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# Anti-Cancer efficacy of Umbelliferone against Benzo(a)pyrene-induced Lung Carcinogenesis in Swiss Albino Mice

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## ABSTRACT

Coumarins and coumarin-related compound are a plentiful source of latent drugs candidate in relation to its protection and effectiveness. The recent study was deliberate to observe the therapeutic result of Umbelliferone (UMB) against Benzo(a)Pyrene induced carcinogenesis in Swiss albino mice. Administration of B(a)P to mice caused in augmented relative lung weight and serum marker enzymes (AHH ,λ-GT and LDH). The levels of lipid peroxides important (in both serum and tissue) with a subsequent reduction in the final body weight and tissue antioxidants like (SOD, CAT, GPx, GR and GST non enzymic antioxidants (GSH, vitamin C, vitamin E and vitamin A).UMB supplementation knowingly reduced these alterations, thereby showing prevailing anticancer effect in lung cancer. Histology examination also clearly showed that UMB significantly inhibited the lung carcinogenesis induced by B(a)P. Based on the results, this study clearly indicates the UMB used as an effective compound against lung carcinogenesis induced by B(a)P.

***Keywords***: Lung carcinoma, Umbelliferone, Benzo (a) pyrene, Antioxidant and LPO.

## 1. INTRODUCTION

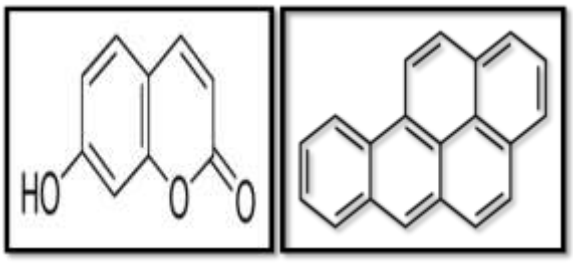
Lung cancer is a major cause of morbidity and mortality worldwide in both men and women, accounting for 29% of all cancers [1]. According to National Cancer Institute, USA, 1.4 million new cases are diagnosed and 1.2 million death rates are recorded every year globally [2].Tobacco smoking is well established as the major etiological risk factor for lung cancer, contributing to a tenfold proliferation in risk in long-term smokers compared with non-smokers [3]. Tobacco smoke contains over 60 known carcinogens. Among the constituents of smoke, the polycyclic aromatic hydrocarbons (PAHs) such as benzo (a) pyrene [B(a)], play a major role in lung carcinogenesis [4]. It is metabolically activated into benzo (a) pyrene 7,8-diol-9,10-epoxied that reacts with DNA predominantly to form an adduct and progression of the disease [5].

Several synthetic and naturally occurring compounds have been tested as chemo preventive agents against B(a)P-induced lung tumoregenesis [6]. Among them flavonoids are important substances that are

commonly present in the daily human diet. A variety of compounds have undergone clinical trials against lung cancer based on this strategy [7]. Flavanoids are naturally occurring molecules abundant in fruits, vegetables, nuts, seeds and beverages such as tea and wine. Benzo(a)pyrene (B(a)P) is a prototypical polycyclic aromatic hydrocarbon, formed by the deficient combustion of several organic materials and is ubiquitously there in our environment. A variety of anatomical sites of cancer are interrelated to ingestion and inhalation of polycyclic aromatic hydrocarbons. Benzo(a)pyrene (B(a)P) is one among the chief constituents of smoke components, and in vivo metabolic activation of (B(a)P) by xenobiotic enzymes leads to highly reactive metabolic activation predominantly by cytochrome P450 generates 7,8-diol-9,10-epoxide-benzo(a)pyrene, which is believed to be the ultimate carcinogenic metabolite of benzo(a)pyrene that leads to the development of DNA adducts [8]. If DNA adducts are not efficiently removed and repaired prior to DNA replication, mutations, DNA strand breaks, or other genetic alterations may result, which contribute to the process of carcinogenesis [9]. The bioactivation of benzo(a)pyrene, the enzymes implicated moreover the subsequent binding to DNA has been well-studied, including studies in both human liver and lung cells. Numerous synthetic and naturally stirring compounds have been tested as chemo preventive agents against B(a)P-induced lung carcinogenesis.

