



Antioxidant activity of berberine on benzo (A) pyrene induced experimental lung carcinogenesis in mice

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ABSTRACT

Currently, search of new plant compounds possessing anticancer and antioxidant activity is a vital area of research in plant medicine, as plant products are safe and relatively low of cost. Berberine, an isoquinoline alkaloid derived from plants, is a traditional medicine for many diseases in India. In recent times, berberine has been revealed to have potent anticancer activity against various cancers. Information regarding the effect of berberine on experimental lung cancer is limited. The objective of the present study to evaluate the antioxidant potential of berberine on experimental lung carcinogenesis. Thirty Swiss albino mice were divided into five groups of six animals each. Animals in group I received corn oil orally and served as control. Group II were administered with 50 mg/kg b.wt. of benzo(a)pyrene (B(a)P) twice a week for 4 consecutive weeks to induce lung cancer by the end of 16th week. Group III received 50 mg/kg b.wt. berberine on alternate days for 16 weeks immediately after the first dose of carcinogen. Group IV also received berberine as in group III but from the sixth week of B(a)P induction till the end of the experiment. Group V animals constituted the drug control and received berberine alone. The results showed that berberine treatment remarkably down regulated lipid peroxidation and unusually improved the antioxidant defense. The reason for this noteworthy restoration might be due to the effect of the interference approach on the down regulation of the peroxidation reaction through the potent antioxidant nature, which ultimately reflected in the down regulation of lipid peroxidation. The histopathological study of lung tissues also confirms the biochemical changes. It can be concluded that berberine shows evidence of anticarcinogenic activity by reducing peroxidation reaction through enhancing antioxidant defense. The results justify the therapeutic applications of the active compound in the indigenous system of medicine, augmenting its therapeutic value.

Key words: Berberine, Benzo (a) Pyrene, lung cancer, antioxidants, lipid peroxidation

INTRODUCTION

Cancer is one of the leading causes of morbidity and mortality throughout the world and it is the second leading cause of death in most of the developed countries.^[1] Lung cancer is one of the commonest malignant neoplasms seen all over the world. It accounts for more cancer deaths than any other cancer, increasingly being recognized in India.^[2] Ineffectiveness, lack of safety, low therapeutic index, adverse effects and high cost have lead to a lack of faith and limited the usage of synthetic drugs.^[3] The constant search for new anticancer compounds from plants is a pragmatic and promising strategy for its treatment and prevention. Medicinal plants are the major source of biologically active principles. Vinblastine and Vincristine are the two plant derived alkaloids have been extensively used in the treatment of various cancers.^[4] Berberine is an isoquinolone alkaloid isolated from many medicinal

herbs such as *Hydrastis canadensis* (goldenseal), *Cortex hellodendri* (Huangbai) and *Rhizoma coptidis* (Huanglian) used to cure various diseases. Interestingly, berberine revealed tumour specific cytotoxicity, selective apoptosis inducing activity and potent anticancer activity against various cell lines.^[5-9] Our previous finding also proved the antitumour activity of berberine against human lung cancer cell line A549.^[10] Keeping this in view, this study has been undertaken to investigate antioxidant potential of berberine B (a) P induced lung cancer in mice.

EXPERIMENTAL SECTION

Chemicals

Berberine and Benzo(a)pyrene were purchased from Sigma Chemicals Co. St. Louis, MO, USA and all other chemicals used in this study were of high purity and analytical grade

Animals

Swiss albino mice weighing between 20 -25 g were procured from “Tamil Nadu Veterinary and Animal Science University”, Chennai, India. The animal house was well ventilated and the animals had 12 ± 1 h day and night rhythm throughout the experimental period. The animals were housed in large spacious polypropylene cages. As per the guidelines for breeding and experiments on Animals, 1998 defined by the Ministry of Social Justice and Empowerment of India was followed (IAEC No.07/040/05). The animals received a balanced commercially available pelleted rat feed and were provided with clean drinking water *ad libitum*.

Experimental Design

The animals were divided into five groups and each group consists of six animals. Group I- Control animals were treated with corn oil (vehicle) orally. Group II- B(a)P (50 mg/kg body weight dissolved in corn oil, treated orally) was administered twice weekly for 4 successive weeks to induce lung cancer. Group III-Berberine (50 mg/kg body weight dissolved in corn oil, orally) was administered on alternate days for 16 weeks after they were treated with the first dose of B(a)P (as in Group II). Group IV-Berberine (as in Group-III) was administered but from the sixth week of B(a)P induction (as in Group II) till the end of the experiment. Group V- animals treated with berberine alone (Drug control).

