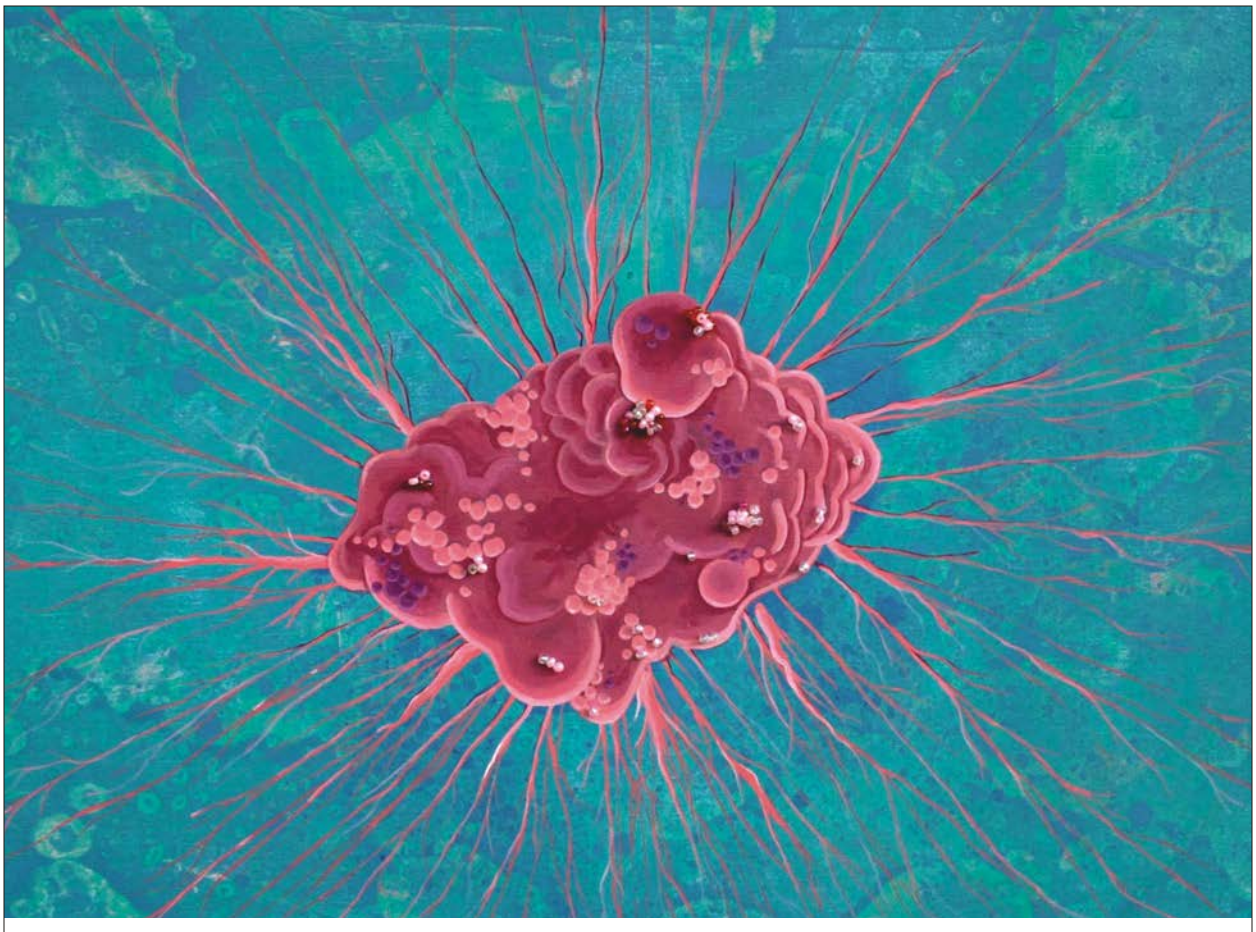

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Protective Effect of Flavanoidal Fraction of *Indigofera tinctoria* on Benzo (α) Pyrene Induced Lung Carcinogenicity in Swiss Albino Mice