Figure 1 Coumarin, a benzopyrone compound is a plant-derived natural product widely distributed in numerous species belonging to different botanical families with the richest source being the Umbelliferae and Rutaceae families [10]. Umbelliferone (UMB) or 7- hydroxy coumarin, the major biotransformation product (75%) of coumarin is a widespread natural antioxidant with a short half-life [11] found predominantly in the edible fruits of golden apple (Aegle marmelos Correa) [12] and bitter orange (Citrus aurantium) [13].It is a yellowish-white crystalline solid which has a slight solubility in hot water, but high solubility in ethanol [14].UMB has been found to exhibits a wide range of pharmacological activities, such as antioxidant, anti-hyperglycmic [15], anti-tumor, anti-fungal[16], anti-asthmatic[17], ant-hyperglycemic[18], anti-tumor, antifungal[19], anti-pyretic, anti-inflammatory[20] and anti-nociceptive [21] effects. UMB displays anti-mutagenic and anti-carcinogenic effects against a variety of carcinogens including carbon tetrachloride, gamma radiation, diethyl nitrosamine, and 1,2-dimethylhyrazine [22-23]. Although it was reported to have many health benefits, the effect of UMB on lung cancer was limited. To date, although various biological activities of UMB have been demonstrated, its anticancer mechanism against carcinogenesis has never been explored. It has been indicated that UMB treatment prevents liver damage, lipid per oxidation and protects the antioxidant defense system. Further, it has been used in the synthesis of anticancer drug and in the treatment of asthma and allergic disorders. The effects of umbelliferone were also evaluated in order to elucidate in mechanism of action. To the best of our knowledge, the present study was aimed to elucidate the protective role of umbelliferone on B(a)P induced lung cancer by assessing lipid peroxidation (LPO) antioxidant tissue defense system, tumor marker enzymes. Histopathological study in lung tissue was done to substantiate the anti-cancer effect of umbelliferone against B(a)P induced lung carcinogenesis in Swiss albino mice.

**Figure 1.** Chemical Structure of Umbelliferone (A) and Benzo(a)pyrene (B)




## 2. MATERIALS AND METHODS

### 2.1 Chemicals

Benzo(a)pyrene and Umbelliferone were purchased from Sigma Chemicals, St Louis, USA. All other chemicals were of analytical grade procured from SRL Chemicals Pvt. Ltd, Mumbai, India.

### 2.2 Experimental animals

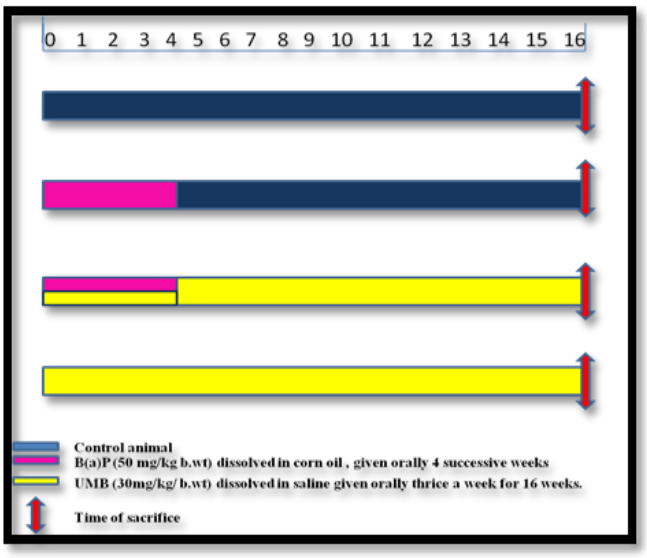
Healthy male Swiss albino mice weighing about 20-25 g (8–10 weeks old) were obtained from the Veterinary College (TANUVAS), Chennai, and all the experiments were deliberate and conducted according to the Institutional Animal Ethics Committee agreed guidelines. The animals were maintained in clean, hygienic cages, well ventilated room with forbidden temperature (25±2°C) and were acclimatized to 12-h light and dark cycles. Animals were fed with commercially available standard rat pellet feed (M/S Hindustan Foods Ltd, Bangalore, India) right

throughout the experimental period. The animals were free admittance to food and water. All the experiments were designed and conducted according to the ethical norms approved by Institutional animal ethics committee guidelines (IACE No:01/11/2018).