Sample Collection

All the experimental animals were killed by cervical decapitation. Blood samples were collected with ethylene diamine tetra acetic acid (EDTA) and without anticoagulant for the separation of plasma and serum respectively to determine blood parameters. The lung and liver tissues were removed, washed with ice-cold buffer and blotted individually on filter paper. One portion of lung and liver were placed in 10% formalin for histopathological observations. The remaining tissues were used for further analysis.

Preparation of Tissue Homogenate

100 mg of lung and liver samples were homogenized in 1 ml of ice-cold 0.1 M Tris-HCl buffer (pH 7.4) to give 10% homogenate. It was subjected to differential centrifugation, and the mitochondria and cytosolic fractions were isolated. Total homogenate and sub-cellular fractions were used for the assay the following parameters in serum, lung and liver samples.

Estimation of Enzymic Antioxidants

Superoxide Dismutase (SOD) was determined by the method of Marklund and Marklund 1974.^[11] Catalase activity was assayed by the method of Sinha 1972.^[12] Glutathione Peroxidase (Gpx) activity was assayed by the method of Rotruck *et al.*, 1973.^[13]

Estimation of Non-Enzymic Antioxidants

Glutathione level was estimated by the method of Moron *et al.*, 1979.^[14] Ascorbic Acid (Vitamin C) level was estimated by the method of Omaye *et al.*, 1979.^[15] γ -Tocopherol (Vitamin E) level was estimated by the method of Desai 1984.^[16]

Estimation of Macromolecular Damages - Lipid Peroxidation (LPO)

The level of lipid peroxides was assayed by the method of Ohkawa *et al.*, 1979.^[17] 1.5 ml of TBA, 0.2 ml of Sodium dodecyl sulphate and 1.5 ml of Glacial acetic acid were added to the test tubes containing 0.1 ml of samples. The test tubes were heated in the water bath for 1 h and then cooled and 1 ml of distilled water was added. The

optical density was determined at 532 nm using a reagent blank. Standard malondialdehyde was also processed in a similar fashion. The results were expressed as n mol of malondialdehyde liberated/mg protein.

Tissue processing for histological studies

Immediately after sacrifice, the organs such as lung was rapidly dissected out and washed in saline and fixed in 10% formalin. The samples were dehydrated in alcohol series 30%, 50%, 70%, 90% and 100% and cleared in xylene. The cleaned tissues were embedded with molten paraffin at 58°C. Consecutive sections were taken at 7µ thickness and stained with heamatoxylin and eosin (H and E).

RESULTS

Enzymic and Non-Enzymic Antioxidants

Table 1 represents the changes of enzymatic and non-enzymatic antioxidants of lung tissues in control and experimental animals. The enzymic and nonenzymic antioxidants such as superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase, reduced glutathione, vitamin C and vitamin E levels were significantly reduced in Group II cancer bearing animals when compared with Group I control animals ($p<0.05$). On simultaneous treatment with berberine Group III animals and post treatment with berberine Group IV animals have showed a significant increase in the activities of SOD, CAT, GPx, GR and in the levels of GSH, vitamin C and vitamin E ($p<0.05$). On the other hand, simultaneous treatment with berberine (Group III) showed an improved revival of lung tissue than the post treated with berberine (Group IV), and there was no considerable change between the control animals (Group I) and the animals treated with berberine alone (Group V). Table 2 represents the activities of enzymatic and non-enzymatic antioxidants of liver in control and experimental animals. Significant reduction in the levels of antioxidants by enzymic and non-enzymic activities in cancer bearing animals (Group II) was observed ($p<0.05$). These changes were reverted back to near normal values in simultaneous treatment with berberine (Group III) animals ($p<0.05$). Post treatment with berberine (Group IV animals) has showed a significant increase in all the level of antioxidants by enzyme and non enzymic activities ($p<0.05$). However, simultaneous treatment with berberine (Group III) has showed a noticeable increase ($p<0.05$) in the activity levels of these antioxidant system than the post treatment of berberine (Group IV animals). There was no substantial alteration between the control animals (Group I) and the animals treated with berberine alone (Group V).