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Abstract: The chemopreventive effect of the flavanoidal fraction of *Indigofera tinctoria* were evaluated in Swiss albino mice using 16 week medium term model of benzo (a) pyrene (BP)-induced lung tumor. Lung tumor was induced biweekly through oral incubation of BP for 4 weeks. The oral administration of flavanoidal fraction of *I. tinctoria* (100 mg kg^{-1} body weight) showed a significant increase in antioxidant enzymes like superoxide dismutase (SOD), glutathione peroxidase (GPx), glutathione reductase (GR), catalase (CAT) and non enzymic antioxidants like reduced glutathione (GSH), vitamin C and vitamin E were significantly increased when compared to BP treated groups. The effect is much more pronounced in pretreatment regime than in the post treatment regime. The levels of lung marker enzymes were significantly decreased in both the treatment regimes when compared to cancer induced group. The levels of lipid peroxidation were significantly decreased in the flavanoidal fraction of *I. tinctoria* treated regimes. The elevated levels of glycoproteins were normalized in the animals subjected to flavanoidal fraction of *I. tinctoria* treatment. The histopathological studies confirmed the protective effect of the extract by showing the reappearance of alveolar spaces in the treatment regimes, where the effect is pronounced much in the pre treatment regime than the post treatment regime. From the results obtained it is concluded that flavanoidal fraction of *I. tinctoria* had protective effects on BP induced lung cancer.

Key words: *Indigofera tinctoria*, benzo (a) pyrene, lung tumor, marker enzymes, lipid peroxidation, glycoproteins, histopathology

INTRODUCTION

Lung cancer has become a leading cause of mortality, largely because of difficulty in its detection at a curable stage (Earle, 2004; Deslauries, 2002). A five year survival rate of only ten percent illustrates this poor prognosis. The major cause of lung cancer is cigarette smoking leading to a high incidence of morbidity and mortality worldwide (Mountain, 1997). Nearly 85% of lung cancer is caused by smoking (Shopland, 1995). The major carcinogens in tobacco smoke are polycyclic aromatic hydrocarbons, typified by benzo (a) pyrene (Hecht, 1998). When, microsomal enzymes act on the carcinogen BP, it gets metabolized to arene oxides, phenols, quinones, dihydrodiols and to diol epoxides. The reactive metabolite BP-7, 8-diol-9, 10-epoxide exists in both cis and trans form. The principle ultimate carcinogenic metabolite of BP is a BPDE (Sticha *et al*., 2000).

Since, prehistoric times, man has been trying to identify plants that can be exploited as food and medicine. Traditional medicines utilizing natural products have been shown to contain bioactive compounds *in vitro* (Courreges and Benencia, 2002). *Indigofera tinctoria* is a widely distributed small erect medicinal shrub belonging to the family of Fabaceae, found throughout India. The plant has

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proved to be more effective against chronic myelogenous and other leukemia's (Steriti, 2002). Isolation of flavanoids apigenein, kaempferol, luteolin and quercetin from the plant has been reported (Kamal and Mangla, 1990). These flavanoids may have beneficial health effects because of their various biological effects, immunomodulating activity, antioxidant properties and inhibitory role in various stages of tumor development (Lee *et al*., 2003).

The present study is to evaluate the protective effect of flavanoidal fraction of the aerial parts of *I. tinctoria* on BP induced lung carcinogenicity in Swiss albino mice.

MATERIALS AND METHODS

Chemicals

Benzo (a) pyrene was obtained as a gift from Dr. Bieland Fredrick, US,FDA. All other chemicals like used were of analytical grade.

Animals

Healthy male Swiss albino mice (6-8 weeks old) were used throughout the study. The animals were purchased from King Institute of Preventive medicine, Chennai-600034 and maintained in a controlled environmental condition of temperature and humidity on alternatively 12 h light/dark cycles. All animals were fed with standard pelleted diet (Purchased from TANUVAS, Chennai) and water ad libitum. This research work on Swiss albino mice was sanctioned and approved by Institutional Animal Ethical Committee (IAEC-03-005-04).

