ORIGINAL ARTICLE

Inhibitory effect of a mixture containing vitamin C, lysine, proline, epigallocatechin gallate, zinc and alpha-1-antitrypsin on lung carcinogenesis induced by benzo(a) pyrene in mice

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Background: This study was aimed to evaluate protective and therapeutic effects of a specific mixture, containing vitamin C, lysine, proline, epigallocatechin gallate and zinc, as well as alpha-1-antitrypsin protein on lung tumorigenesis induced by benzo(a) pyrene [B(a)P] in mice. **Materials and Methods:** Swiss albino mice were divided into two main experiments, experiment (1) the mice were injected with 100 mg/kg B(a)P and lasted for 28 weeks, while experiment (2) the mice were injected with 8 doses each of 50 mg/kg B(a)P and lasted for 16 weeks. Each experiment (1 and 2) divided into five groups, group (I) received vehicle, group (II) received the protector mixture, group (III) received the carcinogen B(a)P, group (IV) received the protector together with the carcinogen (simultaneously) and group (V) received the carcinogen then the protector (consecutively). **Results:** Total sialic acid, thiobarbituric acid reactive substances, vascular epithelial growth factor, hydroxyproline levels, as well as elastase and gelatinase activities showed significant elevation in group (III) in the two experiments comparing to control group ($P < 0.001$). These biochemical alterations were associated with histopathological changes. Administration of the protector in group IV and group V causes significant decrease in such parameters with improvement in histopathological alterations with improvement in histopathological alterations when compared with group III in the two experiments ($P < 0.001$). **Conclusion:** The present protector mixture has the ability to suppress neoplastic alteration and restore the biochemical and histopathological parameters towards normal on lung carcinogenesis induced by benzo(a) pyrene in mice. Furthermore, the present mixture have more protective rather than therapeutic action.

Key words: Alpha-1-antitrypsin, benzo(a) pyrene, epigallocatechin gallate, lung tumorigenesis, lysine, proline, protector, vitamin C, zinc

INTRODUCTION

Lung cancer is one of the most lethal cancers of the 20th century and still the most common cancer in the world causing up to 3 million deaths annually, and it is increasing at a rapid rate.[1,2] In Egypt, official statistics showed that lung cancer is the second most common cancer in men and second leading cause of cancer death, after bladder cancer.[3] Lung cancer often takes many years to develop. It usually starts with premalignant changes in the epithelium and develops from squamous metaplasia through carcinoma *in situ* to frank invasive cancer, which can break away and spread to other parts of the body.[4]

Lung cancer is largely attributable to environmental carcinogens. By far, tobacco smoke is the most important environmental carcinogen leading to lung cancer. Today, the epidemiology of lung cancer is the epidemiology of smoking. Experts estimate that 85-90% of lung cancers are caused by tobacco smoke.[5,6] Tobacco smoke contains over 4,000 chemical compounds. More than 50 chemical compounds have been recognized as human carcinogens. However, the major tobacco-related carcinogens are polycyclic aromatic hydrocarbons typified by benzo(a) pyrene and nitrosamine, which are likely to play major roles in lung cancer induction in smokers.[7]

Treatment for lung cancer revolves around surgery, radiotherapy and chemotherapy used either alone or in combination. All these forms have their downsides as they focuses exclusively on destroying the cancerous cells, but fail to deal with the principal anabolic imbalance that set the stage for the cancer development.[8]Moreover, the high mortality and the marginal improvement in the survival, taken together with the toxic side effects of these therapeutic approaches necessitate the sighting of novel agents with the potential to reduce the risk of lung cancer.[9]

The search for new chemopreventive and antitumor agents that are more effective and less toxic has kindled great interest in the naturally occurring constituents

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mainly in the nutritional substances.[10] Nutrients can directly affect tumor growth and metastases and a good nutritional plan can complement conventional cancer treatments by helping to strengthen the immune system, avoiding malnutrition, selectively starve the cancer cells and reduce the toxicity of medical therapies with enhancement of the anticancer activity of chemotherapy drugs. Also, nutrients act as biological response modifiers and can protect living cells against damage from free radicals. In addition, nutrients can revert cancer cells back to healthy cells in the early stages of cancer.[11] Researches have shown that nutritional supplements, such as vitamins, essential fatty acids, essential amino acids, bioflavonoids and minerals may play a vital role in filling the nutrient gap and can help the body to fight cancer.[12-14] Previously an international report demonstrated that a unique formulation composed of lysine, proline, vitamin C and epigallocatechin gallate exerts a chemopreventive effect on several types of cancer.[15] Also, earlier studies illustrate that zinc, an essential trace mineral; can play an important role in prevention and treatment of various types of cancer.[16,17] Moreover, the deficiency of alpha-1-antitrypsin protein can greatly increase the risk for the development of lung cancer.[18]

Based on the foregoing, a mixture composed of vitamin C, lysine, proline, zinc and epigallocatechin gallate along with alpha-1-antitrypsin protein was formulated in the present work to study its protective effects on different physiologic aspects involved in experimental lung tumorigenesis induced by B(a)P carcinogen in Swiss albino mice.

