activities at low doses (Table 2). Whereas at higher doses cytochrome P450 was reduced significantly, by 0.85 fold ($P < 0.05$) of the BPM extract at 400 mg/kg body weight.

Cytochrome b5, there is a less significant was observed at low dose of BPM extract by 0.87 folds ($P < 0.005$) and at the higher dose of the BPM extract have shown significant reduction of 0.73 fold ($P < 0.005$) in the cytochrome b5 activity.

Phase II enzymes

Specific activities of both phase II enzymes studied, glutathione S-transferase (GST) and DT-diaphorase (DTD), showed significant increases at all the dose levels of treatment with BPM extract, with respect to the control group.

Glutathione S-transferase

Relative to the level in untreated control mice, glutathione S-transferase enzyme activity was enhanced by 1.34 fold ($P < 0.005$) at low dose of BPM. 1.61 fold ($P < 0.005$) in higher dose of BPM extract, when compared to that of control group.

DT-diaphorase

Specific DTD activity was increased by 1.14 fold ($P < 0.05$) at low dose of BPM extract and 1.47 fold ($P < 0.001$) in higher dose of the BPM extract, as compared to that of control group.

Table 1: Modulatory influence of the methanolic extract of *Bidens pilosa* on weight, protein levels and toxicity related parameters of mice

Groups and treatment	Body weight (g)		Liver weight × 100/final body weight	LDH ^a	Protein (mg/ml)	
	Initial	Final			Microsome	Cytosol
Control	31.31 ± 1.28 (100)	34.12 ± 0.92 (100)	6.61 ± 0.51 (100)	2.06 ± 0.16 (100)	6.93 ± 1.47 (100)	9.94 ± 1.99 (100)
BPME 200 mg/kg	36.18 ± 3.22 (115.43)	39.05 ± 2.35 (114.45)	6.95 ± 0.87 (105.14)	1.57 ± 0.16 ^a (76.21)	7.51 ± 1.87 (108.37)	10.12 ± 1.51 (101.30)
BPME 400mg/kg	30.09 ± 1.85 (95.85)	37.21 ± 1.88 (109.06)	5.08 ± 0.65 ^a (76.85)	1.79 ± 0.17 ^b (86.89)	10.26 ± 2.36 ^a (148.05)	11.60 ± 1.69 (116.12)

Values are expressed as mean ± sd, n=6. a: mole/mg of protein, b: significant changes against vehicle treated negative control (P<0.005), a: significant changes against vehicle treated negative control (P<0.001), BPME: *Bidens pilosa* methanolic extract, LDH: Lactate dehydrogenase

Table 2. Modulatory influence of the methanolic extract of *B. pilosa* on mice hepatic phase I and II drug-metabolizing enzyme levels

Groups	Cyt P450 ^[1]	Cyt b5 ^[1]	GST ^[2]	DTD ^[3]
Control	0.463±0.035 (100)	0.287±0.021 (100)	2.50±0.35 (100)	0.021±0.001 (100)
BPME 200 mg/kg	0.361±0.016 ^c (77.97)	0.259±0.018 (90.24)	3.37±0.46 ^c (134.80)	0.023±0.002 (109.52)
BPME 400 mg/kg	0.397±0.038 ^a (85.75)	0.212±0.025 ^c (73.87)	4.04±0.58 ^c (161.60)	0.031±0.0017 ^d (147.62)

Values are expressed as mean ± SD, n=6. [1]: nmole/mg protein, [2]: μmole CDNB-GSH conjugate formed/min/mg protein, [3]: μmole of DCPIP reduced/min/mg protein, a: significant changes against control (P < 0.05), c: significant changes against control (P < 0.005), d: significant changes against control (P < 0.001), Cyt P450: Cytochrome P450, Cyt b5: Cytochrome b5, BPME: *Bidens pilosa* methanolic extract, DTD: DT-diaphorase, GST: glutathione

Antioxidant parameters

Superoxide dismutase

The hepatic superoxide dismutase activity in extract treated mice was increased by 1.10 fold (P < 0.05) at low dose for the extract of BPM and 1.12 fold (P < 0.05) of increase at higher dose of the extract of BPM, when compared to control group (Table 3).

