

Tricholoma giganteum Ameliorates Benzo[A]Pyrene – Induced Lung Cancer in Mice

Chatterjee S¹, Chatterjee A¹, Chandra S¹, Khatua S¹, Saha G K², Acharya K^{1*}

¹Molecular and Applied Mycology and Plant Pathology Laboratory, Department of Botany, University of Calcutta, 35, Ballygunge Circular Road, Kolkata, 700019, West Bengal, India.

²Entomology and Wildlife Biology Research Laboratory, Department of Zoology, University of Calcutta, Kolkata, India.

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ABSTRACT

Tricholoma giganteum is a wild edible mushroom, consumed in tropical region of the world and demonstrated to have multiple beneficial effects. We investigated the chemopreventive effect of ethanolic fraction of *T. giganteum* in mice with acute lung injury induced by Benzo[a]Pyrene. Oral administration of Benzo[a]Pyrene (1 mg/20g body weight) induced marked lung injury, evident from histological and biochemical data. Intraperitoneal administration of ethanolic fraction of *T. giganteum* (50 mg/kg body weight) significantly overcame the side effect of benzo[a]pyrene by modulation of different antioxidant enzyme and pro/anti-apoptotic gene ratio. Therefore, it could be inferred that alcoholic extract of *T. giganteum* may hold promise as a chemopreventive agent during the early phases of carcinogenesis.

Keywords: Benzo[a]pyrene, Lung cancer, Mushroom, *Tricholoma giganteum*, Tropical medicine.

INTRODUCTION

Tricholoma giganteum is a wild edible mushroom, placed in the family Tricholomataceae. It is the most conspicuous in the tropical region during rainy season and robust in size. *T. giganteum* is considered to be a healthy food because it is low in fat and calories but rich in amino acids, vitamins, and dietary fibres. For its gastronomic and nutritional delicacy, it is also very popular among the people of tropical region¹. From very long time, dietary mushrooms have been used globally for millennia to promote health and treat disease primarily via their multitude of medicinal qualities. There are many reports on the use of mushrooms and their metabolites for the treatment of a variety of human ailments²⁻¹⁰. On this light, Wang et al.,¹¹ showed immunomodulatory and antitumor activities of polysaccharide-peptide complex from a mycelial culture of *T. giganteum*. Whereas, Ooi¹² showed hepatoprotective activity of *T. giganteum* against paracetamol induced liver injury in mice. Research on *T. giganteum* has given light on its various medicinal aspects¹³⁻¹⁹. Earlier investigation from this laboratory showed that ethanolic fraction from *T. giganteum* possessed potent *in vitro* antioxidant property²⁰ and offers hepatoprotection²¹. 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 model²² and chemopreventive effect against benzo[a]pyrene-induced forestomach cancer in Swiss albino mice¹. Cancer is the leading cause of death in economically developed countries and the second leading cause of

death in developing countries. The global burden of cancer continues to increase largely because of the aging and growth of the world population alongside an increasing adoption of cancer causing behaviors, particularly smoking, in economically developing countries. The WHO estimates that lung cancer is the most common cancer in the world and the global incidence of lung cancer is increasing at the rate of 0.5% per year due to the fact that the smoking epidemic continues to spread to developing countries²³. Lung cancer is the major cause of morbidity and mortality worldwide both in men and women accounting for 29% of all other cancers²⁴. Totally 90% of lung cancer cases are related to tobacco smoking, even though other etiological agents are implicated which include commercial and industrial chemical pollutants, minerals and organic solvents, air pollutants and radiation. Polycyclic aromatic hydrocarbons (PAHs) are common products of incomplete burning of fossil fuel, tobacco and other organic matter. Among them benzo[a]pyrene is one of the most prominent and established carcinogens, and plays a major role in lung carcinogenesis²⁵. In this study, we attempt to develop lung cancer in Swiss albino mice by oral administration of Benzo[a]Pyrene and evaluate chemopreventive effects of ethanolic fraction of *T. giganteum* with reference to lipid peroxidation, antioxidant defense status and apoptosis associated genes.