### *2.3 Experimental Design*

The experimental animals were divided into four groups, each group comprising of six animals (Figure 2). Group I Normal control mice were provided with corn oil through the experimental period along with standard diet and drinking water. Group II Mice were administered with B(a)P (50 mg/kg.bw, dissolved in corn oil, orally) twice a week for 4 successive weeks to induce lung cancer by 16$^{th}$ week [24]. Group III Mice were administered with UMB (30mg/kg.bw) to the B(a)P induced group of mice from the day1 till the end of the experimental period. Group IV Mice were administered with UMB orally (30mg/kg body weight, dissolved in corn oil) on all the days of the experimental period. At the end of the experimental period, the animals were anesthetized with ketamine (90 mg/kg) and xylazine (10 mg/kg) and sacrificed by cervical decapitation. Blood was collected and allowed to coagulate at room temperature for 30 min. Serum was detached by centrifugation at 3000 rpm for 15 min at 4˚C. The lung tissue was immediately separated and washed in ice-cold saline. The tissues were sliced and homogenized in 0.1 M Tris–HCL buffer (pH 7.4). The homogenates were centrifuged at 1000 rpm for 10 min at 4˚C in a cooling centrifuge. The supernatants were separated and used for analyzing various parameters.

**Figure 2:** Schematic representation of the experimental design




### *2.4 Biochemical Parameters*

Lung mitochondria were isolated by the method of Johnson and Lardy [25] and the following parameters were analyzed. Protein was estimated by the method of Lowry et al. [26] LPO was assayed by the method of Ohkawa et al [27] in which the malondialdehyde (MDA) released to serve as the index of LPO. Superoxide dismutase (SOD) was assayed according to the method of Marklund and Marklund [28] Catalase (CAT) activity was assayed by the method of Sinha [29], glutathione peroxidase (GPx) was determined by the method of Rotruck et al. [30]. Glutathione reductase (GR) was assayed by the method of Beutler [31]. Glutathione-Stransferase (GST) was assayed by the method of Habig et al. [32]. Reduced glutathione (GSH) was assayed by the method of Moron et al. [33], vitamin E was estimated by the method of Desai [34], vitamin C was measured by the method of Omaye et al. [35] and vitamin A was determined by the method of Bayfield and Cole [36]. The marker enzyme aryl hydrocarbon hydroxylase (AHH) was estimated by Mildred et al.[37], gamma glutamyl transpeptidase (λ-GT) [38], and lactate dehydrogenase (LDH) [39].

### *2.5 Histological Examination*

Histopathological assessment was performed on the lung and a portion of the specimen was fixed in 10% formalin and embedded in paraffin wax. Sections were cut at 4 µm in thickness, stained with hematoxylin and eosin and viewed under a light microscope for histological changes.

### *2.6 Statistical analysis*

The data were analyzed with SPSS/10 Software. Hypothesis testing methods integrated one way analysis of variance (ANOVA) followed by least significant difference (LSD) test. P values of <0.05 were considered to indicate the statistical significance. All the results were expressed as mean ± standard error (SE) for six animals in each group.

## 3. RESULTS

### *3.1 Effect of UMB on Body weight, Lung weight and tumor incidence in control and experimental group of animals*

Table 1 shows the effect of UMB on body weight, lung weight and tumor incidence in control and experimental group of animals. Significantly (P<0.05) increased lung weight and tumor incidence together with decreased body weight were observed in lung cancer-bearing animals. UMB treatment

markedly (P<0.05) increased body weight and reduced lung weight and tumor incidence in Group III animals.

### 3.2 *Effect of UMB on Lipid Peroxidation in control and experimental group of animals*

Figure 3 shows the extent of LPO in the serum and lung of control and experimental groups of animals. In B(a)P induced (Group II) animals, there was a significant (P<0.05) increase in the levels of lipid peroxides when compared with normal control (Group I) animals. Whereas UMB treated group III animals, there is a significant decrease in the levels of lipid peroxides when compared with group II induced animals. However, animals treated with UMB alone group IV did not show any significant changes when compared with Group I animal.

### *3.3 Effect of UMB on serum marker enzymes in control and experimental group of animals*

Figure 4 (A&B) shows the activities of marker enzymes AHH, LDH, γ-GT were found to be significantly (p<0.05) increased in lung cancer bearing animals (group II) animals administrated with B(a)P when compared with control (group I)animals, whereas treatment with UMB to the cancer bearing animals significantly (p<0.05) restores the level of these marker enzymes to their near normal values indicating it's anti-proliferative effect on lung carcinogenesis. However, the status of these marker enzymes in the UMB alone-administrated (Group IV) animals does not exhibit any significant alterations.