Glutathione peroxidase

At higher dose of BPM extract was investigated, augmented the specific activity of glutathione peroxidase enzyme significantly, relative to control basal levels. On treatment with lower dose, an induction of 1.2-folds (P < 0.005) of the extract of BPM. At high dose treatment, an induction of 1.4 fold (P < 0.005) was evident in higher dose of the extract of BPM, when compared to control group.

Catalase

The catalase activity was increased in low and high doses of the extract of BPM. 1.43-folds ($P < 0.005$) at low dose and 1.63-folds ($P < 0.005$) at higher dose of BPM extract was observed relatively compared to control group.

Glutathione reductase

Both the doses of BPM extract was investigated for the glutathione reductase. Increased glutathione reductase activity was observed in the liver cytosol of mice over that of the control group by 1.17 fold ($P < 0.005$) at lower dose of the BPM extract. Whereas at high dose 1.29 fold ($P < 0.005$) of significant activity was evident for the BPM extract, relatively compared to that of control group.

Reduced glutathione

The level of GSH, the non-enzymatic antioxidant protein, was enhanced in the BPM extract treated groups. At low dose of the extract, the basal reduced glutathione was increased by 2.06 fold ($P < 0.005$), whereas at the high dose induced 1.99 fold ($P < 0.005$) increase in reduced glutathione concentration.

Protein

Results of the analysis of cytosolic protein from different experimental groups showed no significant statistical difference with BMP extract treated groups. There was, however, a significant increase caused at high dose of BPME treated group (1.57 fold, $P < 0.005$) in the microsomal protein value.

Extrahepatic studies

Reduced glutathione

Pre-treatment with entire plant extract of BPM caused a significant in the reduced glutathione level in all the extrahepatic organs examined. In the forestomach, the activity of GSH was increased by 1.26 fold at low dose of the BPM extract. 1.97 fold ($P < 0.001$) increase was noticed in higher dose of the BPM extract, when compared to control group (Table 4). In kidney there was no significant increase at the

low dose level but a significant increase of 1.39 folds ($P < 0.001$) was revealed at the high dose of treatment relative to control group. In the lung GSH activity was augmented by 1.75 folds ($P < 0.01$) by the low dose. A 1.72 fold ($P < 0.005$) was observed in higher dose for the BPME, when compared to that of control group.

DT-diaphorase

Relative to control mice the specific enzyme activity was maximally induced in the fore-stomach being 1.65 folds ($P < 0.005$) at low dose of the BPM extract and 1.60 folds ($P < 0.001$) at high dose for the extract of BPM, when compared to control group. In kidneys of the treated mice dose dependent increases of 1.79-folds ($P < 0.005$) at low dose of the BPM extract. 1.72 fold ($P < 0.005$) was evident in higher dose of the BPM extract, as compared to that of control group. In the lung an induction of 1.23 fold ($P < 0.005$) at low dose for the extract of BPM. 1.28 fold ($P < 0.005$) above basal activity was noted in high dose for the extract of BPM, as compared to that of control group

Glutathione S-transferase

The specific activity of glutathione S-transferase as determined in the extrahepatic supernatant of the experimental mice revealed a significant increase above the basal level of activity in the respective organs investigated. In the fore-stomach GST activity was increased by 1.47-folds ($P < 0.005$) at low dose for the extract of BPM. 1.65-fold ($P < 0.005$) was noticed in higher dose for the extract of BPM, as compared to that of control group. In kidneys of the treated mice had the same increase of 1.44 fold ($P < 0.005$) was observed at low dose for the extract of BPM. 1.44-fold ($P < 0.005$) was evident in high dose for the extract of BPM, as compared to that of control group. In lung, a dose dependent increase of GST activity was noticed; being 1.43 ($P < 0.005$) was evident at low dose of BPM extract. 1.41 folds ($P < 0.005$) have shown in high dose of BPM extract, when compared to that of control group

Table 3: Modulatory influence of two different doses of methanolic extract of *B. pilosa* on mice hepatic antioxidant related parameters and lipid peroxidation