MATERIALS AND METHODS

Extraction procedure

Powdered *T. giganteum* (100 g) was extracted with 80% ethanol at room temperature overnight and was repeated 4

*Author for Correspondence: krish_paper@yahoo.com

times, and then freeze-dried to obtain ethanolic fraction (Fa). The freeze-dried fraction 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 use²⁶.

Phenolic Compounds Analysis

For HPLC analysis, 10 mg dried extract was dissolved in 1 mL of HPLC grade methanol and then was filtered through a 0.2 µm membrane, 20 µL of the filtrate was analysed by HPLC (Agilent, USA). Separation was performed with Agilent Eclipse Plus C18 column (100 mm×4.6 mm, 3.5 µm) using a flow rate of 0.8 mL/min at room temperature. The mobile phase consisted of eluent A (acetonitrile) and eluent B (aqueous phosphoric acid solution, 0.1% v/v). Elution was carried out by using a gradient procedure: 0-2 min, 5% A; 5-10 min, 15% A; 10-15 min, 40% A; 15-20 min, 60% A; 20-22 min, 90% A. The compounds were identified by their UV spectra recorded with diode array detector and retention time of authentic standards. The results were expressed in µg per g of extract²⁷. For quantitative analysis of phenolic compounds, a 3-level calibration curve was obtained by injection of known concentrations (10–50 µg/ml) of eleven standard compounds: gallic acid ($y = 34.773x - 9.2238$; $R^2 = 0.9991$), *p*-hydroxybenzoic acid ($y = 45.79x - 7.3583$; $R^2 = 0.9995$), chlorogenic acid ($y = 13.776x - 2.9025$; $R^2 = 0.9993$), vanillic acid ($y = 19.225x + 0.2588$; $R^2 = 0.9994$), *p*-coumaric acid ($y = 49.773x - 10.541$; $R^2 = 0.9994$), ferulic acid ($y = 30.425x - 2.8188$; $R^2 = 0.9995$), myricetin ($y = 5.0676x - 6.0375$; $R^2 = 0.9937$), salicylic acid ($y = 4.4974x - 0.4763$; $R^2 = 0.9994$), quercetin ($y = 5.2478x - 5.9763$; $R^2 = 0.9954$), cinnamic acid ($y = 108.07x - 111.55$; $R^2 = 0.9979$) and pyrogallol ($y = 10.8x + 0.3333$; $R^2 = 0.9999$).

Animals

Healthy male Swiss albino mice of approximately of the same age 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 temperature, 35-60% relative humidity). The animals were maintained according to the guidelines recommended by the Animal Welfare Board and approved by our institutional animal ethical committee.

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) 1mg/20g body weight dissolved in peanut oil orally] twice a week for four successive weeks to induce lung cancer by 16th week. Group III animals received B[a]P (as in group II) along with fraction Fa of *T. giganteum*

(50 mg/kg body weight ip.). Fa fraction treatment was started 30 days prior the first dose of B[a]P administration and continued for 16 weeks.

Histopathological analysis

At the end of the experimental period (16th week), animals were sacrificed by cervical decapitation under ether anaesthesia and lungs were excised immediately and washed with ice-cold saline. Histological evaluation was performed on the lung tissue and a portion of specimen was fixed in 10% neutral buffered formalin, dehydrated in ethanol gradient (30-100%), cleared in xylene and then embedded in paraffin. Sections were cut at 4-5 µm in 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 and viewed under light microscope for histological changes.

Preparation of homogenates, cytosol and microsome fractions

After sacrifice, excised part of lung tissues was washed with ice-cold saline. Tissues were weighed and then homogenized in Tris-HCl buffer 0.1 M (pH 7.4) to yield a 10% (w/v) homogenate. 0.5 ml aliquot of this homogenate was used for assaying reduced glutathione. The homogenate was then centrifuged at a speed of 12,000 × g for 30 min in a refrigerated high-speed centrifuge at 4°C, the resulting supernatant obtained was used for further analysis.

