

Research Article



Chemopreventive effect of *Tricholoma giganteum* against benzo[a]pyrene-induced forestomach cancer in Swiss albino mice

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ABSTRACT

A convenient animal model of forestomach cancer is compelling for development of chemopreventive strategies. The present study was an effort to identify the chemopreventive role of *Tricholoma giganteum* Fa fraction (80% ethanolic extract) against benzo[a]pyrene-induced forestomach cancer in Swiss albino mice. B[a]P-induced large numbers of tumors at forestomach and also caused abnormal alterations in the activities of superoxide dismutase, catalase, glutathione S-transferase, reduced glutathione and lactate dehydrogenase. Fa fraction administration effectively suppressed forestomach cancer as revealed by the decrease in lipid peroxidation and all the above alterations were observed to return towards normal. The preventive effect could be occurring possibly by regulating expression of some critical genes associated with cell cycle and apoptosis such as p53, p21, Bcl-2, Bcl-xl, Bax and cyclinD1. The treatment profoundly reverted back the histopathological changes observed in cancerous animals and thus further supports our findings. Our present data suggests that the Fa fraction of *T. giganteum* might extend its chemopreventive potential by modulating lipid peroxidation, augmenting antioxidant defense system, inhibiting cell proliferation and inducing apoptosis.

Keywords: Benzo[a]pyrene, Chemoprevention, Forestomach, Mushrooms, *Tricholoma giganteum*.

INTRODUCTION

Benzo[a]pyrene (B[a]P), a prototypical polycyclic aromatic hydrocarbon is a widespread environmental pollutant and thought to be one of the etiological factors in human chemical carcinogenesis. B[a]P-induced carcinogenesis of the forestomach in female Swiss albino mice has been used extensively as an experimental model to identify potential chemoprotectors¹. Over time the murine model system has become one of the ideal systems to work with in the field of cancer biology. This system has helped in increasing our understanding of various steps in the process of carcinogenesis and its prevention. Forestomach papillomagenesis is initiated by benzo[a]pyrene and involves an endogenous promoter for tumor growth and progression². It is associated with alterations in oxidant-antioxidant status, increased cell proliferation, angiogenesis and dysregulation of apoptosis³. Even though the mechanism by which chemoprotectors attenuate the carcinogenic effects of B[a]P is poorly understood, there is sufficient evidence to suggest that their effect may, at least in part, be due to induction of the antioxidant enzymes, apoptosis and inhibition of cell proliferation⁴. Stomach cancers are generally poorly responsive to chemotherapy and radiotherapy⁵, suggesting chemoprevention to be a practical strategy. Chemoprevention by dietary constituents has emerged as a novel approach to control stomach cancer incidence. Dietary mushrooms have been used globally for millennia to promote health and prevent and treat disease primarily via their multitude of medicinal qualities. Attempts have been made in many

parts of the world to explore the use of mushrooms and their metabolites for the treatment of a variety of human ailments⁶⁻⁸. A number of wild edible mushrooms of West Bengal have been extensively studied and found promising for the treatment of diseases like cancer⁹, microbial diseases¹⁰⁻¹³, cardiovascular diseases¹⁴, ulcer¹⁵, diabetes¹⁶, hepatic injury¹⁷ etc. *Tricholoma giganteum* of the family Tricholomataceae, a wild edible mushroom is most conspicuous in the tropical region during rainy season. They are robust in size and popular among the people of these areas because of their being a gastronomic and nutritional delicacy. Our earlier investigation showed that Fa fraction from *T. giganteum* possessed potent *in vitro* antioxidant property¹⁸ and offers hepatoprotection¹⁹. Recently we demonstrated that this fraction could even induce the expression of p53, p21 and modify Bax/Bcl-2 ratio suggesting apoptosis and blocking of cell cycle progression in Ehrlich's ascites carcinogenesis model²⁰.