Group	GSH ^[1]	SOD ^[2]	CAT ^[3]	GPx ^[4]	GR ^[4]	LP ^[5]
Control	2.15 ± 0.51 (100)	4.84 ± 0.44 (100)	54.35 ± 9.36 (100)	17.07 ± 1.13 (100)	29.91 ± 2.30 (100)	1.02 ± 0.072 (100)
BPME 200mg/kg	4.45 ± 0.77 ^c (206.98)	5.34 ± 0.34 ^a (110.33)	86.29 ± 6.56 ^c (158.77)	21.21 ± 1.48 ^c (124.25)	35.02 ± 1.43 ^c (117.08)	0.813 ± 0.11 ^b (79.70)
BPME 400mg/kg	4.28 ± 0.85 ^c (199.07)	5.46 ± 0.45 ^a (112.81)	88.01 ± 6.59 ^c (161.93)	24.30 ± 1.76 ^c (142.35)	38.77 ± 1.63 ^c (129.62)	0.747 ± 0.041 ^c (73.24)

Values are expressed as mean ± s.d. of six mice. [1]: nmole GSH/g of tissue, [2]: specific activity expressed as mole/mg of protein, [3]: μmole H₂O₂ consumed/min/mg of protein, [4]: nmole of NADPH consumed/min/mg of protein, [5]: nmole malondialdehyde formed/mg of protein., a: significant changes against control (P < 0.05); b: significant changes against control (P < 0.001), c: significant changes against control (P < 0.005), CAT: catalase, GSH: reduced glutathione, GPx: glutathione peroxidase, GR: glutathione reductase, LP: lipid peroxidation,

Mouse skin papillomagenesis experiment

Tables 5 and Fig. 1 depict results of skin papillomagenesis obtained from treatment of BP entire plant during peri-initiation period. No adverse effect on body weight gain, during the observation period was noticeable. Moreover no evidence of development of spontaneous tumor including skin lesions in the colony of nu/nu mice has been encountered. In the DMBA-induced skin papillomagenesis study the mean number of papillomas per animal (tumor burden) in control mice was 5.46 ± 3.98, whereas it was 3.87 ± (P < 0.005) and 1.98 ± 1.37 (P < 0.001) in lower and higher doses of the extract of BPM respectively, as compared to that of control group. The tumor incidence was only by 80.25% and 76% at lower and higher doses of the extract of BPM respectively, treated groups against the 100% tumor incidence of the control group.

Mouse forestomach papillomagenesis experiment

Table 6 shows the result obtained from BPM extract supplementation on TPA induced forestomach papillomagenesis. No difference was noticeable in the weight gain profile of mice treated with both the doses of BPM extracts or in the positive control group of mice. 93% of the control mice developed

forestomach papilloma with TPA treatment, only 77.25% (22.75% reduction) at low dose

of the extract of BPM and 61.50% (39.5% reduction) of mice developed stomach tumors at higher dose of the extract of BPM, when compared to that of control. The mean number of papilloma/mouse in this group of mice was 4.065 ± 2.56. In contrast, among mice pre-treated and post-treated with 200 mg/body weight of BPM extract, the mean number decreased to 1.223 ± 1.32 (P < 0.005) and at 400 mg/body weight of BPM extract, the mean number decreased to 1.023 ± 1.21 (P < 0.001).

DISCUSSION

Results obtained from the present study have shown that on administration of entire plant extract to mice affected liver enzyme activities, as well as lipid peroxidation and lactate dehydrogenase related changes, which correlated with attenuating the risk of chemical carcinogenesis.

Cytochrome P450 (CYP) isoenzymes, are necessary to begin the conversion of metabolize lipophilic carcinogens compounds to more water soluble metabolites, which are then acted upon by phase II enzymes to promote their polarity and assisting in their excretion [19]. Similarly, the production of phase I enzymes is measured as a cancer risk factor because of starting carcinogens to ultimate carcinogens [20]. BPM extract pretreatment was significant in reducing the basal level activity of hepatic phase I enzymes, comparatively with the control group.

Table 4: Modulatory influence of both the doses of methanolic extract of *B. pilosa* on detoxifying and antioxidant enzyme profiles in extrahepatic organs of mouse.