Biochemical analysis

Glutathione S-transferase Assay

The glutathione S-transferase (GST) activity was determined spectrophotometrically at 37°C according to the procedure of Habig et al.,²⁸. 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 mM⁻¹ cm⁻¹.

Reduced glutathione Assay

Reduced glutathione (GSH) was estimated as total non-protein sulphhydryl group by the method as described by Moron et al.,²⁹. GSH was used as a standard to calculate µmole GSH per g tissue.

Catalase Activity

Catalase (CAT) activity was estimated at 240 nm by monitoring the disappearance of H₂O₂ as described by Aebi³⁰. Catalase enzyme activity has been expressed as µmoles of H₂O₂ consumed per min per mg protein.

Superoxide dismutase Activity

Superoxide dismutase (SOD) activity was estimated according to the method described by Kono³¹. The activity of SOD was expressed as U per mg protein.

Estimation of Lipid peroxidation

Lipid peroxidation (LPO) was estimated spectrophotometrically by the thiobarbituric acid reactive substances (TBARS) method³². The extent of lipid peroxidation was calculated using molar extinction coefficient of 1.56 × 10⁵ M⁻¹cm⁻¹ and expressed as nmoles MDA produced per mg protein.

Lactate dehydrogenase Assay

Lactate dehydrogenase (LDH) was assayed by measuring the rate of oxidation of NADH at 340 nm according to the

USA) and 200 units RevertAid M-MuLV Reverse Transcriptase. The reaction was carried out at 45°C for 60