Macromolecular Damage

The level of lipid peroxidation in lung and liver tissues of control and experimental animals is depicted in Fig.1 and Table.3 There was a noticeable increase in LPO of Group II cancer bearing animals when compared to control animals ($p<0.05$). Significant increase in the levels of LPO was reversed to near normal value with simultaneous treatment of berberine (Group III) and berberine post treated (Group IV) animals. However, simultaneous treatment with berberine, Group III has showed a noticeable decrease ($p<0.05$) in the activity of LPO. There was no significant change between the control animals (Group I) and the animals treated with berberine alone (Group V).

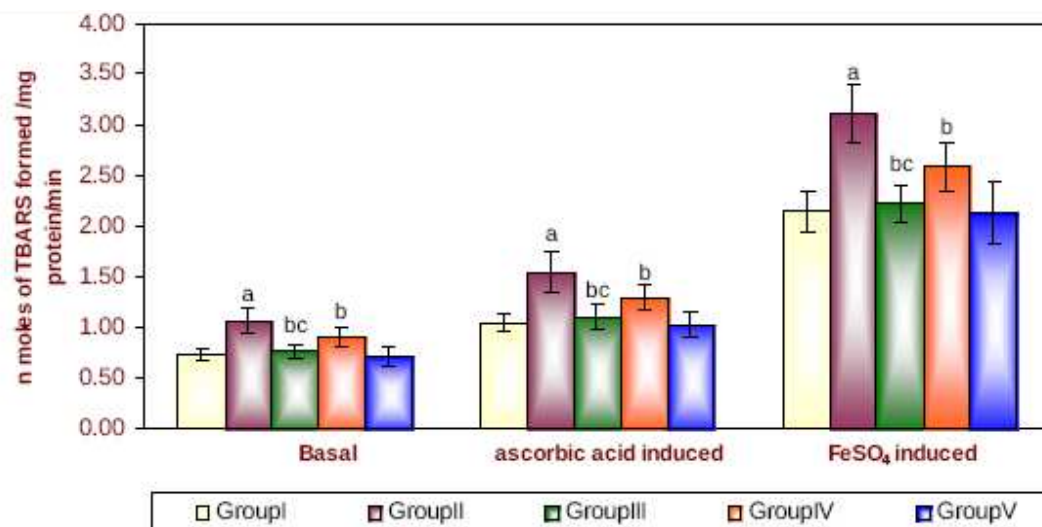
Histopathological Examination

In histopathological examination (Figure 2), Group I animals showed normal architecture of bronchioles and alveoli whereas Group II animals showed marked destruction of alveoli with stromal proliferation and infiltration by inflammatory cells with alveolar wall thickening. On the other hand Group III simultaneous treatment with berberine showed a significant reduction in stromal proliferation with near normal appearing alveoli with peribronchiolar inflammation and Group IV Post treatment with berberine showed a moderate reduction in stromal proliferation and inflammation. Normal architecture of lung with bronchiole and alveoli were observed in Group V berberine alone treated animals confirming the non toxic nature of the active compound.

Statistical Analysis

Data are presented as the mean \pm standard deviation (SD). One way analysis of variance (ANOVA) followed by LSD was used to compare the means of different groups of by using SPSS 7.5 student version. Comparisons are made between groups: a-Groups I & II; b-Groups II & III, IV; c-Groups IV & III The symbols a, b and c also represents statistical significance at $P<0.05$.

Fig.1. Effect of berberine on lipid peroxidation in lung of control and experimental animals



Each value is expressed as mean \pm SD of six mice in each group.

Comparisons are made between groups: a - Groups I & II; b - Groups II & III, IV; c - Groups IV & III. The symbols a, b and c also represents statistical significance at $P < 0.05$.

Table 1. Effect of berberine on enzymic and non-enzymic antioxidants in the lung of control and experimental animals