The extent of lipid peroxidation, the status of the antioxidants superoxide dismutase (SOD), catalase (CAT), reduced glutathione (GSH), glutathione S-transferase (GST), and profile of the marker enzyme, lactate dehydrogenase (LDH) were used to biomonitor chemoprevention against neoplastic development²¹. An increasing body of evidence indicates that oxidative stress is an important element of mutagenesis, which is a basis for carcinogenesis. Hydroxyl radical produced as a result of oxidative stress initiates a chain of reactions that leads to the process of lipid peroxidation and generates in turn the highly mutagenic singlet oxygen. GSH is known to be an effective quencher of this singlet oxygen²². GSH is one



of the major cellular defenses against ROS, generated endogenously, or the electrophilic metabolites of carcinogen biotransformation. The electrophilic functional groups are conjugated with glutathione through GST²³. The other major cellular defense against oxidative stress is the SOD-CAT system and is closely related to the modulation of carcinogenesis. SOD dismutates pairs of superoxide anions by oxidizing one to oxygen and reducing the other to hydrogen peroxide. Hydrogen peroxide, which is also considered to be mutagenic, is degraded to H₂O and oxygen by CAT. LDH, a cytoplasmic marker enzyme, is well known as an indicator of cell damage induced by several factors, including xenobiotic compounds²⁴.

Tumors can arise from alterations in the activity of genes that result in accelerated rates of cell division, decreased rate of cell death or both. In this context, it is noteworthy that apoptosis-inducing ability seems to have become a primary factor in considering the efficacy of anti-tumor agents. Emphasis has been laid on the mechanisms that regulate apoptosis pathways. p53, the tumor suppressor protein, suppresses tumor growth through two mechanisms, cell cycle arrest and apoptosis²⁵. p53-mediated growth arrest involves p21 as a major effector and thus up-regulation of p21 results in blocking of cell cycle progression²⁶. Even the members of Bcl-2 family regulate the cellular life span. Over-expression of the Bcl-2 gene prevents or delays normal cell turnover caused by programmed cell death, contributing to the clonal expansion of neoplastic cells²⁷. Bcl-2 associated protein (Bax) forms heterodimers with Bcl-2 *in vivo*. Over-expressed Bax can counter the activity of Bcl-2, and accelerate apoptotic cell death²⁸. The most abundant form of Bcl-2 protein is Bcl-xl, which is regarded as the blocker of cell death⁵. Overexpression of cyclin D1 shortens the G1 phase and occurs in many types of human cancer, whereas inhibition of cyclin D1 expression blocks G1-S transition²⁹.

Here, an attempt had been made to develop murine model systems of forestomach tumors by oral administration of B[a]P and evaluate chemopreventive effects of Fa fraction of *T. giganteum* with reference to lipid peroxidation and antioxidant defense status. In addition, differences in the expression of cell cycle regulation and apoptosis associated genes using RT-PCR analysis were analyzed and, further to determine the clinical status, histopathological parameters were assessed.

MATERIALS AND METHODS

Chemicals

Benzo[a]pyrene (B[a]P), bovine serum albumin (BSA), 1-chloro-2,4-dinitrobenzene (CDNB), dithiobis-2-nitrobenzoic acid (DTNB), reduced glutathione (GSH) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). All the other chemicals used were of analytical grade.

Extraction procedure

Powdered *T. giganteum* (100 g) was extracted with 80 % ethanol at room temperature overnight and was repeated 4 times, and then freeze-dried to obtain the Fa fraction as reported earlier¹⁸. The freeze-dried fraction Fa was reconstituted in distilled water at a concentration of 10 mg/ml. This stock solution was kept in the dark at 4 °C for further use.

Animals

Healthy female Swiss albino mice of approximately the same age (6-8 weeks old) weighing about 20 g were used for the study. They were fed with standard diet and water *ad libitum*. They were housed in polypropylene cages maintained under standard condition (12 h light/dark cycle; 25 ± 3 °C, Relative humidity 35-60 %). The animals were maintained according to the guidelines recommended by the Animal Welfare Board and approved by our institutional animal ethical committee. All procedures complied with the Declaration of Helsinki, as revised in 1996.