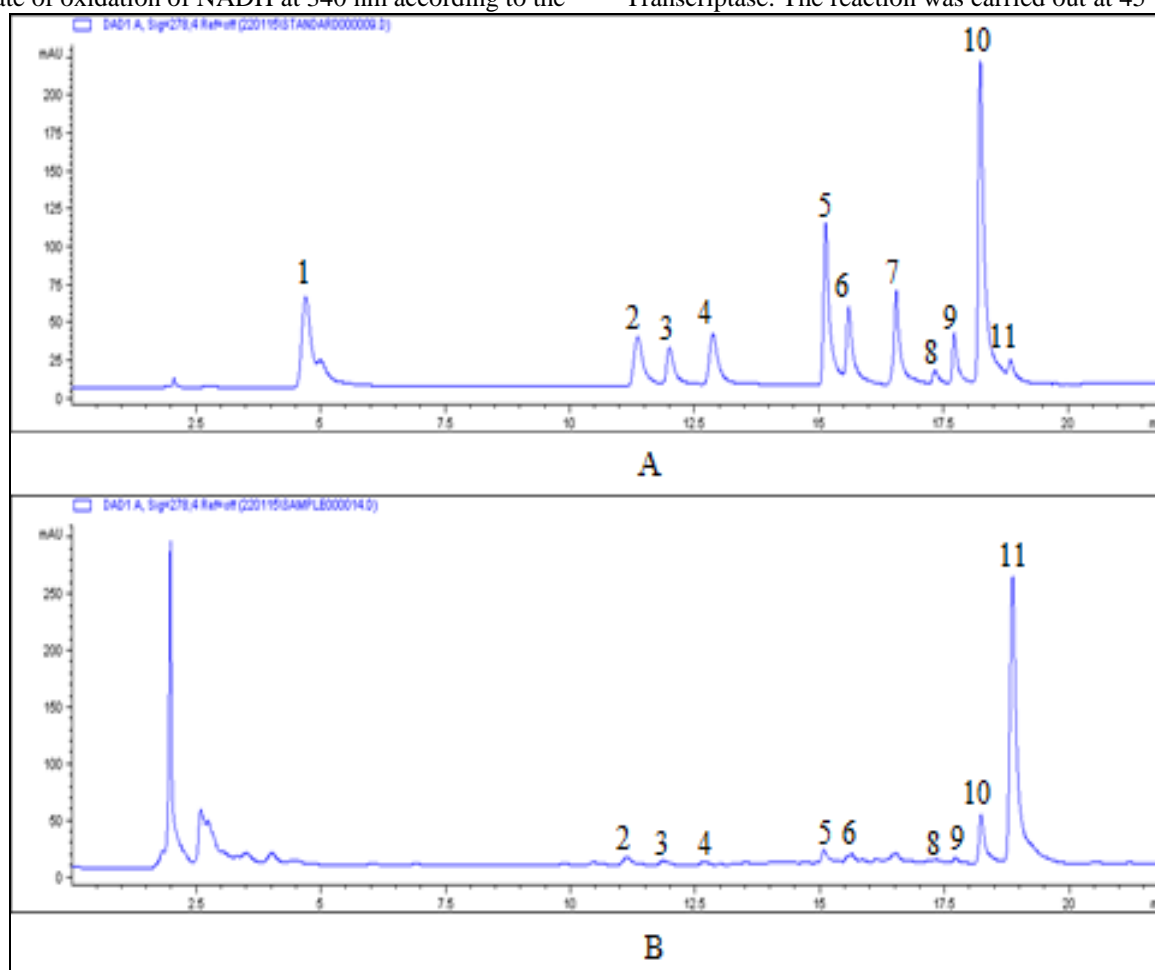


Figure 1: HPLC chromatogram of standards and ethanolic extract of *Tricholoma giganteum*. A. Standards B. Phenolic profile of extract (peaks: MP: mobile phase; 1: gallic acid; 2: *p*-hydroxybenzoic acid; 3: chlorogenic acid; 4: vanillic acid; 5: *p*-coumaric acid; 6: ferulic acid; 7: myricetin; 8: salicylic acid; 9: quercetin; 10: cinnamic acid; 11: pyrogallol)

method of Bergmeyer and Bernt³³. 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 μmol of NADH oxidized per min per mg protein.

Protein content determination

Protein was determined by the method of Lowry et al.,³⁴ using BSA as the standard at 660 nm.

Analysis of gene expression by semi-quantitative RT-PCR

Expression of apoptosis and cell cycle associated genes, Bcl-2, Bcl-xL, Bax, p53, p21 and cyclin D1 was studied using reverse transcriptase-PCR (RT-PCR). The housekeeping gene β -actin was used as control. Total cellular RNA was extracted from lung tissues with TRIzol Reagent (Invitrogen, USA). The cDNA was generated by reverse transcription of the total denatured RNA using RevertAid M-MuLV Reverse Transcriptase (Fermentas, USA). 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,

min followed by 70°C for 10 min. The above RT product was used for PCR reaction. PCR was carried out using the gene specific upstream and downstream primers. Primers used for RT-PCR analyses follows Chatterjee et al.,¹. 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). Initial denaturation at 95°C for 4 min was followed by a 30 PCR cycles of denaturation at 94°C for 30 s, annealing at 60°C for 30 s and strand extension at 72°C for 60 s with a final extension step of 7 min at 72°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.

Statistical analysis

Data are expressed as the mean \pm SD unless mentioned otherwise. Comparisons were made between different treatments using one-way analysis of variance followed by an error protecting multiple comparison procedure, namely Tukey-Kramer post hoc test by Graph Pad InStat

(GraphPad Software Inc., San Diego, USA) software for the analysis of significance of all the data (In all the cases

20th century, epidemiological studies and related meta-analyses strongly suggested that long term consumption