Plant Material and Extract Preparation

The aerial parts of the plant were used. The methanolic extract is referred to as the crude extract and it is prepared according to the standard method described with slight modification (Sreepriya *et al*., 2001). It is then run in TLC using benzene and ethyl acetate as mobile phase. The flavanoidal fraction of the plant was taken for the study.

Induction of Lung Cancer

Lung cancer was induced orally by injecting benzo (α) pyrene [50 mg (dissolved in corn oil) kg^{-1} b.wt.$^{-1}$] twice weekly for 16 weeks (Selvindran *et al*., 2003).

Experimental Protocol

The animals were divided into five groups and each groups consisted of six animals. Group I served as control animals and was given corn oil (vehicle) (50 mL kg^{-1} body weight) orally for 16 weeks. Group 2 animals were treated with BP (50 mg kg^{-1} body weight) dissolved in corn oil orally twice weekly for 4 successive weeks to induce lung cancer. Group 3 animal's pre treated with flavanoidal fraction of *I. tinctoria* (100 mg kg^{-1} body weight) orally alternative days for 4 successive. Group 4 animals were post treated with flavanoidal fraction of *I. tinctoria* as for 4 successes. Group 5 Control animals treated with flavanoidal fraction of *I. tinctoria* alone as above.

At the end of the experimental period, the animals were fasted over night and killed by cervical decapitation. Lung tissues were removed from all animals and washed with ice cold saline, were used for analysis. Total protein was estimated by the method of Lowry *et al*. (1951), the level of lipid peroxides (LPO) was measured according to Ohkawa *et al*. (1979), superoxide dismutase (SOD) was determined by Misra and Fridovich (1972), catalase activity (CAT) was estimated by Sinha (1972), glutathione peroxidase (GPx) was estimated by Rotruck *et al*. (1973). Reduced glutathione (GSH) levels were measured according to Moron *et al*. (1979), ascorbic acid levels were assayed by Omaye *et al*. (1979) and Vitamin E levels were determined by Desai (1984).

Lung Marker Enzymes

The AHH assay was modified from the method of Mildred *et al*. (1981), γ-glutamyl transpeptidase levels were determined by Orlowski and Meister (1965), 5′-Nucleotidase levels were measured by Luly *et al*. (1992) and lactate dehydrogenase levels were determined by King (1965).

Glycoprotein Levels

Hexose level estimation was performed according to protocol given by Neibes (1972). Hexosamine levels were determined by Wagner (1974) and sialic acid levels was estimated as according to Warren (1959).

Histopathological Studies

The lung tissues of all the five groups were fixed in 10% formaldehyde. After several treatments in different concentrations of alcohol, the dehydrated tissue was embedded in paraffin wax. Sections were cut (4 μm) and stained with eosin and haematoxylin.

Statistical Analysis

Statistical Analysis was calculated by using Statistical Package for the Social Sciences package(SPSS). Values are mean±SD for six specimens in each group and the significance of difference between mean values were determined by One-Way Analysis of Variance (ANOVA) followed by Duncan multiple comparison tests.

RESULTS AND DISCUSSION

The mice were observed for 1 h continuously and intermittently for 4 h and further for 72 h for any mortality. None of the mice showed changes and there was no mortality up to 72 h period of observation and even up to a dose of 100 mg of the crude flavanoidal fraction, thereby showing the non toxic nature of the flavanoidal fraction. The safety of the drug was evaluated and it was found that at 100 mg kg^{-1} b.wt. was found to be optimal dosage.