### *3.4 Effect of UMB on enzymic and non enzymic antioxidants in lung tissue of control and experimental mice*

Antioxidant status has been recommended as a useful tool in estimating the risk of oxidative damage induces carcinogenesis. Table II represents the activities of both enzymic antioxidants SOD,CAT,GPx GR, and GST and non-enzymatic antioxidants GSH, Vitamins C,E and A B(a)P induced group II animals exhibited a considerable reduced in compared with group I control animals of enzymic antioxidants SOD,CAT,GPx GR, and GST and non-enzymatic antioxidants GSH, Vitamins C,E and A. UMB treated Group III showed a significant increase in the activities of SOD and CAT when compared with Group II B(a)P induced animals. The activities of GPx, GR, and GSH also considerably decreased in B(a)P induced Group II tumor-bearing animals when compared with Group I control animals. However, the UMB treated animals Group III, there is a significant enhance in the activities of GPx, GR and GSH when compared with Group II B(a)P induced animals. No significant change was observed in UMB alone treated Group IV animals when compared with control animals.

### *3.5 Histology Examination*

Figure 5 shows the histological analysis of lung tissue section of control and experimental groups of animals. Group I control animals revealed normal architecture and small identical nuclei (5A). Lung cancer induced Group II animals showed loss of architecture with distorted alveoli as seen from increased number of hyper chromatic nuclei in the cells of alveolar wall (5B). Group III animals treated with B(a)P and UMB showed slightly reduced alveolar damage (5C) Group IV animals exhibited normal architecture indicating the anti-proliferative nature of UMB (5D).

## 4. DISCUSSION

Lung cancer is currently a leading cause of death all over the world. In recent years, considerable attention has been given to increased dietary intake of phytochemicals, since numerous epidemiological as well as experimental studies gave positive correlation between reduced risk of cancer and intake of phytochemicals [40].Benzo(a)pyrene, a well identified environmental carcinogen is known to produce enormous amounts of free radicals and these free radicals and non-radical oxidizing species are highly reactive, toxic and mutagenic [41]. These toxic radicals are involved in mediating tissue lipid per oxidation. Lipid per oxidation – induced tissue damage is the sensitive feature in the cancerous conditions and any deterioration of destruction of the membrane can lead to the leakage of these enzymes from the tissues [40]. Free radical mediated oxidative stress leading to lipid peroxidation is usually pronounced in all stages of development of cancer as well as in the genesis of other diseases [42]. Mitochondrion is now gaining importance in cancer research because of its central role as a regulator of energy balance and mitochondria appears to be the primary target for oxidative stress induced damaged during cancers as it has been suggested to be the main source of free radical production [43]. Our results in agreement with the above findings where we observed a increase in mitochondrial LPO in B(a)P induced lung cancer animals( Group II). It is chiefly accepted that the induction of antioxidant enzymes is a major approach for protecting cells against a variety of endogenous and exogenous toxic

**Table 1.** Effect of benzo(a)pyrene and UMB on body weight, lung weight, tumor incidence, inhibition rate in experimental group of animals.

| Parameters | Group I (Control) | Group II (Induced) | Group III (Treated) | Group IV (Drug alone) |
|---|---|---|---|---|
| Number of mice examined | 6 | 6 | 6 | 6 |
| Body weight (g) | 26.2 ± 2.80 | 17.8 ± 1.86 [a] | 25.5 ± 2.6[b] | 27.1 ± 2.9 |
| Lung weight (mg) | 286 ± 26.1 | 427 ± 51.3 [a] | 294 ± 3.11[b] | 284 ± 26.1 |
| Tumour incidence/mice (%) | 0 | 84.4 | 15.7 | 0 |