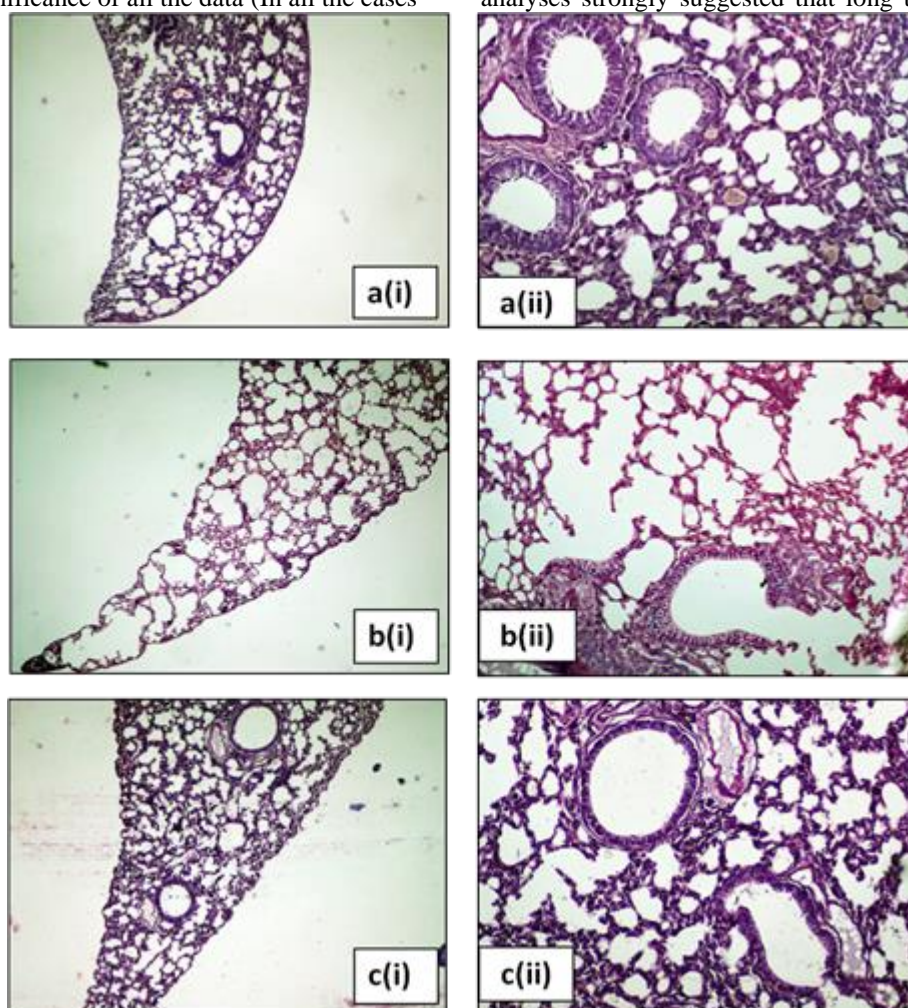


Figure 2: Histopathological investigation of the lungs of control and experimental mice. a(i) and (ii) Group I, control mice show normal architecture; b(i) and (ii) Group II, B[a]P only treated animals revealed alveolar damages and irregular hyperchromatic cells in the alveolar wall; c(i) and (ii) Group III, Fa fraction of *Tricholoma giganteum* treated set animals, the lung illustrated almost normal architecture with reduced alveolar damage

result is the mean \pm SD (standard deviation) of at least three individual experimental data.)

RESULTS AND DISCUSSION

In this study, the molecular phenolic profile of the ethanol fraction of *T. giganteum* is reported first time. Figure 1A depicts a typical HPLC chromatogram of eleven phenolic compounds and Figure 1B represents HPLC chromatogram of the extract consisting of 17 phenols of which 9 have been identified (Table 1). The major component in the fraction was found to be pyrogallol. Other detected phenols were presented in the order of cinnamic acid > chlorogenic acid > salicylic acid > *p*-coumaric acid > quercetin > *p*-hydroxybenzoic acid > ferulic acid. Thus it can be assumed that the fraction might be enriched with flavonols, hydroxycinnamic acid derivatives and hydroxybenzoic acid derivatives. Polyphenols and other food phenolics are the matter of growing scientific interest because of their promising beneficial effects on human health³⁵. Towards the end of