Table 1 represents the changes in the levels of enzymic and nonenzymic antioxidants in lung tissues of experimental animals. The enzymic antioxidants such as superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase and non-enzymic antioxidants such as glutathione, vitamin E and vitamin C were found to be significantly reduced in group 2 animals ($p<0.01$). In treated animals (group 3 and group 4) the antioxidant enzymes levels were significantly corrected to near normal levels and having much effect compared to group 5 animals ($p<0.01$). No adverse effect was

Table 1: Effect of flavanoidal fraction of *I. tinctoria* on enzymic and nonenzymic antioxidants in the lung of control and experimental animals

Particulars	Group 1	Group 2	Group 3	Group 4	Group 5
SOD	$6.68\pm0.31^{c**}$	$4.71\pm0.29^{a**}$	$6.44\pm0.31^{c**}$	$5.98\pm0.30^{b**}$	$6.59\pm0.32^{c**}$
CAT	$358.17\pm29.4^{c**}$	$235.67\pm29.6^{a**}$	$346.83\pm30.5^{c**}$	$274.83\pm29.2^{b**}$	$348.10\pm29.3^{c**}$
GPx	$64.96\pm7.90^{c**}$	$38.78\pm7.89^{a**}$	$62.05\pm7.82^{c**}$	$49.88\pm7.85^{b**}$	$63.97\pm7.90^{c**}$
GR	$3.91\pm0.30^{c**}$	$2.68\pm0.31^{a**}$	$3.62\pm0.31^{c**}$	$3.25\pm0.27^{b**}$	$3.81\pm0.30^{c**}$
GSH	$2.55\pm0.30^{c**}$	$1.49\pm0.29^{a**}$	$2.05\pm0.29^{c**}$	$2.41\pm0.31^{b**}$	$2.45\pm0.32^{c**}$
Vit E	$1.47\pm0.29^{c**}$	$0.53\pm0.28^{a**}$	$1.41\pm0.30^{c**}$	$0.91\pm0.29^{b**}$	$1.38\pm0.27^{c**}$
Vit C	$1.49\pm0.30^{c**}$	$0.63\pm0.29^{a**}$	$1.50\pm0.19^{c**}$	$0.99\pm0.28^{b**}$	$1.39\pm0.30^{c**}$

Each value is expressed as mean±SD for six mice in each group. Since p value is less than 0.01, there is a significant difference between groups with regard to antioxidant enzymes. Based on Duncan Multiple Range test, the group 2 is significant with group 3, 4 and group 1. Group 3 is significant to Group 4. Group 1 and Group 5 are not significant. Different alphabet between groups denotes significance at 5% level. SOD-Units/min/mg protein; CAT-μmoles of H_2O_2 liberated/min/mg protein; GPx-μmoles of GSH oxidized min mg^{-1} protein; GSH, vitamin C and vitamin E-μg mg^{-1} protein; GR-nmoles of NADPH oxidized/min/mg protein.**Denotes significance at 1% level; *Denotes significance at 5% level

observed in group 5 animals. Normal human lung is efficiently protected and buffered against exogenous free radicals. Besides classical antioxidant enzymes (AOEs), epithelial lining fluid contains lower molecular weight antioxidants and proteins including a tripeptide glutathione (GSH) (Cantin *et al*., 1987), mucin GSH dependent enzymes and catalase that are located in the cytosolic compartment and peroxisomes. Among these antioxidants GSH is abundantly localized to the epithelial lining fluid of human lung (Kosower and Kosower, 1978) and its reaction pathways are tightly linked to the reactions of other thiol containing proteins, that participate not only in scavenging of H_2O_2 but also in the regulation of the redox balance of the cells.

Reduced glutathione, chemically γ-glutamyl cysteinyl glycine is a predominant non-protein thiol present in virtually all cell types. Glutathione is ubiquitous in animals (Duke *et al*., 1996). Glutathione often attains millimolar levels inside cells, which makes it one of the most highly concentrated intracellular antioxidants. It fulfills a wide variety of important functions such as detoxification of electrophiles, serves as a transfer vehicle for cystenine and renders protection against ROS conjugation. The reduced glutathione in tissues keeps up the cellular level of vitamin C and vitamin E in active forms. These vitamins also exist in inter convertible form and participate in neutralizing free radicals. When there is reduction in the level of GSH, the cellular levels of vitamin C and vitamin E are also lowered. Intracellular GSH status appears to be sensit4e indicator of the cell's overall health, and of its ability to resist toxic challenge. Experimental GSH depletion can trigger suicide of the cell by a process known as apoptosis (Cameron *et al*., 1979).