Acute toxicity studies

The standard conditions of the mice were maintained during the experiment. They were housed in polypropylene cages maintained under standard condition (12 h light/dark cycle; 25 ± 3 °C temperature, 35-60 % relative humidity). The Fa fraction was fed orally with increasing dose up to 3,000 mg/kg body weight.

Experimental design

Experimental animals were divided into three groups of six mice each as follows. Group I, treated with vehicle (peanut oil 5 ml/kg body weight orally), throughout the course of the experiment, served as control. Group II animals were treated with benzo[a]pyrene [(B[a]P) 50 mg/kg body weight dissolved in peanut oil] orally twice a week for four successive weeks to induce forestomach cancer by 16th week³⁰⁻³¹. Group III animals received benzo[a]pyrene (as in group II) along with fraction Fa of *T. giganteum* intraperitoneally (ip). Fa fraction treatment was started 30 days prior the first dose of B[a]P administration and continued for 16 weeks.

Dose optimization

Dosing regimen for the experimental animals was optimized on assessment of tumor burden in forestomach after treatment with Fa fraction, doses ranging from 10 to 100 mg/kg body weight, ip. At the end of the experiment (after 16 weeks), the animals were sacrificed. The forestomach was cut opened longitudinally, separated from the glandular stomach and cleaned of all its contents by flushing with and changing the buffer 5–6 times. The tumor count and gross visible cancerous lesions were determined under a dissecting microscope. Tumor volume was also determined by measuring three dimensional sizes of all tumors using the average of the three measurements to calculate radius.



Tumor volume was calculated as: $\text{Volume} = 4\pi r^3/3$

Preparation of homogenates and cytosol and microsomal fractions

At the end of the experimental period (16th week), animals which were treated with the standardized optimum dose of Fa were killed by cervical decapitation under ether anaesthesia. The entire liver was then perfused immediately with cold 0.9 % NaCl, 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, frozen in liquid nitrogen 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 for assaying 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 $105,000 \times g$ for 60 min in an ultracentrifuge. The supernatant (cytosol fraction), after discarding any floating lipid layer and appropriate dilution, was used for the assay of glutathione S-transferase, lactate dehydrogenase and antioxidant enzymes, whereas the pellet representing microsomes was suspended in homogenizing buffer and used for assaying lipid peroxidation.

Biochemical analysis

Lipid peroxidation

Lipid peroxidation (LPO) in the microsomes was estimated spectrophotometrically by the thiobarbituric acid reactive substances (TBARS) method. 0.3 ml of the homogenate was mixed with 2 ml of TCA-TBA-HCl [trichloroacetic acid (TCA) 15 % w/v, thiobarbituric acid (TBA) 0.375 % w/v, and hydrochloric acid (HCl) 0.25N], heated for 15 min in a boiling water bath, cooled; the flocculent precipitates were removed, and the absorbance was recorded at 535 nm. The extent of lipid peroxidation was calculated using molar extinction coefficient of $1.56 \times 10^5 \text{ M}^{-1}\text{cm}^{-1}$ and expressed as nmoles MDA produced per mg protein³².

Glutathione S-transferase

The glutathione S-transferase (GST) activity was determined spectrophotometrically at 37°C according to the procedure of Habig et al.³³. 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. Reaction mixture without the enzyme was used as blank. The specific activity of glutathione S-transferase is expressed as μmoles of CDNB-GSH conjugate formed per min per mg protein using an extinction coefficient of $9.6 \text{ mM}^{-1}\text{cm}^{-1}$.

Reduced glutathione

Reduced glutathione (GSH) was estimated as total non-protein sulphhydryl group by the method as described by Moron et al.³⁴. Homogenates were immediately precipitated with 0.1 ml of 25 % trichloroacetic acid and the precipitate was removed after 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 0.1 ml of the supernatant. Absorbance was read at 412 nm. GSH was used as a standard to calculate μmole GSH per g tissue.

Catalase

Catalase (CAT) was estimated at 240 nm by monitoring the disappearance of H_2O_2 as described by Aebi³⁵. 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 μmoles of H_2O_2 consumed per min per mg protein.