of diets rich in plant polyphenols offered some protection against development of cancers, cardiovascular diseases, diabetes, osteoporosis and neurodegenerative diseases^{36,37}. Apart from plant polyphenols, mushroom's polyphenol also may act directly as antioxidant or prevent underlying oxidative stress related pathological conditions such as cancer²², heart ailments⁴, diabetes⁷ etc. On the basis of this concept in this present study, the modulatory effect of Fa fraction of *T. giganteum* on organ specific carcinogenesis was assessed. In lung cancer bearing animals, the significant tumor progression may be due to enormous proliferation of the cancer cells. At the end of the experimental period (16 weeks), animals were sacrificed. After excision of lung tissues, studied different antioxidant enzyme status, marker enzyme profile and the extent of lipid peroxidation. Further, the lung tissue was subjected for histopathological observations and analysis of transcript level expression of cell cycle and apoptosis associated genes by semi-quantitative RT-PCR. Figure 2 portrays the histopathological analysis of lung section of

control and experimental groups. The lung cancer-bearing animals (Group II) revealed loss of architecture, alveolar

treated mice [Figure 2 a(i) and 2 a(ii)]. On Fa treatment, the significant tumor regression may be due to inhibitory

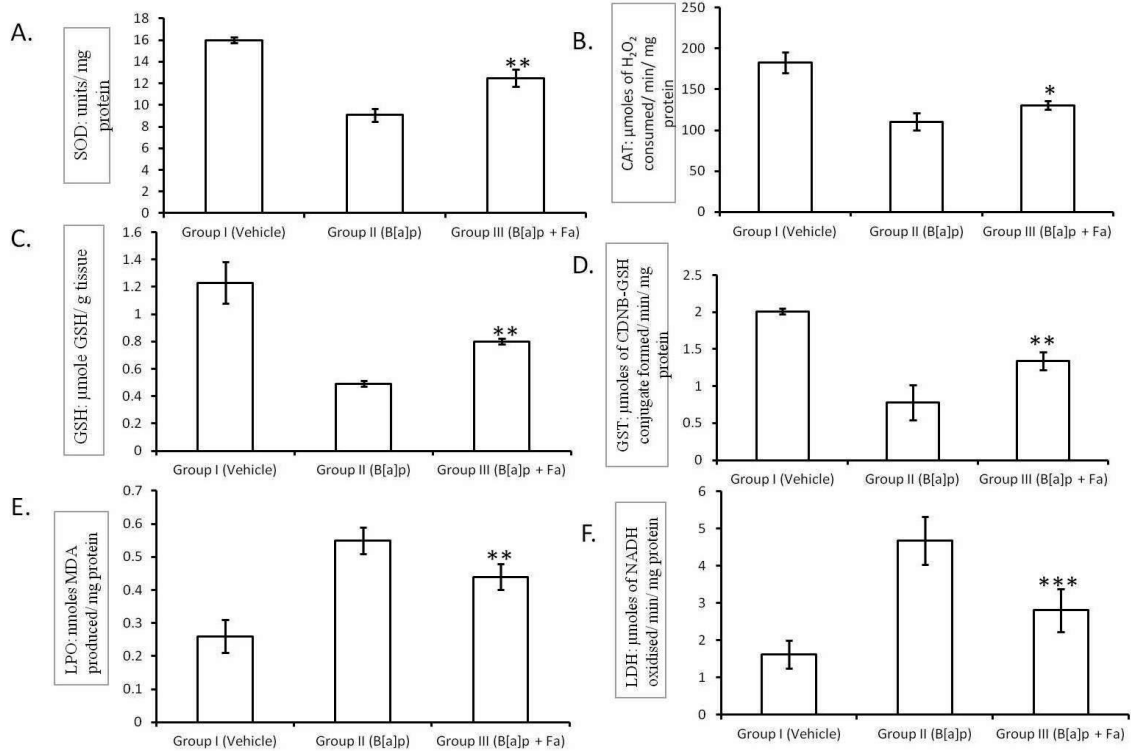


Figure 3: Effect of Fa fraction of *Tricholoma giganteum* on lipid peroxidation, antioxidant enzymes profiles and marker enzyme status in the lung of experimental mice. Values are mean ± SD, n = 6 animals. ***P < 0.001, **P < 0.01, *P < 0.05 when compare the group II(B(a)p) to group III(B(a)p + Fa)