Groups and Treatment	Organ wt. X 100/ Final body wt.	GSH ^[1]	GST ^[4]	DTD ^[5]	Protein (mg/ml)
Lung					
Control vehicle	0.94 ± 0.29 (100)	1.04 ± 0.14 (100)	0.55 ± 0.022 (100)	0.021 ± 0.0004 (100)	5.16 ± 0.80 (100)
BPME (200 mg/kg body wt.)	0.85 ± 0.14 (90.43)	1.83 ± 0.23b (175.96)	0.79 ± 0.094c (143.64)	0.026 ± 0.0012c (123.81)	5.12 ± 0.71 (99.22)
BPME (400 mg/kg body wt.)	0.87 ± 0.18 (92.55)	1.78 ± 0.27c (172.12)	0.78 ± 0.06c (141.82)	0.027 ± 0.0016c (128.57)	5.10 ± 0.71 (98.88)
Kidney					
Control vehicle	1.92 ± 0.38 (100)	1.09 ± 0.18 (100)	0.46 ± 0.085 (100)	0.029 ± 0.0024 (100)	6.21 ± 1.24 (100)
BPME (200 mg/kg body wt.)	1.91 ± 0.28 (99.48)	1.23 ± 0.14c (112.84)	0.82 ± 0.079c (146.43)	0.042 ± 0.0051c (144.61)	5.69 ± 1.36 (91.63)
BPME (400 mg/kg body wt.)	1.99 ± 0.22 (103.64)	1.58 ± 0.25c (139.45)	0.81 ± 0.10b (146.43)	0.042 ± 0.0027c (144.41)	5.41 ± 0.56 (87.12)
Fore-stomach					
Control vehicle	0.29 ± 0.043 (100)	0.67 ± 0.14 (100)	0.38 ± 0.024 (100)	0.041 ± 0.0016 (100)	4.92 ± 0.65 (100)
BPME (200 mg/kg body wt.)	0.28 ± 0.058 (96.55)	0.85 ± 0.162 (126.87)	0.56 ± 0.034c (147.37)	0.068 ± 0.004c (165.85)	3.98 ± 0.95 (78.86)
BPME (400 mg/kg body wt.)	0.27 ± 0.031 (93.10)	0.88 ± 0.11a (131.34)	0.63 ± 0.055c (165.79)	0.063 ± 0.0045d (160.98)	4.11 ± 1.02 (83.54)

Values are expressed as mean ± SD; n=6, a: significant changes against control (P < 0.05), b: significant changes against control (P < 0.01), c: significant changes against control (P < 0.005), d: significant changes as against control (P < 0.001), [1] nmole GSH/g tissue, [4] μmole CDNB-GSH conjugate formed/min/mg protein, [5] μmole of DCPIP reduced/min/mg protein, wt: weight

Phase II detoxifying enzymes that detoxify carcinogens either by destroying their reactive centers or by conjugating them with endogenous ligands, facilitating their excretion. Glutathione S-transferase and DT-diaphorase are two major phase II enzymes. GSTs are a family of enzymes that catalyze the conjugation of reactive chemicals with GSH and play a major role in protecting cells [21]. Glutathione S-transferase predominantly participates in detoxification of xenobiotics,

and the ability to induce glutathione S-transferase is a property found in many of the chemopreventive agents ameliorating toxicity and carcinogenicity [22]. GST catalyses the conjugation of a variety of endogenous and exogenous compounds with the non-protein thiol, glutathione. This reaction inhibits reactive electrophiles from reaching cellular targets and results in the production of a thio-ether linked glutathionyl conjugate that is less cytotoxic. DT-diaphorase is a flavoprotein that

Table 5: Effect of two different doses of the methanolic extract of *B. pilosa* on DMBA-induced skin papillomagenesis in mice.

Groups and treatment	Body weight (mg)		Total tumor incidence (%)	Tumor Burden (tumor/mouse)
	Initial	Final		
Only vehicle + DMBA + Croton oil.	29.32 ± 1.11	31.14 ± 1.64	94.15	5.46 ± 3.98
BPME (200 mg/kg body wt) + DMBA + Croton oil.	25.97 ± 1.22	32.36 ± 1.16	80.25	3.87 ± 2.0b
BPME (400 mg/kg body wt) + DMBA + Croton oil.	25.20 ± 1.38	33.16 ± 1.65	76.00	1.98 ± 1.37 d

Values are expressed as mean ± SD; n=6, a: significant changes against control (P < 0.005), b: significant changes against control (P < 0.01), d: significant changes against control (P < 0.001)

Table 6: Effect of two different doses of the plant of *B. pilosa* on TPA induced forestomach papillomagenesis in mice.