Superoxide dismutase

Superoxide dismutase (SOD) activity was estimated according to the method described by Kono³⁶, wherein reduction of nitroblue tetrazolium mediated by superoxide anions generated by photo oxidation of hydroxylamine hydrochloride to blue formazon was measured at 560 nm. The activity of SOD was expressed as U per mg protein. A single unit of enzyme activity is defined as the quantity of SOD required for 50 % inhibition of reaction.

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 Bernt³⁷. 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 to make 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 spectrophotometrically. The enzyme activity was calculated using an extinction coefficient of $6.22 \text{ mM}^{-1}\text{cm}^{-1}$. The activity of lactate dehydrogenase was expressed as μmoles of NADH oxidized per min per mg protein.

Protein quantification

Protein was estimated by the method of Lowry et al.³⁸ using BSA as the standard at 660 nm.

Statistical analysis

In all the cases result was the mean \pm SD (standard deviation) of at least three individual experimental data. Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by the Tukey's test. The levels of significance were evaluated with p -values.



Histopathological analysis

Tissue samples collected for the forestomach tumorigenesis study were processed conventionally using paraffin-embedded sections. Sections were cut at 4-5 μm thickness. These sections were deparaffinized in xylene and rehydrated in descending concentrations of ethanol, and stained with hematoxylin and eosin. Sections were then dehydrated in ascending concentrations of ethanol followed by xylene, mounted and viewed under a light microscope for histopathological examination for histological changes.

Table 1: Primer sequences used in RT-PCR analysis³⁹

Gene of interest	Sequences of primer
p53	FP 5' ATG ACT GCC ATG GAG GAG TCA CAG T 3' RP 5' GTG GGG GCA GCG TCT CAC GAC CTC C 3'
Bax	FP 5' AAG CTG AGC GAG TGT CTC CGG CG 3' RP 5' GCC ACA AAG ATG GTC ACT GTC TGC C 3'
Bcl-2	FP 5' CTC GTC GCT ACC GTC GTG ACT TCG 3' RP 5' CAG ATG CCG GTT CAG GTA CTC AGT C 3'
Bcl-xl	FP 5' TGG ATC CTG GAA GAG AAT CG 3' RP 5' AGA TCA CTG AAC GCT CTC CG 3'
p21	FP 5' AAT CCT GGT GAT GTC CGA CC 3' RP 5' AAA GTT CCA CCG TTC TCG G 3'
cyclin D1	FP 5' AAC ACC AGC TCC TGT GCT GCG AA 3' RP 5' GTC TCC TTC ATC TTA GAG GCC ACG 3'
β -actin	FP 5' GTG GGC CGC TCT AGG CAC CAA 3' RP 5' CTC TTT GAT GTC ACG CAC GAT TTC 3'

Analysis of gene expression by semi-quantitative RT-PCR

Removed forestomachs were stored at $-80\text{ }^{\circ}\text{C}$ until further processing. Expression of the genes was analyzed by semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR). Total cellular RNA was extracted from forestomachs with TRIzol Reagent (Invitrogen, USA). The cDNA was synthesized from the total RNA using RevertAid M-MuLV Reverse Transcriptase (Fermentas, USA) according to the manufacturer protocol. 20 μl reaction volume contained 1 μg of RNA, 0.5 μg of Oligo(dT), 20 units of RiboLock RNase Inhibitor (Fermentas, USA), 4 μl of 5x reaction buffer (250 mM Tris-HCl (pH 8.3), 250 mM KCl, 20 mM MgCl_2 , 50 mM DTT), 2 μl of 10 mM each deoxynucleoside triphosphates (dNTP Mix; Fermentas, USA) and 200 units RevertAid M-MuLV Reverse Transcriptase. The reaction was carried out at $45\text{ }^{\circ}\text{C}$ for 60 min followed by $70\text{ }^{\circ}\text{C}$ for 10 min. To analyze the expression of specific gene, 1 μl of the cDNA was taken in a 50 μl PCR mixture containing 1x DreamTaq PCR buffer, 0.2 mM of each dNTPs, 1 μM of each gene specific primer and 1.25 units DreamTaq DNA polymerase (Fermentas, USA). p53, Bax, Bcl-2, Bcl-xl, p21 and cyclin D1 genes were co-amplified with the β -actin gene as control. PCR was carried out using the gene specific upstream and downstream primers (Table 1). Initial denaturation at $95\text{ }^{\circ}\text{C}$ for 4 min was followed by 30 PCR cycles of denaturation at $94\text{ }^{\circ}\text{C}$ for 30 s, annealing at $60\text{ }^{\circ}\text{C}$ for 30 s and strand extension at $72\text{ }^{\circ}\text{C}$ for 60 s with a final extension step of 7 min at $72\text{ }^{\circ}\text{C}$ in a thermal cycler (Applied BioSystem, USA). The PCR products were electrophoresed in 2 % agarose gel, stained in ethidium