MATERIALS AND METHODS

Animals

The animal care and handling was done according to the guidelines set by the World Health Organization, Geneva, Switzerland and according to approval from the ethical committee for animals care at the National Research Centre, Egypt. Healthy male Swiss albino mice (6-7 weeks old) weighing 17-20 g were used in the present study. The animals were purchased from the animal house laboratory of the National Research Center, Cairo-Egypt. They were maintained under standard laboratory conditions of temperature and humidity on alternatively 12-h light-dark regimen and monitored for the duration of the study.

Experimental design

After housing the animals for a week, the mice were divided into two experiments. Eighty mice were used in experiment (1), which lasted for 28 weeks. Those animals were subdivided into five groups, Group I_1 (15 mice) served as control and were given vehicle only. Group II_1 (15 mice) were injected interproteneal (i.p.) twice weekly with the protector mixture till the end of the experiment. Group III_1 (20 mice) were gavaged orally once with (100 mg B(a)P/kg body weight dissolved in corn oil).[19] Group IV_1 (15 mice) were treated with the protector mixture as group II_1 simultaneously with B(a)P dose as group III_1. Group V_1 (15 mice) were treated with B(a)P dose as group III_1 then after 7 weeks received the protector mixture as group II_1.

In experiment, (2) 50 mice were used and the experiment lasted for 16 weeks. Animals were subdivided into five groups each contain 10 mice. Group I_2 served as control and was given vehicle only. Group II_2 animals were injected i.p. twice weekly with the protector mixture till the end of the experiment. Group III_2 animals were gavaged orally with 8 doses of B(a)P (each dose has 50 mg/kg body weight dissolved in corn oil).[20] Group IV_2 animals were gavaged with B(a)P as group III_2 and treated i.p. with the protector mixture as group II_2 after the first carcinogen dose. Group V_2 animals were gavaged with B(a)P as group III_2 then treated i.p. with the protector mixture after the last carcinogen dose till the end of the experiment.

Protector composition

The constituents of the protector mixture for each mouse are 1 mg lysine, 0.75 mg proline, 0.7 mg vitamin C, 0.1 mg epigallocatechin gallate, 0.2 mg $ZnCl_2$ and 0.1 mg alpha-1-antitrypsin. The selected dose based on preliminary study performed in our department.

Biochemical analysis in serum

Total sialic acid (TSA) level was estimated by periodate-resorcinol microassay according to Surangkul *et al*.[21] vascular epithelial growth factor concentration (VEGF) was determined using ELISA kit (Koma Biotech Inc., Korea) according to Kim,[22] alanine and aspartate transaminases (ALT and AST) were estimated as liver function tests, also urea and creatinine were estimated as kidney function tests were determined calorimetrically according to standard procedures using commercially available diagnostic kit (Biodiagnostic, Egypt).

Biochemical analysis in lung tissues

Lipid peroxidation was estimated by thiobarbituric acid reactive substances (TBARS) assay according to Lef'evre *et al*.[23] the gelatinases was estimated by zymography method according to Frederiks and Mook,[24] the elastase activity was assayed according to the method of Zay *et al*.[25] collagen breakdown was determined by hydroxyproline assay by the method of Edwards and O'Brien.[26]

Histopathological examination

For histopathological examination, portions of lung tissues were fixed in 10% formalin in saline, then embedded in paraffin wax, serially sectioned and stained according to Conn *et al*.[27] using a standard method of hematoxylin and eosin (H and E).

Statistical analysis

The statistical analysis of data were carried out by the aid of excel data sheets and statistical package for the social sciences software program (Texas, USA) (SPSS) version 9.0 the data then analyzed statistically using one-way analysis of variance (ANOVA); the levels of significance were evaluated with *P* values less than 0.05.

RESULTS

The mean serum total sialic acid levels of study groups are shown in Figure 1. TSA levels were significantly ($P < 0.001$) elevated in groups received B(a)P when compared with control animals of experiment (1) and (2). On administration of the protector mixture in Group IV and Group V of both experiments there was a significant ($P < 0.001$) decrease in TSA levels when compared with Group III. Where TSA level was improved in Group IV_1 and V_1 by approximately 31% from Group III_1, and improved in Group IV_2 by 41% and 35% in Group V_2 from Group III_2.