**Figure 3.** Effect of UMB on Lipid Peroxidation in control and experimental group of animals

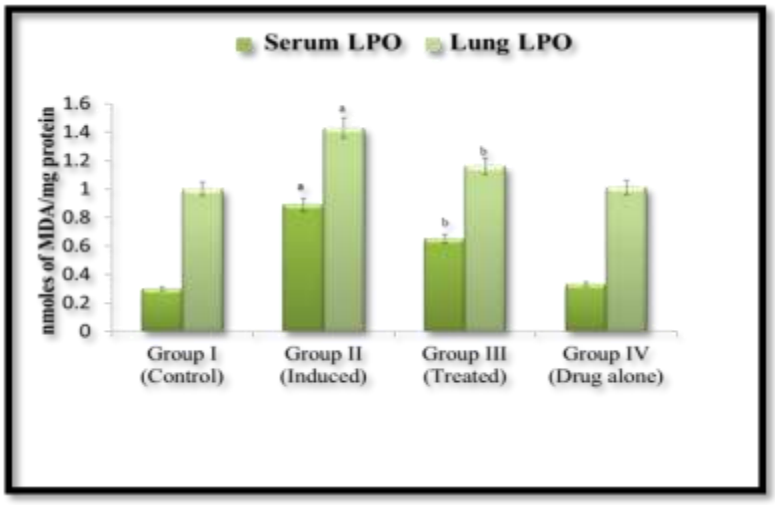
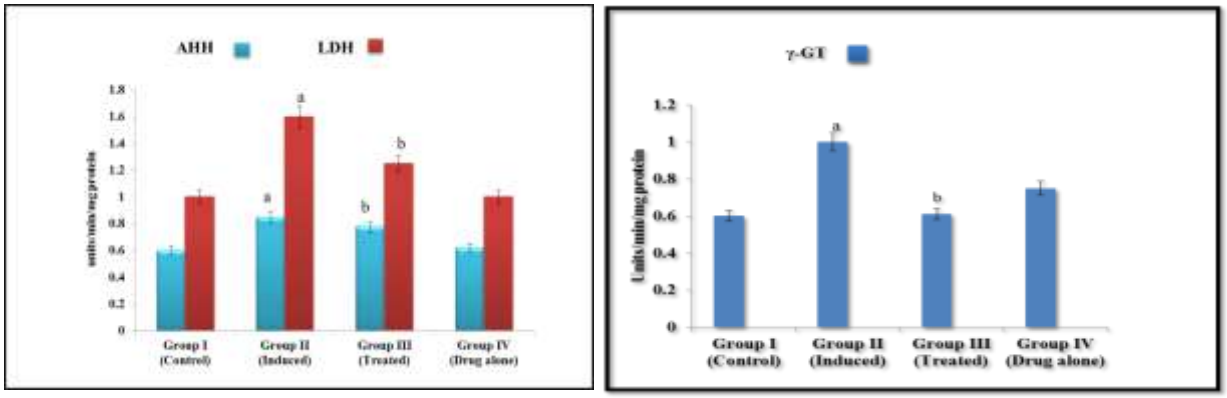



**Figure 4.** Effect of UMB on serum marker enzymes in control and experimental group of animals.




Effect of UMB on the activities of marker enzymes in the serum of control and experimental animals. Each value is expressed as mean ± S.D for (n=6). AHH, µmoles of fluorescent phenolic metabolites formed/min/mg protein; LDH, µmoles of pyruvate liberated/min/mg protein and γ-GT, nmoles of p-nitroaniline formed/min/mg/protein. Statistical significance at $p<0.05$. Group II[a] compared with Group I, Group II[b] compared with Group II[a].