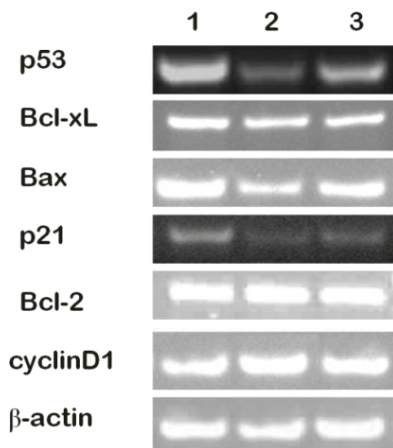


Figure 4: Semi-quantitative RT-PCR of p53, Bcl-xL, Bax, p21, Bcl-2, cyclin D1 gene expressions as represented in Lane 1: Control; Lane 2: B[a]p treated set; Lane 3: B[a]p + Fa treated set. β-actin used as loading control

damage as seen from hyperchromatic and irregular nuclei in the cells of alveolar wall [Figure 2 b(i) and 2 b(ii)]. In the treated set animals, the lung illustrated almost normal architecture (Group III) showing reduced alveolar damage as evident from reduced number of hyper chromatic irregular cells in the alveolar [Figure 2 c(i) and 2 c(ii)]. Group I showed no appreciable change of histopathological abnormalities as that of B[a]P only

action of the drug on tumor growth as revealed from tissue histology. Assessment of the enzymatic profile is the key step to examine the possibility of probable chemopreventive potential of any compound (Figure 3). Figure 3 shows the levels of lipid peroxidation, cellular antioxidant enzymes such as SOD, CAT, GST, non-enzymatic antioxidant (GSH), LDH was estimated in lung tissues of various experimental groups. The extent of lipid peroxidation (LPO) and the status of antioxidant defense arsenal comprising of superoxide dismutase (SOD), catalase (CAT), reduced glutathione (GSH) are closely related to the modulation of carcinogenesis. Under excessive oxidative stress, the level of LPO becomes very significant, and a growing body of evidence has shown that excessive LPO may be involved in carcinogenesis and malondialdehyde (MDA) a product of lipid peroxidation was observed to be mutagenic and carcinogenic³⁸⁻⁴¹. In our study, the significant increase in the levels of LPO was observed in the lung tissue of cancer bearing mice. This may be due to either a consequence of increased level of superoxide radicals, which are produced in significant amounts in response to B[a]P exposure⁴². SOD is the only enzyme that scavenge superoxide radicals and are present in all cells⁴³. It protects the cells against superoxide- and hydrogen peroxide-mediated LPO. Administration of Fa fraction markedly elevated the SOD and CAT activities, suggesting that it had the ability to restore the activities of

these two enzymes. GST activity has been shown to be decreased by B[a]P administration, whereas treatment

systems during p53-mediated apoptosis⁵¹. Bcl-2 over expression results in formation of homodimers and

Table 1: Phenolic profile of ethanol extract of *Tricholoma giganteum*. ND: Not determined.