bromide, visualized in UV transilluminator and then photographed.

RESULTS

Dose dependent inhibitory effect of *T. giganteum* Fa fraction on mouse forestomach tumorigenesis

Figure 1 and Table 2 depict the dose dependent effects of Fa fraction on B[a]P-induced forestomach tumorigenesis. No significant difference was noticed in the weight gain profile of animals treated with any dose of the fraction when compared with the positive control group of mice. In the B[a]P only treated mice, forestomach displayed large number of fused and solitary papillomas and/or carcinomas. Carcinogen administration of B[a]P at a dose of 50 mg/kg body weight given orally twice a week for four weeks resulted in 38 tumors/mouse (tumor burden) after 16th week, with average tumor volume/tumor of $14.33 \pm 3.01\text{ mm}^3$. Administration of Fa fraction at a dose of 10 mg/kg body weight, ip, resulted in reduction of tumor burden and tumor volume by 34.21 % and 50.17 % respectively. When Fa fraction was administered with a dose of 25 mg/kg body weight, ip, the areas of the formation of the papillomas and/or carcinomas in the forestomach of the animals were decreased and localised with reduction in tumor burden and tumor volume by 65.79 % and 75.16 % respectively. In comparison, when treatment was carried out with a dose of 50 mg/kg body weight, ip, the percentage inhibition of tumor burden and tumor volume was 81.58 % and 91.14 % respectively. At this high dose, only some lesions of the squamous epithelial cells at the Squamo Columnar Junction (SCJ) of the forestomach were noticed. At a higher dose of 100 mg/kg body weight, ip, dose dependent response was found to be more or less same relative to the previous dose. Therefore, for further experiments the dose to be taken into account was 50 mg/kg body weight, ip.

Hepatic Studies

Biochemical parameters

The modulatory effect of *T. giganteum* Fa fraction on the enzymes involved in antioxidant function and levels of non-enzymatic antioxidant (GSH) in mouse liver is given in (Table 3). In group III, Fa fraction treatment at a dose of 50 mg/kg body weight, ip, resulted enhanced activity of SOD and CAT by 1.40 and 1.34-fold, respectively when compared with cancer bearing animals (Group II) and thus retained to near normal levels. The GSH content and level of GST was found to be induced by 1.22 and 1.42-fold respectively in Group III animals with the same dose, in comparison with tumor bearing mice (Group II). Table 3 also shows the levels of lipid peroxidation and activity of marker enzyme (LDH) estimated in liver tissues of various experimental groups. On Fa fraction treatment, notable lipid peroxidation inhibition was observed with reduction by 1.29-fold in comparison with the B[a]P only treated group (Group II). In case of LDH, the activity was reduced by 1.18-fold in Fa fraction treated set compared with the positive control set.