Groups and treatment	Body weight (mg)		Total tumor Incidence (%)	Tumor Burden (tumor/mouse)	% inhibition of tumor burden
	Initial	Final			
TPA + (only vehicle)	22.13 ± 1.23	20.58 ± 3.06	93	4.065 ± 2.56	--
TPA + BPME (200 mg/kg body wt.)	23.06 ± 1.37	17.81 ± 1.79	77.25	1.223 ± 1.32c	69.91
TPA + BPME (400 mg/kg body wt.)	27.66 ± 1.30	17.01 ± 3.0	61.50	1.023 ± 1.21d	74.83

Values are expressed as mean ± SD; n=6,c : significant changes against control (P < 0.005), d: significant changes against control (P < 0.001).

catalyzes the two electron reduction of quinones and other nitrogen oxides. This reaction prevents one-electron redox cycling of these groups, thereby preventing the formation of DNA-damaging reactive oxygen species. BPM extract treatment increased the activities of both of these phase II enzymes in the liver and in all the extrahepatic organs examined when compared to the control group.

The antioxidant enzymes in liver including glutathione peroxidase, glutathione reductase, catalase and superoxide dismutase, were found to be elevated above the control basal values. Superoxide dismutase (SOD) plays an important role in the antioxidant enzyme defense system. SODs convert superoxide radicals into hydrogen peroxide [3]. Because the activity of SOD was induced in mice by

the entire plant extract of BP treatment, the generation of reactive oxygen species is inhibited and the dismutation of super oxide radicals may well be accelerated by the catalyzing role of SOD. Catalase, the activity of which has also been augmented by BPME treatment, helps in removing the hydrogen peroxide produced by the action of SOD. Indeed, SOD activity, along with that of catalase, explains the significant decrease in lipid peroxidation, which is an indicator of oxidative stress that persists in the cell.

Glutathione reductase (GR) is another major antioxidant enzyme that catalyzes the NADPH-dependent reduction of glutathione disulfide to glutathione, thus maintaining GSH levels in the cell [23]. Significant elevation of the activity of GR following BPME treatment was evident, thereby helping the cell to

maintain the basal level of GSH, which is important for many other GSH-dependent detoxification reactions. Reduced glutathione (GSH) participates in spontaneous scavenging of electrophiles or free radicals and in reactions catalyzed by enzymes like GPX and GST [24, 25]. GSH activity was increased above basal level in the liver, as well as in all the extrahepatic organs investigated. The activity of lactate dehydrogenase, which is an indicator of cell damage, has decreased significantly at both the dose levels used for the present experiment, implying a cytoprotective effect exerted by entire plant extract.

Reference to antioxidant enzyme status in liver, the specific activity of almost all the antioxidant related enzymes including glutathione peroxidase, glutathione reductase, catalase, and superoxide dismutase were found elevated above the control basal values either in dose-dependant or dose-independent manner. Superoxide dismutase plays an important role in catalyzing the dismutation of superoxide radicals increased SOD activity should accelerate the removal of the reactive oxygen species. Catalase, whose activity has also been augmented by the BPM extract, helps in removing the hydrogen peroxide produced by the action of SOD, when

compared with the control group. Induced SOD activity along with that of catalase explains the decrease in lipid peroxidation, which is an indicator of oxidative stress that persists in the cell. Glutathione reductase, another major antioxidant enzyme catalyzes the NADPH-dependent reduction of glutathione disulfide to glutathione thus maintaining the GSH level in the cell [24]. BPM extract treatment has significantly increased the activity of GR, when compared to that of control group, thereby helping the cell to maintain the basal level of GSH, which is important for many other GSH-dependent detoxification reactions [26].