Particulars	Group I	Group II	Group III	Group IV	Group V
Superoxide dismutase(IU /mg Protein/min)	5.30 \pm 0.50	3.50 \pm 0.47 ^a	5.14 \pm 0.63 ^{bc}	4.27 \pm 0.41 ^b	5.41 \pm 0.67
Catalase (μ moles of H ₂ O ₂ liberated /mg protein/min)	272.51 \pm 28.40	186.23 \pm 22.44 ^a	258.88 \pm 20.84 ^{bc}	215.65 \pm 28.32 ^b	276.30 \pm 27.05
Glutathione peroxidase (μ moles of GSH oxidized /mg protein/min)	45.35 \pm 4.73	29.21 \pm 4.37 ^a	44.01 \pm 4.12 ^{bc}	36.51 \pm 4.93 ^b	45.26 \pm 5.48
Glutathione reductase (nmoles of NADPH oxidized /mg protein/min)	2.48 \pm 0.28	1.56 \pm 0.12 ^a	2.36 \pm 0.29 ^{bc}	1.96 \pm 0.28 ^b	2.53 \pm 0.28
Glutathione (μ g/mg protein)	1.58 \pm 0.15	0.98 \pm 0.12 ^a	1.53 \pm 0.13 ^{bc}	1.28 \pm 0.14 ^b	1.62 \pm 0.23
Vit.E (μ g/mg protein)	0.68 \pm 0.06	0.41 \pm 0.05 ^a	0.65 \pm 0.08 ^{bc}	0.53 \pm 0.05 ^b	0.69 \pm 0.09
Vit.C (μ g/mg protein)	0.55 \pm 0.07	0.35 \pm 0.03 ^a	0.53 \pm 0.07 ^{bc}	0.44 \pm 0.05 ^b	0.58 \pm 0.04

Comparisons are made between groups: a-Groups I & II; b - Groups II & III, IV; c- Groups IV & III. The symbols a, b and c also represents statistical significance at $P < 0.05$. ^{NS}, Not significant

Table2. Effect of berberine on enzymic and non-enzymic antioxidants in the liver of control and experimental animals

Particulars	Group I	Group II	Group III	Group IV	Group V
Superoxide dismutase (Units /mg/protein/min)	5.98 \pm 0.62	3.63 \pm 0.51 ^a	5.68 \pm 0.46 ^{bc}	4.66 \pm 0.49 ^b	6.02 \pm 0.81
Catalase (μ moles of H ₂ O ₂ liberated /mg protein/min)	349.53 \pm 42.21	240.63 \pm 22.24 ^a	339.04 \pm 41.70 ^{bc}	292.36 \pm 31.21 ^b	353.61 \pm 33.85
Glutathione peroxidase (μ moles of GSH oxidized /mg protein/min)	49.75 \pm 5.99	31.71 \pm 2.56 ^a	48.26 \pm 6.37 ^{bc}	39.09 \pm 4.69 ^b	50.27 \pm 3.77
Glutathione reductase (nmoles of NADPH oxidized /mg protein/min)	3.79 \pm 0.43	2.46 \pm 0.19 ^a	3.62 \pm 0.44 ^{bc}	3.10 \pm 0.42 ^b	3.81 \pm 0.42
Glutathione (μ g/mg protein)	1.92 \pm 0.21	1.12 \pm 0.16 ^a	1.86 \pm 0.25 ^{bc}	1.51 \pm 0.18 ^b	2.01 \pm 0.15
Vit.E (μ g/mg protein)	0.82 \pm 0.09	0.52 \pm 0.06 ^a	0.78 \pm 0.06 ^{bc}	0.65 \pm 0.06 ^b	0.85 \pm 0.11
Vit.C (μ g/mg protein)	1.93 \pm 0.20	1.25 \pm 0.19 ^a	1.86 \pm 0.17 ^{bc}	1.52 \pm 0.21 ^b	1.92 \pm 0.23

Comparisons are made between groups: a-Groups I & II; b - Groups II & III, IV; c- Groups IV & III. The symbols a, b and c also represents statistical significance at $P < 0.05$.

Table 3. Effect of berberine on the levels of lipid peroxidation in basal and in the presence of inducers in liver of control and experimental animals

Parameters (nmol of TBARS formed/mg protein/min)	Group I	Group II	Group III	Group IV	Group V
Basal	0.95 \pm 0.08	1.44 \pm 0.19 ^a	1.00 \pm 0.12 ^{bc}	1.18 \pm 0.11 ^b	0.94 \pm 0.12
Ascorbic acid induced	1.92 \pm 0.20	2.88 \pm 0.35 ^a	2.05 \pm 0.29 ^{bc}	2.40 \pm 0.25 ^b	1.90 \pm 0.25
FeSO ₄ Induced	2.98 \pm 0.36	4.31 \pm 0.35 ^a	3.07 \pm 0.41 ^{bc}	3.59 \pm 0.43 ^b	122.96 \pm 0.22

Each value is expressed as mean \pm SD of six mice in each group.

Comparisons are made between groups:

a - Groups I & II

b - Groups II & III, IV

c - Groups IV & III

The symbols a, b and c also represents statistical significance at $P < 0.05$.