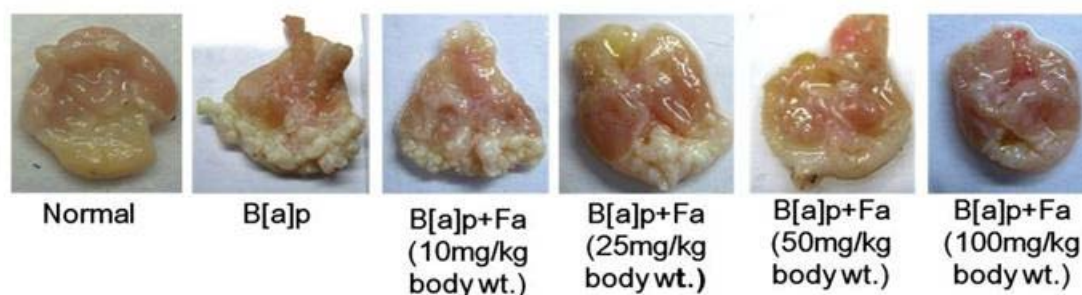


Figure 1: Effect on tumor incidence with dose related treatment of *T. giganteum* Fa fraction on B[a]P-induced forestomach tumorigenesis

Table 2: Effect of different doses of *T. giganteum* Fa fraction on tumor inhibition rate in experimental group of animals. Results are mean \pm SD of three separate experiments, each in triplicate

Groups and treatment	Tumor burden (Tumors/mouse)	Mean Tumor volume/tumor (mm ³)
Control (only vehicle)	0	0
B[a]P	38	14.33 \pm 3.01
B[a]P + Fa (10 mg/kg body wt)	25	7.14 \pm 2.68
B[a]P + Fa (25 mg/kg body wt)	13	3.56 \pm 2.18
B[a]P + Fa (50 mg/kg body wt)	7	1.27 \pm 0.59
B[a]P + Fa (100 mg/kg body wt)	6	1.07 \pm 0.58

Table 3: Effect of Fa fraction of *T. giganteum* on lipid peroxidation, antioxidant enzymes profiles and marker enzyme status in the liver of experimental mice

Parameters	Group I (Control)	Group II (B[a]p)	Group III (B[a]p + Fa)
SOD	19.37 \pm 0.54	12.22 \pm 1.5 ^a	17.13 \pm 0.12 [#]
CAT	299.40 \pm 59.21	188.61 \pm 41.34 [#]	253.44 \pm 35.86 [#]
GSH	4.64 \pm 0.27	3.19 \pm 0.17 ^a	3.90 \pm 0.13 ^b
GST	16.05 \pm 1.87	8.82 \pm 1.88 ^b	12.56 \pm 2.76 [#]
LPO	0.32 \pm 0.04	0.53 \pm 0.03 ^a	0.41 \pm 0.07 [#]
LDH	9.57 \pm 0.60	14.16 \pm 1.01 ^c	12.05 \pm 0.23 ^a

Values are mean \pm SD, n = 6 animals. Superscript letters a (p<0.01), b (p<0.05) and c (p<0.001) indicates significant changes compared with control. #: indicates not significant.

SOD: units/ mg protein; **CAT:** μ moles of H₂O₂ consumed/ min/ mg protein; **GSH:** μ mole GSH/ g tissue; **GST:** μ moles of CDNB-GSH conjugate formed/ min/ mg protein; **LPO:** nmoles MDA produced/ mg protein; **LDH:** μ moles of NADH oxidised/ min/ mg protein.

Effect of the fraction on some cell cycle progression and apoptosis associated genes

To illustrate the effect of Fa fraction on selected apoptosis and cell cycle arrest related genes, their transcript level expression were elucidated (Figure 2). Administration of Fa fraction with the optimized dose in the experimental animals (Group III) was shown to markedly increase the expression level of p53 in the forestomach tissue as compared with group II, which is suggested to cause cell cycle arrest mediated by p21, moderate elevation of which was observed as well in antitumor therapy. Bax level was up-regulated compared to the untreated tumor-bearing set, which is suggested to induce apoptosis. However, the expression status of Bcl-2, Bcl-xl remained relatively unchanged on treatment when compared with positive control. The expression of

cyclin D1 was lowered in the Fa treated set (Group III) when compared to the cancer bearing set (Group II).