The ascorbate molecules are involved in the feedback inhibition of the lysosomal glycosidases responsible for the malignant invasiveness (Buettner, 1993). Vitamin C protects cell membrane and lipoprotein particles from oxidative damage by regenerating the antioxidant from vitamin E (Wiseman, 1996). Thus vitamin C and vitamin E act synergistically in scavenging wide variety of ROS.

Vitamin E is the major lipid soluble peroxy radical scavenger, which can limit LPO, terminating chain reactions initiated in the membrane lipids (Das, 1994). Decreased vitamin E content in lung cancer bearing animals might be due to excessive utilization of this antioxidant for quenching enormous free radicals produced in these conditions. Vitamin E acts a chain breaking antioxidant by donating its labile hydrogen atom from phenolic-OH group to prorogating lipid peroxyl and alkoxyl radicals intermediates of LPO, thus terminating the chain reactions (Gulliame *et al*., 2001). The supplementation of flavanoids containing quericitin, indirubin, naringeinin and to the experimental animals would have improved various cellular antioxidants and thiol content in tissues, which in turn reduces free radical formation during lung carcinogenesis induced by BP.

Antioxidant enzymes are the main scavengers of free radicals and function as the inhibitors at both initiation and promotion or transformation stages of carcinogenesis (Thirunavukkarasu and Sakthisekaran, 2001). The antioxidant enzymes SOD, CAT and GPx play an important role as protective enzymes against reactive oxygen species in tissues and also comprise the cellular antioxidant defense system (Kinnula *et al*., 1995).

Our present study shows a reduction in the activities of SOD, CAT and GPx in lung cancer bearing animals. All flavanoids can absorb free electrons also known as free radicals, and hold them. This stops further free radical damage to cells. Thus, flavanoids known for its chain breaking antioxidant property breaks the chain caused by free radicals. On flavanoidal treatment, the activities of these enzymes inclined to near normal. This may be due to the reaction of flavanoids on Xanthine-xanthine oxidase system (Hu *et al*., 1995).

Table 2 shows the effect of flavanoidal fraction of *I. tinctoria* on the activities of marker enzymes (AHH, LDH, γGT and 5'nucleotidase) in the lung of control and experimental animals. The levels of enzymes in group 2 ($p<0.01$) cancer bearing animals were increased when compared to group 1 control animals. This increase in the activities was significantly decreased in group 3 ($p<0.01$) and group 4 ($p<0.01$) flavanoidal fraction of *I. tinctoria* treated animals when compared to group 2 benzo(a)pyrene

Table 2: Effect of flavanoidal fraction of *I. tinctoria* on lung marker enzymes of control and experimental animals

Particulars	Group 1	Group 2	Group 3	Group 4	Group 5
AHH	$0.75\pm0.08^{a**}$	$1.02\pm0.11^{d**}$	$0.64\pm0.06^{a**}$	$0.93\pm0.04^{c**}$	$0.69\pm.064^{ab**}$
LDH	$1.23\pm0.30^{a**}$	$2.01\pm0.29^{c**}$	$1.20\pm 0.29^{a**}$	$1.65\pm0.27^{b**}$	$1.12\pm0.29^{a**}$
γ-GT	$1.17\pm0.30^{a**}$	$2.12\pm0.29^{c**}$	$1.31\pm0.31^{a**}$	$1.73\pm0.28^{b**}$	$1.07\pm0.30^{a**}$
ADA	$247\pm29.24^{a**}$	$391\pm29^{d**}$	$288.67\pm30.7^{b**}$	$327.33\pm24.86^{c**}$	$237\pm28.4^{a**}$
5'-nucleotidase	$1.92\pm0.27^{a**}$	$3.0\pm0.29^{c**}$	$2.49\pm0.28^{b**}$	$1.94\pm0.26^{a**}$	$1.82\pm0.3^{a**}$