The activity of lactate dehydrogenase, which is an indicator of cell damage, decreased significantly at the higher dose levels of BPM extract implying the cytoprotective effect exerted by *B. pilosa* entire plant. Apart from the numerous functions like transport of ions/sugars, synthesis of protein and DNA, maintenance of membrane integrity, reduced GSH participates in spontaneous scavenging of electrophiles or free radicals produced in reactions catalyzed by GPX and GST [27]. GSH activity was increased in above basal level in the liver as well as all the extrahepatic organs investigated. Thus, the induction of GSH by BPM extract at higher dose facilitates

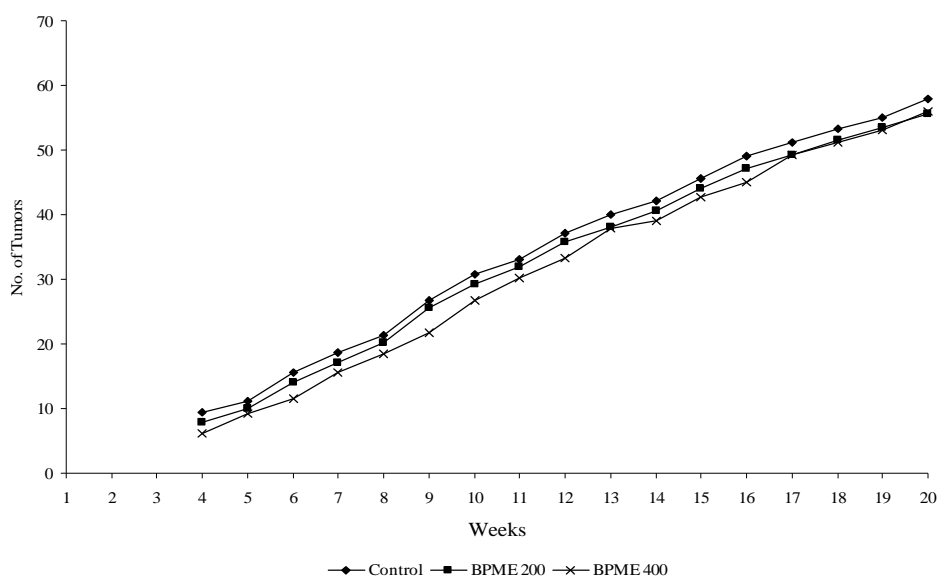


Figure 1: Effect of two different doses of BPME on DMBA-induced skin papillomagenesis.

the protection of the cell against free radical-induced damage.

Numerous reports strongly support the correlation between the induction of phase I, phase II, and antioxidant enzymes and cancer incidence [3, 22]. In the present work, administration of at the higher dose of BPM extract have shown a significant induction of mainly phase II enzymes and antioxidative parameters, along with a significant decrease in the specific activity of LDH and the level of lipid peroxidation. These effects when considered together presumably resulted in enhanced carcinogen detoxification by both the doses of plant extract and its blocking/suppressing effect on "initiation" stages of carcinogenicity.

Various approaches to cancer chemoprevention exist. For example, the inducer of GSTs has received much attention as a potential chemopreventive agent, because the ability to induce GST is a property found in many chemopreventive agents, ameliorating toxicity and carcinogenicity [22]. Moreover many naturally-occurring chemopreventive agents have been reported to convert DNA-damaging entities into excretable metabolites through induction of GST. Another approach/mechanism of chemoprevention is the elevation of antioxidant defense that can combat the oxidative stress produced by reactive oxygen species (ROS), which often leads to mutation and cancer.

The present investigation has demonstrated that at high dose of (400 mg/kg of body weight) BPM extract can be used as a cancer chemopreventive agent by virtue of its efficacy in inducing drug detoxification enzymes such as GST and DTD, as well as in blocking carcinogen-activating phase I enzymes [19]. It also protects against oxidative stress through the elevation of antioxidative defense enzyme, while significantly reducing the specific activity of LDH and the level of lipid peroxidation. All these effects considered together might result in significant reduction of DMBA-induced and TPA promoted papillomagenesis in nu/nu mice at the peri-initiation period. Since the plant has shown no toxic effect at the tested doses, it could well be applied in cancer chemoprevention, to reduce the risk of cancer.

CONCLUSION

The present study evaluated the carcinogen-induced stomach and skin cancer chemoprevention potentials of *B. pilosa*. The results obtained suggest that *B. pilosa* can significantly inhibit the chemical carcinogenesis at peri-initiation stages of carcinogenesis. In the case of modulation of phase II detoxification enzymes, higher doses of *B. pilosa* methanolic extract elevated the antioxidant enzymes level and by inhibiting lipid peroxidation and lactate dehydrogenase induced damages when compared to that of its lower dose and control groups.

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