**Table 2.** Effect of UMB on enzymic and non enzymic antioxidants in lung tissue of control and experimental mice.

Enzymic antioxidants

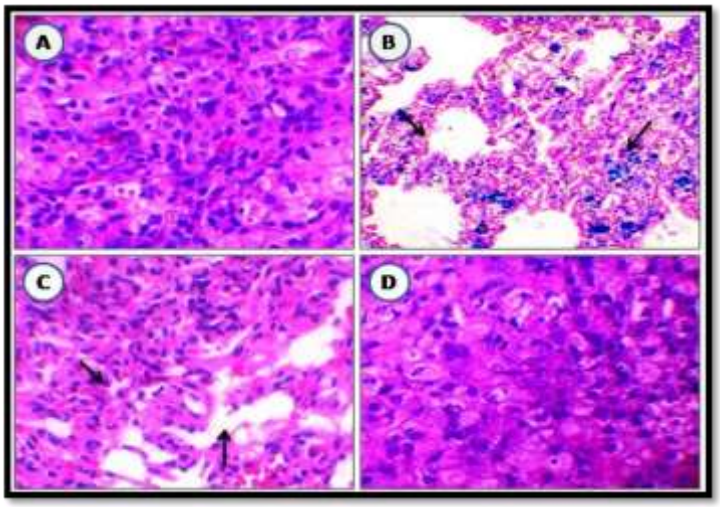
| Parameters | Group I (Control) | Group II (Induced) | Group III (Treated) | Group IV (Drug alone) |
|---|---|---|---|---|
| SOD | 6.20 ± 0.23 | 3.80 ± 0.21 [a] | 5.27 ± 0.29 [b] | 6.21± 0.25 |
| CAT | 159 ± 12.8 | 90 ± 8.68 [a] | 138 ± 12.5 [b] | 158 ± 12.7 |
| GPx | 4.83 ± 0.56 | 1.92 ± 0.18 [a] | 5.30 ± 0.19 [b] | 4.84 ± 0.57 |
| GR | 4.31 ± 0.16 | 1.25 ± 0.12 [a] | 3.05 ± 0.08 [b] | 4.30 ± 0.17 |
| GST | 43.24 ± 0.45 | 25.09 ± 0.39 [a] | 40.10 ± 0.67 [b] | 42.15 ± 0.44 |

Non-enzymic antioxidants

| Parameters | Group I (Control) | Group II (Induced) | Group III (Treated) | Group IV (Drug alone) |
|---|---|---|---|---|
| GSH | 1.48 ± 0.18 | 0.63 ± 0.05 [a] | 1.39 ± 0.08 [b] | 1.46 ± 0.16 |
| Vitamin C | 1.87 ± 0.07 | 0.79 ± 0.03 [a] | 1.01 ± 0.04 [b] | 1.68 ± 0.07 |
| Vitamin E | 1.65 ± 0.10 | 0.42 ±0.02 [a] | 0.79 ± 0.07 [b] | 1.64 ± 0.09 |
| Vitamin A | 1.64 ± 0.08 | 0.83 ± 0.03 [a] | 1.21 ± 0.05 [b] | 1.63 ± 0.06 |

Each value is expressed as mean ± SD for six mice in each group. Statistical significance at P<0.05 compared with a Compared with control and b Compared with B(a)P SOD - units/mg protein (one unit is equal to the amount of enzyme required to inhibit autoxidation of pyrogallol by 50%); CAT - µmoles of H2O2 consumed/min/mg protein; GPx: µmol of GSH oxidized/min/mg protein and GST: µmol of 1-chloro-2,4 dinitrobenzene conjugated/min/mg protein; GR: µmol of NADPH oxidised/min/mg protein; GSH, vitamin E,C and A:µg/mg protein.

**Figure 5.** Presents the histological examination of lung section (haematoxylin and eosin, 400x) of control and experimental group of animals.




Control animals showing lung parenchymal cells with normal alveoli (5A). Benzo(a)pyrene (B(a)P)-induced animals showing proliferation of closely packed alveolar cells with hyperchromatic nuclei (5B). B(a)P along with UMB- treated animals showing normal alveoli on one side with reduced number of hyperchromatic nuclei on the adjacent areas (5C). Umbelliferone (UMB) alone treated animals showing normal architecture (5D).

-compounds and chemical carcinogens [44]. Antioxidant status has been suggested as a useful tool in estimating the risk of oxidative damage induced carcinogenesis. Enzymatic antioxidants like SOD, CAT and Gpx synergistically scavenge reactive oxygen species (ROS) and prevent LPO. SOD is the only enzyme that disrupts superoxide radicals and protects the cells against superoxide and hydrogen peroxide-mediated LPO [45]. SOD, which converts superoxide radicals to hydrogen peroxide, is widely distributed in cells having oxidative metabolism and is thought to protect such cells against the toxic effects of superoxide anion [46]. CAT is a heme protein that catalyses the direct degradation of hydrogen to water .It protects the cellular constituents against oxidative damage [47] and it have been determined to be the most important members of enzymatic antioxidant defenses against ROS and they are closely related to the modulation of cytotoxicity [48].