Each value is expressed as mean±SD for six mice in each group. Since p value is less than 0.01, there is a significant difference between groups with regard to lung marker enzymes. Based on Duncan Multiple Range test, the group 2 is significant with group 3,4 and group 1. Group 3 is significant to Group 4.Group 1 and Group 5 are not significant. AHH-µmoles of fluorescent phenolic metabolites formed/min/mg protein. LDH-µmoles of pyruvate liberated/min/mg/protein. 5'-nucleotidase -nmoles of Pi liberated/min/mg protein. γ-GT- nmoles of p-nitroaniline formed/min/mg protein. ADA-µmoles NH_3 liberated/mg protein/hour.**Denotes significance at 1% level. *Denotes significance at 5% level. Different alphabet between groups denotes significance at 5% level

treated animals. The group 3 is more significant compared to group 4 animals. Group 5 showed no significant difference in the activities of marker enzymes when compared with group 1 control animals. The activities of marker enzymes were found to be elevated in tissues of lung carcinoma bearing animals, which could be due to the destruction of the neoplastic tissue. The abnormal variations in the marker enzymes reflect the overall change in metabolism that occurs during malignancy (Stefanini, 1985).

The marker enzymes such as AHH, ADA, GGT, 5'-ND and LDH are specific indicators of lungdamage (Durak *et al*., 1993). The increase in the activities of these enzymes may be due to the increased tumour incidence. The AHH and ADA activities were increased in lung cancer animals.

GGT is a broad specificity transferase that catalyses the transfer of gamma glutamyl groups from a large variety of peptide donors to a wide range of aminoacids and peptide receptors (Valentich and Moris, 1992). γ-Glutamyl transpeptidase activity serves as a marker for the progress of carcinogenic events. The enzyme is membrane bound and its active site is oriented on the outer surface of cell membrane. γ-Glutamyl transpeptidase is a cell surface enzyme that cleaves extra cellular glutathione thereby providing the increased intracellular glutathione synthesis (Durhan *et al*., 1997). This deviation shows the progress of carcinogenic process, since its ability correlated with growth rate, histological differentiation and survival time of the host (Koss and Greengard, 1982). LDH is a tetrameric enzyme and is recognized as a potential tumour marker in assessing the progression of the proliferating malignant cells. LDH is a fairly sensitive marker for solid neoplasm (Lippert *et al*., 1981). Activity of LDH is found to be higher in malignant tissues (Rogers *et al*., 1981). This may be due to the higher glycolysis in the cancerous condition, which is the only energy yielding pathway for the uncontrolled proliferating malignant cells.

The decrease in the activities of above mentioned marker enzymes on treatment with flavanoids suggests that flavanoids offers some protection against abnormal cell growth by changing the permeability or affecting cellular growth. This may be due to the antineoplastic property of flavanoids.

The levels of lipid peroxidation in lung mitochondria of control and experimental animals are shown in Fig. 1. There found to be an increase in LPO in group 2 ($p<0.01$) cancer bearing mice when compared with control animals ($p<0.01$). Flavanoidal portion of *I. tinctoria* treatment resulted in significant decrease in the activities of these enzymes in group 3 ($p<0.01$) and group 4 ($p<0.01$) animals where the effect in group 3 is much more pronounced than group 4. However the extract alone treated group 5 animals when compared with group 1 control animals did not show any significant difference in the LPO levels. The significant increase in the levels of LPO was observed in animals bearing lung cancer .