Histological analysis

Figure 3 depicted the histological observation of the forestomach tumor sections of the experimental animals. Group II showed extensive infiltration as prevalent in tumors of the positive control group of mice (Group II), (Figure 3b). Conversely, histopathological changes in the treated set animals (Group III) revealed almost normal architecture in the forestomach epithelium with marginal infiltration (Figure 3c). They had the similar histopathological appearance as normal mice (Group I), treated with peanut oil (Figure 3a). This evidence supported the hypothesis that Fa fraction of *T. giganteum* could inhibit B[a]P-induced forestomach carcinogenesis in mice.

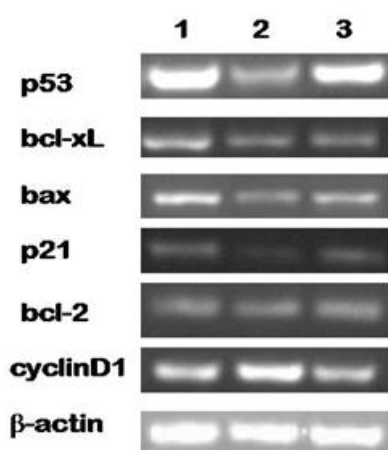


Figure 2: Semi-quantitative RT-PCR of p53, Bcl-xL, Bax, p21, Bcl-2 and cyclin D1 gene expressions as represented in Lane 1 Control; Lane 2 B[a]p treated set; Lane 3 B[a]p + Fa treated set. β-actin band represented equal loading

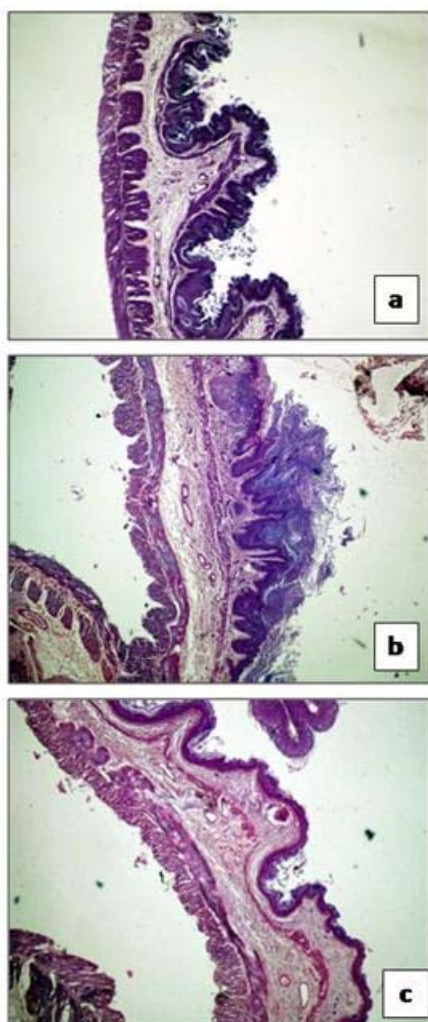


Figure 3: Histopathologic features in forestomach. **a** Group I, the control set showing normal mucosa of forestomach; **b** Group II, BaP-induced forestomach cancer bearing animals revealed extensive infiltration in forestomach carcinogenesis; **c** Group III, Fa fraction