GPx is a well-known first line of defense against oxidative stress, it catalyze the transformation of hydrogen peroxide to harmless byproducts, thereby curtailing the quantity of cellular destruction and several studies have reported the decreased activities of GPx in various cancerous conditions [49]. GR plays a major role in regenerating GSH from GSSG, thus maintain the balance between the redox couple [50]. Its a important enzyme for maintain the intracellular concentration of reduced glutathione. GST is a group of multifunctional proteins that total tasks ranging from catalyzing the detoxification of electrophilic compounds to protection against peroxidative damage. GST has been used as an important parameter for diagnosis and monitoring of lung malignancy, and it protects cells from mutagens and carcinogens as a free radical scavenger along with glutathione [51]. GSH plays a vital role in detoxification of xenobiotic compounds, in the antioxidant ion of ROS and free radicals [52]. Decreased GSH levels signify increased oxidative stress. In our present study, we have observed a decline in GSH levels in lung cancer bearing animals (Group II), which may be due to the excess utilization of this antioxidant for tumor cell proliferation. Antioxidant Vitamins have a number of biological activities such as immune stimulation, scavenging the free radicals and alteration in metabolic activation of carcinogens [53]. The availability of vitamin C is a determining factor in controlling and patenting many aspects of host resistance to cancer. Vitamin C, which prevents oxidative damage to cell membrane induced by aqueous radicals also exist d in interconvertable forms and participates in neutralizing free radicals [54] by regenerating the antioxidants from vitamin E [55]. Vitamin E is a principal lipid soluble antioxidant in cell membranes that protects critical cellular structures against oxidative damage [56]. The concentration of vitamin E has been inversely correlated to LPO. Vitamin A at pharmacological doses is reported to ameliorate oxidative stress mediated membrane lipid peroxidation and membranes enriched with vitamin A are protected against oxidative stress. Vitamin A as an antioxidant is involved in counteracting free radicals and is known to help in the repair of damaged tissues [57]. Vitamin C,E and A levels were found to be reduced in B(a)P administered groups suggesting an increase in LPO in these animals. The levels of these vitamins in mice challenged with B(a)P were almost normalized upon UMB treatment. In this study, B(a)P treated animals showed a significant decrease in the activities of enzymic antioxidant (SOD, CAT, GPx, GST, and GR) and non-enzymic antioxidant (GSH, vitamins C and E) deplicts the utilization of these molecules against lipid hydroperoxides and it turn shift redox balance to oxidative stress [58]. Analysis of serum marker enzymes serves as an indicator of cancer response to therapy. Distribution of many biochemical, immunological and molecular properties of the host has been observed in B(a)P mediated cancer conditions [59]. Marker enzymes such as AHH, λ-GT, LDH are serves as marker index of lung damage. AHH is a useful biomarker in the early diagnosis of lung cancer. λ-GT, activity is ubiquitously present in the plasma membrane of lung cells virtually and found in serum as marker of pathological state.λ-GT, catalyses the cleavage of λ-glutamyl peptide bond of GSH and various GSH conjugates [60]. LDH is essential for continuous glycolysis necessary for accelerated tumor growth, and is recognized as a potential tumor marker enzyme in assessing the proliferation of malignant cells. From these observations it can be concluded that the anticancer effect of UMB against B(a)P induced lung carcinogenesis in mice is due to ability to increase antioxidants and thereby inhibiting proliferation. Biochemical data were further confirmed the Histopathological studies were performed to further confirm the occurrence of apoptotic morphological changes at the cellular level. The Control animals showed normal nuclei and cytoplasm. The animals induced with B(a)P showed the occurrence of several irregular shaped nuclei was very close to each other in that irregular cytoplasm were also seen and which might be due to the extreme free radical generation during B(a)P administration. UMB alone treated animals showed normal architecture, so did not induce any intracellular morphology of lung tissue, which

ultimately shows its nontoxic nature at a given dosage.

## 5. CONCLUSION

Induction of antioxidant enzymes in the lung by UMB seen in the present study may contribute in protecting against oxidative stress induced carcinogenesis in Swiss albino mice. Being major constituents of golden apple and bitter orange, the potential role of UMB in human health warrants further investigation, including factors that influence uptake from the diet, metabolism and molecular basis of direct and indirect modulation of cancer cascades. Therefore, the present study concludes that UMB has potent chemo preventive due to its anti-lipid peroxidative, antioxidant potential modulating effect of phase I and phase II detoxification enzymes, as well as anti-cell proliferative and induced apoptosis in Benzo(a)Pyrene–induced lung carcinogenesis in Swiss albino mice.

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## Conflicts of Interest

There are no conflicts of interest.

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