In our present study the flavanoidal fraction of *I. tinctoria* altered these macromolecular damage mediated through free radicals thereby displaying the protective role of flavanoidal fraction of *I. tinctoria* in inhibiting free radical mediated cellular damages. The suppressive action of flavanoidal fraction of *I. tinctoria* on lung lipid peroxidation observed in *in vivo* systems suggests that the drug may have a direct effect on the membranes and these may decrease the susceptibility of the membranes to lipid peroxides. The present study indicates that oral administration of flavanoidal fraction of *I. tinctoria* significantly suppresses the LPO formation in lung tissues of animals bearing cancer.

Table 3 shows the effect of flavanoidal fraction of *I. tinctoria* on the levels of glycoproteins in lung of control and experimental groups. There were found to be an increase in levels of glycoproteins in group 2 ($p<0.01$) of animals bearing cancer when compared with group 1 control animals. These changes were significantly reduced in group 3 and group 4 ($p<0.01$) animals on treatment with flavanoidal fraction of *I. tinctoria*. Group 5 animals showed no significant difference in the levels of glycoproteins when compared with group 1 control animals. Glycoproteins are essential for cell to cell communications which are found on the surface of all the cells and some are released in to the blood stream and other body fluids (Dennis *et al*., 1981). Malignant transformation of normal cell may be accompanied by changes in the (CH_2O) n of glycoprotein viz., hexose, hexosamine and sialic acid in the plasma membrane.

Sialic acid is widely distributed in mammals and occurs as a terminal component at the non reducing end of the carbohydrate side chains of glycoproteins and glycolipids. Sialic acid has been implicated in the number of conditions including metasatic spread, contact phenomenon, tumour antigenecity, transport processes and viral receptors (Emmelot, 1973). Flavanoidal fraction of *I. tinctoria* may alter the cell membrane glycoprotein synthesis and structure, indicating its potent antioxidant property. This reduction in the levels of glycoprotein components indicates that

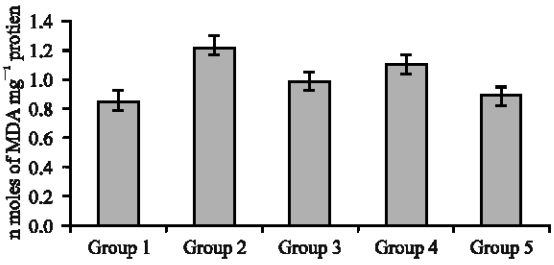

Fig.1: Levels of lipid peroxidation in lung tissues of normal and experimental groups of mice. Each value is expressed as mean±SD for six mice in each group. Since p value is less than 0.01, there is a significant difference between groups with regard to lipid peroxidation. Based on Duncan Multiple Range test, the group 2 is significant with group 3,4 and group 1. Group 3 is not significant to Group 4. Group 1 and Group 5 are not significant

Table 3: Effect of flavanoidal fraction of *I. tinctoria* on glycoproteins of control and experimental animals

Particulars	Group 1	Group 2	Group 3	Group 4	Group 5
Hexose	$1.41\pm0.30^{a**}$	$3.69\pm0.28^{c**}$	$1.61\pm0.27^{a**}$	$2.14\pm0.29^{b**}$	$1.50\pm0.27^{a**}$
Hexosamine	$0.64\pm0.03^{a**}$	$1.14\pm0.03^{b**}$	$0.64\pm0.03^{a**}$	$0.76\pm0.02^{c**}$	$0.63\pm0.03^{a**}$
Sialic acid	$0.69\pm0.09^{a**}$	$0.96\pm0.08^{c**}$	$0.72\pm0.07^{a**}$	$0.83\pm0.07^{b**}$	$0.73\pm0.03^{a**}$

Each value is expressed as mean±SD for six mice in each group. Values are expressed as mg g^{-1} of defatted tissue. Since p value is less than 0.01, there is a significant difference between groups with regard to lung marker enzymes. Based on Duncan Multiple Range test, the group 2 is significant with group 3,4 and group 1. Group 3 is significant to Group 4. Group 1 and Group 5 are not significant. **Denotes significance at 1% level. *Denotes significance at 5% level. Different alphabet between groups denotes significance at 5% level

flavanoidal fraction of *I. tinctoria* has the ability to suppress the malignancy by modulating cell transformation, decreasing the degree of lung cancer growth and controlling cell proliferation.