treated set animals, the forestomach epithelium shows near normal histology

DISCUSSION

The results obtained in this present study demonstrate definite conclusions concerning the nature of the tumor inhibiting potential of Fa fraction of *T. giganteum*. In the case of B[a]P-induced forestomach papillomagenesis, tumor incidence as well as tumor burden were decreased in a dose dependent manner with Fa fraction treatment. B[a]P is metabolized to its oxidized derivatives (epoxides, phenols, diols, tetrols and quinones) by monooxygenases and epoxide hydrolases. The moderately polar metabolites are conjugated with sulfate, glucuronic acid and glutathione by cytosolic enzymes to produce their water-soluble and excretable forms. Metabolism of the carcinogens and the applications of the tumor promoters have been known to generate activated oxygen species. Experimental carcinogenesis and modulatory action on detoxifying and antioxidant enzymes helps to evaluate chemopreventive potential of natural products. We screened the potential of the fraction to induce carcinogen metabolizing enzymes and antioxidant defense mechanisms in experimental animals. Since the fraction moderately increased the activity of drug metabolizing and antioxidant enzymes, it might have facilitated the detoxification and excretion of oxidized metabolites of B[a]P. Because the SOD-CAT system is effective in detoxifying these free radicals, as well as H_2O_2 , agents that cause enhancement in the activities of the SOD-CAT system would be of great use in protection against activated oxygen species and peroxide molecules. Peroxidation, initiated in membranes by hydroxyl radical, is self-propagating and yields mutagenic ROS, including singlet oxygen and lipid hydroperoxides. Because the fraction significantly enhances components of the antioxidant defense mechanism and also the specific activity of GST, a reduction in lipid peroxidation is highly expectable. The suppression of lipid peroxidation on administration of the fraction suggests that it may have a direct effect on membranes as these may decrease susceptibility of the membranes to lipid peroxides. These results are in line with antioxidative property exerted by the fraction as already demonstrated earlier by chelating metal ions, preventing free radical generation and inhibiting lipid peroxidation¹⁸. There are several reports of natural products being potent chemopreventive agents that significantly induce these enzymes⁴⁰⁻⁴². Because antioxidants play an important role in inhibiting and scavenging radicals, thus providing protection to humans against infection and degenerative diseases, their levels in the body can also be used in expressing the status of the diseased body. Enhancement of antioxidant defense is another mechanism by which many chemopreventive agents alter cancer risk. Needless to say that induction of antioxidants correlates with the inhibition of oxidative damage produced by reactive oxygen species (ROS), which is very important to keep a balance between cell regulation and functioning. LDH formation is an indicator

of cellular damage. Repressed activity of LDH in the mice of Fa fraction treated group thus signifies its antineoplastic property.

It is known that mutations affecting regulation of cell cycle and apoptosis lead to carcinogenesis. The diminished lipid peroxidation associated with enhanced activities of GST, SOD and CAT as well as the levels of GSH might attribute protection to B[a]P-induced forestomach tumors, creating a permissive environment for apoptosis. The fraction has been shown to induce apoptosis in EAC previously, thereby suggesting a positive correlation between the antiproliferative and proapoptotic effects of *T. giganteum* Fa fraction²⁰. The genes p53 and Bcl-2 are closely related to the majority of human cancers. The p53 tumor suppressor gene plays an important role in both apoptosis and DNA repair pathways. In various tumors, p53 is an essential gene for inducing apoptosis and the level is increased in antitumor therapy. However, when p53 also mutated in response to intracellular and extracellular stress signals, e.g. chemically induced DNA damage, the level was increased. Many studies have demonstrated that treatment with BaP seemed to increase p53 mRNA by inducing p53 mutation in some models^{43,44}. p53 mutation or modification is observed in most of the human patients suffering from cancer⁴⁵. Thus chemopreventive potential could be associated with the modulation of cellular redox status, modification of cell cycle and induction of apoptotic pathways as evidenced by upregulation of p53 and p21. The tumor suppressor p53 plays critical roles in these stress-induced cellular responses through the activation of its down-stream gene p21 and results in blocking of cell cycle progression by restricting entry of cell in S phase²³. It is well-known that Bax helps the release of cytochrome c from the mitochondrial matrix to cytosol and cytochrome c release activates caspase-3, which, in turn, executes apoptosis, as a primary mechanism of apoptosis⁴⁶. An increase in Bax expression and relatively no change in Bcl-2 level, thereby increasing Bax/Bcl-2 ratio induces mitochondrial mediated apoptosis. Our findings also coincides with the observations of Kim et al.⁴⁶ who demonstrated that conjugated linoleic acid isomers attributed to the induction of apoptosis in similar experimental system mediated by mitochondrial dysfunction, upregulation of Bax gene expression and down regulation of Bcl-2 gene expression.

Further investigation of the fraction's potential to induce check in cell cycle progression is also warranted by the down regulation of cyclinD1 expression. The findings of the present investigation support this fraction to be an effective cancer preventive agent in a murine model system, therefore triggering the urgency of pre-clinical studies and pharmacokinetic profiling to determine its clinical doses for human population.

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