DISCUSSION

It is well established that antioxidant systems are critical in protecting against tumor promoting agents and that one or more components of these systems are deficient in many forms of cancer. Interestingly, cell malignancy or transformation is often accompanied by a decrease in activity of antioxidant enzyme.^[18] Their function is to protect membrane and cytosolic components against damage caused by free radicals. Superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) are endogenous enzymes that function as antioxidants inside the cells. Extracellular antioxidants, such as vitamin C, carotenoids and vitamin E are often of dietary origin and act by directly scavenging oxidants.^[19,20] Different types of antioxidants can act in a complementary or synergistic way to each other so that the reaction of one antioxidant can spare or even regenerate others.^[21] B(a)P is an effective carcinogen which interacts with membrane lipids and consequently induces free radical formation.^[22] Recent evidences show that B(a)P induces free radicals and oxidative DNA damage and forms DNA adducts.^[23] The first line of defense against O₂ and H₂O₂ are enzymic antioxidants such as SOD, CAT and GPx. SOD are a family of metalloenzymes that convert O₂ to H₂O₂. SOD is the most important enzyme because it is found virtually in all aerobic organisms. O₂ is the only known substrate for SOD in aerobic metabolism of the cells. SOD can act as anticarcinogen and inhibitor of initiation and promotion/transformation stage in carcinogenesis. In the present investigation, the decreased activity of SOD was observed in lung and liver of cancer bearing animals (Group II). Catalase is an enzymic antioxidant which catalyzes the decomposition of hydrogen peroxide to water and oxygen. This enzyme is a heme containing protein. It is localized mainly in mitochondria and subcellular respiratory organelles.^[24] A number of workers reported that CAT prevents chromosomal aberration caused by hypoxanthione in Chinese hamster cells.^[25] In the present study, decreased level of CAT was observed in cancer bearing animals. This might be due to the utilization of enzyme to scavenge the B(a)P induced free radicals. Pinto *et al.* 1980^[26] has reported that CAT activity was found to be lower in tumour tissues than in normal tissues. Glutathione peroxidase enzyme is a well-known first line of defense against oxidative stress, which in turn requires glutathione as a cofactor. GPx catalyzes the oxidation of GSH to GSSH at the expense of H₂O₂. Peskin *et al.*^[27] have reported decreased levels of GPx in cancer condition. Generally reduction of SOD and GPx in cancer conditions would be expected to have direct consequences. However, reduction in GPx is found to be more deleterious than SOD. The greater relative importance of GPx over SOD can be attributed to the ability of GPx to detoxify H₂O₂ formed by SOD.^[28] In this context Sivalokanathan *et al.*,^[29] also suggested that GPx and CAT were found to be important for quenching the free radicals. In the present study, the level of GPx was found to be decreased in Group II cancer bearing animals. Simultaneous (Group III) and post (Group IV) treatment of berberine restores the enzymic antioxidants SOD, CAT and GPx levels to near normal and confirmed the protective nature of berberine. Enzymic antioxidants are inactivated by hydroxyl radicals and hence the presence of non-enzymic antioxidant is presumably essential for the removal of these radicals. Non-enzymic antioxidants such as glutathione, alpha-tocopherol (Vitamin E) and ascorbic acid are mostly chain breaking antioxidants which interrupt the autocatalytic spread of radical reactions.^[30] Antioxidant vitamins have a number of biological activities such as immune stimulation, inhibition of nitrosamine formation and an alteration of metabolic activities of carcinogens.^[31] They can prevent genetic changes by inhibiting DNA damage induced by the reactive oxygen metabolites (ROMs).^[32] Lupulescu and Hormone^[33] indicated that cancer cell synthesized increased amount of DNA, RNA and proteins when compared to normal cells which may be controlled by the administration of vitamins. The major protective function of the vitamins against cancer is the scavenging of ROMs. Vitamin C (ascorbic acid) is an important water soluble non-enzymic antioxidant in biological fluids and an essential micronutrient required for normal metabolic functioning of the body.^[34] Vitamin C acts as a reducing agent of the hepatic cytochrome P450 mediated elimination of certain chemicals like aromatic chemicals and carcinogens.^[35] Plasma membrane devoid of vitamin C without other endogenous antioxidant is extremely vulnerable to oxidant stress and susceptible to peroxidative damage of lipids.^[36] In this connection, epidemiological studies have indicated an inverse association between vitamin C intake and the risk of cancers.^[37] Therefore, Vitamin C acts as a co-antioxidant by regenerating α -tocopheroxyl radical produced during scavenging of ROMs.^[38] In the present investigation, decreased level of Vitamin C was observed in cancer bearing animals. Thangaraju *et al.*^[39] have also reported a significant reduction in the concentration of serum Vitamin C in breast cancer patients. Decreased vitamin levels were associated with increased LPO in the serum of the patients with gastric carcinoma.^[40] Berberine increased the Vitamin C levels in simultaneous (Group III) and post (Group IV) berberine treated animals. Vitamin E is a lipid soluble vitamin present in plasma membrane. Among the variety of tocopherols occurred, alpha and gamma isomers are usually the major ones. Vitamin E neutralizes ROMs and reduces oxidative DNA damage and genetic mutations.^[41] Kimmick *et al.*^[42] opinions that Vitamin E is thought to be an important chain-breaking antioxidant which plays an important role in various stages of carcinogenesis through its contribution to immunocompetence, DNA repair and decreasing oxidative DNA damage. The major function of