S. No.	RT (min)	λ_{\max} (nm)	Concentration ($\mu\text{g}/\text{mg}$)	Compound
1	4.004	258	ND	ND
2	4.475	270	ND	ND
3	6.052	285	ND	ND
4	11.123	255	0.213 ± 0.002	<i>p</i> -hydroxybenzoic acid
5	11.855	273	0.386 ± 0.026	Chlorogenic acid
6	12.671	260, 295	0.231 ± 0.012	Vanillic acid
7	13.527	271	ND	ND
8	15.072	310	0.253 ± 0.041	<i>p</i> -coumaric acid
9	15.55	290, 325	0.079 ± 0.037	Ferulic acid
10	15.629	286, 324	ND	Probably naringin
11	16.122	282	ND	ND
12	16.521	375	ND	ND
13	17.171	303	0.348 ± 0.035	Salicylic acid
14	17.318	235, 302	ND	ND
15	17.707	255, 372	0.243 ± 0.02	Quercetin
16	18.226	277	0.419 ± 0.017	Cinnamic acid
17	18.857	276	25.056 ± 0.297	Pyrogallol

with the fraction restored the activity near to vehicle-treated group in lung, making the cells more effective with respect to detoxification of toxic metabolite. The notable change in the drug metabolizing enzyme, glutathione S-transferase (GST) is also a very important parameter to assess the potential of any given compound against neoplastic development⁴⁴. Moreover, the elevated level of GSH could effectively provide thiol group for the possible GST-mediated detoxification reaction. GSH also has profound importance for cellular homeostasis and is one of the major cellular defenses against ROS generation. Xenobiotic metabolizing enzymes play a major role in regulating toxic, oxidative damaging, mutagenic and neoplastic effect of chemical carcinogen⁴⁵. Analysis of cancer marker enzyme lactate dehydrogenase (LDH) serves as an indicator of cancer response to therapy, LDH being a specific indicator of cell damage induced by several factors, including xenobiotic compounds^{46,47}. A possible reason for this elevated LDH activity in cancer-bearing animals may be enhanced glycolysis during tumour growth. Alternately, it may be due to overproduction by tumour cells⁴⁸. The reduction in LDH level during chemotherapy is an important event for the effective treatment of lung cancer. In many lung cancers, there is a loss of function of pro-apoptotic proteins (p21, p53, Bax) and activation or over expression of anti-apoptotic molecules (Bcl₂, Bcl-Xl, cyclin D) by various mechanisms. p53 - mediated growth arrest involves p21 as a major effector and thus up-regulation of p21 results in blocking of cell cycle progression by restricting entry of cell in S phase^{49,50}. Further insight into the mechanism of action suggested that the activation of p53 blocks the cell cycle progression either in G1/S or G2/M checkpoints. Specifically, the fraction increased p53 and p21 expression levels and down regulated cyclin D1 level, which has been also linked to apoptosis and cell cycle arrest. Bax, a pro-apoptotic member of Bcl-2 family, is a p53 target and is transactivated in a number of

offering resistance to apoptosis, while the formation of Bcl-2/Bax heterodimers induces apoptosis⁵². Thus induction of apoptosis by the fraction was indicated by subsequent positive shift of Bax/Bcl-2 ratio, which is a critical determinant of the overall propensity of a cell to undergo apoptosis⁵³. Our results revealed that treatment of Fa fraction increased the level of p53 with related positive shift of the expression of p21. The high basal level of Bax expression was consistent with p53 activation. However, the expression status of anti-apoptotic genes Bcl-2 and Bcl-xl did not change significantly due to Fa fraction treatment, whereas, repression of cyclin D1 was noted in the treatment set when compared to the cancer bearing animals and this decrease appeared to correlate with an increase in apoptosis (Figure 4).

CONCLUSION

Therefore, it could be inferred that ethanolic (Fa) fraction of *T. giganteum* may hold promise as a chemopreventive agent during the early phases of carcinogenesis in B[a]P induced lung cancer in Swiss albino mice by inducing the antioxidant enzymes with reference to histopathological analysis. The selective action of stabilization of membrane bound enzyme profiles and lipid peroxidation in lung cancer may even account for the inhibitory effect of the fraction on cancer cells or may act to re-sensitize cancer cells to apoptosis and chemosensitivity.

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