In the histopathological studies (Fig. 2), shows the control animals (Group 1) having a normal architecture of the lung epithelium with alveolar spaces. In the BP (Group 2) treated group, shows the number of alveolar spaces were reduced due to invasion of alveolar spaces by marginal epithelium. The alveolar spaces reappeared back to near normal in the *I. tinctoria* in both the pre and treated (Group 3 and Group 4) animals. The drug alone treated group shows the effect of *I. tinctoria* (Group 5) alone treated which is similar to control (Group 1). The control animals showed normal architecture in histopathological studies. In BP treated animals, alveolar damages are seen characterized by hyperchromatic nuclei or pyknotic nuclei in the cells of alveolar wall, whereas the flavanoidal fraction reduced the alveolar damage as evident from reduced number of hyperchromatic nuclei which showed the protective nature of the extract. In the flavanoidal fraction alone treated animals normal architecture is seen as compared to that of control animals.

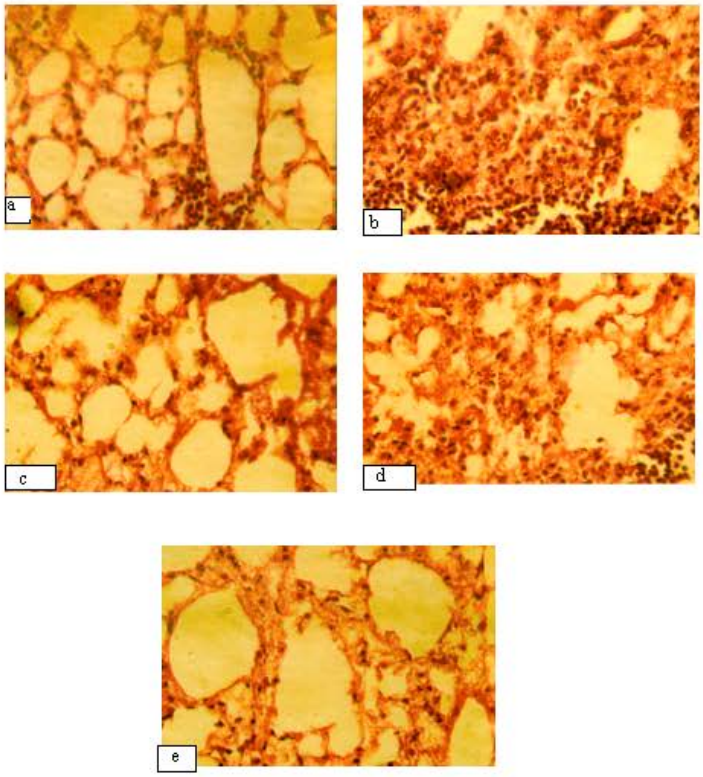

Fig. 2: Histological investigations of the lungs of Control and Experimental animals. (a) Group 1-Control animals showing anormal architecture (40 X). (b) Group 2-Lung cancer bearing animals showing alveolar damages with more number of pyknotic nuclei (40 X). (c) Group 3-Flavanoidal fraction of *I. tinctoria* pre treated showing reduced number of hyperchromatic irregular cells in alveolar wall (40 X). (d) Group 4-Flavanoidal fraction of *I. tinctoria* post treated showing reduced alveolar damage and reduced irregular hyperchromatic cells (40 X). (e) Group 5- Flavanoidal fraction of *I. tinctoria* alone treated animals showing no histological abnormalities (40 X)

The present study demonstrated that oral administration of *I. tinctoria* has chemopreventive effect against BP in Swiss albino mice which may be due to the protective nature of the flavanoidal fraction of *Indigofera tinctoria* attributing to the presence of flavanoids like quericitin, indirubin, Narigeneinin, Kaemferol and Hesperidins.

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