Vitamin E is a physiological membrane bound antioxidant protecting the cell membrane lipids from oxidative damage initiated by ROMs. In the present study, decreased level of Vitamin-E was observed in cancer bearing animals. This might be due to utilization of this enzyme to quench the free radicals formed by the carcinogen. Berberine increased the Vitamin E levels in simultaneous (Group III) and post (Group IV) berberine treated animals. In the present investigation, berberine increased the enzymic and non-enzymic antioxidants levels in berberine simultaneous treated (Group III) and post (Group IV) treated animals. These results were in accordance with the finding of Shirwaikar *et al*.^[43] Thus, the revival of these enzymes in berberine simultaneous and post treated animals might be due to antioxidant activity of berberine and undoubtedly the active compound has favorable biological activity. Considerable attention has recently been focused on the inter-relationship of lipid peroxidation process, free radical reactions, and the development of a variety of pathological events. It is well established that lipid peroxidation (LPO) is the deleterious result of free radical reactions leading to disruption of biomolecules, dysfunction of cells and tissues.^[44] Malondialdehyde (MDA), which is a major end product and an index of LPO that cross-links DNA, protein, and nucleotides on the same and opposite strands.^[45] It is well documented that MDA is mutagenic in mammalian systems, which readily reacts with deoxyribonucleosides to produce adducts.^[46] MDA is a genotoxic agent that may contribute to the development of human cancer.^[47] B(a)P is an effective carcinogen which interacts with membrane lipids and consequently induces free radical formation.^[48] Free radicals react with lipids causing peroxidation which result in release of products such as malondialdehyde, hydro peroxides and hydroxyl radicals. An increase in hydrogen peroxides indicates serious damage to cell membranes, inhibition of several enzymes and cellular functions^[49] by Kim *et al*^[48] illustrated that free radicals participate in B(a)P induced lung carcinogenesis that is measured by over production of 8-hydroxyguanine in lung and the liver of a B(a)P administrated mice. In the present investigation, increased level of LPO was observed in lung and liver of cancer bearing animals. This might be due to the free radicals induced by B(a)P. However, the administration of berberine extract decreased the LPO levels in Group III and Group IV berberine treated animals.

To prove the anticancer activity of berberine, histopathological studies were also carried out. In the present investigation Group II cancer bearing animals showed marked destruction of alveoli with stromal proliferation and infiltration by inflammatory cells with alveolar wall thickening when compared to control animals. Simultaneous treatment with berberine (Group III) showed significant reduction in stromal proliferation when compared with cancer bearing animals and post treatment with berberine (Group IV) also showed marked reduction in stromal proliferation proving the cytoprotective nature of the active compound.

CONCLUSION

Remedial active principles of plant origin have been revealed to heal many diseases without adverse side effects. Berberine shown to be very active against experimental lung carcinogenesis. It significantly improved the antioxidant enzymes and proves its role as key modulator of lipid peroxides through its antioxidant nature and these observations are inexorably suggesting that the berberine could possibly protect against B(a)P induced carcinogenesis most likely through its strong antioxidant nature. Moreover, the Histopathological investigation also proves in cytoprotective nature of the active compound. Furthermore, studies are in progress in order to establish the exact molecular mechanism of the anticancer property of berberine against experimental lung cancer.

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