The mean TBARS levels in lung tissues of various groups are shown in Figure 2 TBARS levels were significantly elevated in groups III_1 ($P < 0.001$), IV_1 ($P < 0.01$) and V_1 ($P < 0.001$) when compared with Group I_1 of experiment (1). Similarly in experiment (2) TBARS levels significantly increased ($P < 0.001$) in groups III_2, IV_2 and V_2 when compared with Group I_2. On the other hand, administration of the protector mixture in Group IV and Group V of both experiments was resulted in a significant ($P < 0.001$) reduction in TBARS levels when compared with Group III. Where TBARS level was improved in Group IV_1 by 55% and 43% in Group V_1 from Group III_1, and improved in Group IV_2 and V_2 by approximately 40% from Group III_2.

The mean VEGF levels of various groups are shown in Figure 3. VEGF level was found to be significantly ($P < 0.001$) increased in Group III, which received B(a)P only of experiment (1) and (2) when compared with control group of experiment (1). This adverse change in VEGF level was reversed to near normal on administration of the protector mixture in either Group IV_1 or Group V_1 with significant decrease ($P < 0.001$) when compared with Group III_1, corresponding to approximately 47% reduction. However, VEGF level decreased significantly, in Group IV_2 ($P < 0.001$) and V_2 ($P < 0.05$) when compared with Group III_2 with 57% reduction in Group IV_2 and 19% in Group V_2.

The mean hydroxyproline levels of various groups are shown in Figure 4. In experiment (1) Group III_1 the hydroxyproline level was significantly increased ($P < 0.001$) when compared with Group I_1. However, administration of the protector in groups IV_1 and V_1 showed a significant reduction ($P < 0.001$) when compared with Group III_1 corresponding to 40% and 28%, respectively. In experiment, (2) hydroxyproline level also significantly increased ($P < 0.001$) in the group received carcinogen when compared with control animals. However, protector administration in both Group IV_2 and Group V_2 showed a significant decrease ($P < 0.001$) with approximately 37% reduction when compared with Group III_2.

The mean elastase activity of various groups is shown in Figure 5. Elastase activity in both experiments was significantly increased ($P < 0.001$) in groups received carcinogen when compared with control groups. On the other hand, administration of the protector in Group IV_1 showed a significant decrease ($P < 0.001$) with 25% reduction in elastase activity when compared with Group III_1 in experiment (1). Also, protector treated groups (IV_2 and V_2) in experiment (2) showed a significant decrease ($P < 0.001$) in the activity of elastase enzyme when compared with Group III_2, corresponding to 38% and 35% reduction.

A representative gelatin zymogram comparing gelatinolytic activity between the different groups is shown in Figure 6.

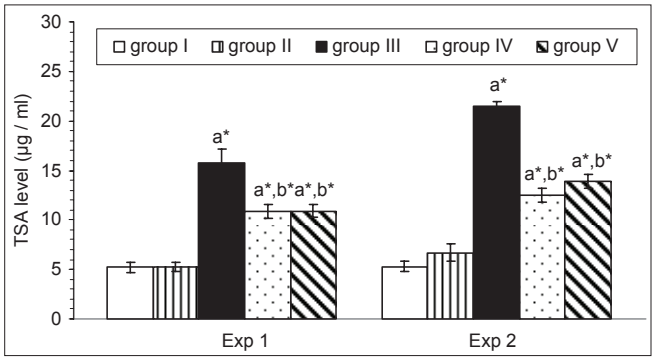

Figure 1: Effect of the protector mixture on TSA levels in serum of various studied groups of experiment (1) and (2). Data are presented as Mean ± SE, [a]significant difference from control group (I), [b]significant difference from B(a)P treated group (III), *statistical significance at $P < 0.001$

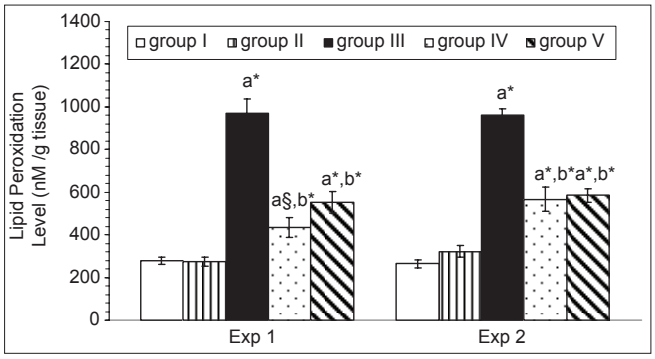

Figure 2: Effect of the protector mixture on lipid peroxidation as expressed as TBARS in the lung tissue of various studied groups of experiment (1) and (2). Data are presented as Mean ± SE for each group, [a]significant difference from control group (I), [b]significant difference from B(a)P treated group (III), *and § are statistical significance at $P < 0.001$ and $P < 0.01$ respectively

Gelatin zymography identified distinct gelatin lysis bands, corresponding to expression of MMP-9 and MMP-2 enzymes either latent or active forms. Interestingly, the gelatinase profile of both MMP-9 and MMP-2 was altered by treatment of the mice with the protector mixture.

The histological investigation of lung tissues from different studied groups revealed that, lung section taken from the control group demonstrating normal alveolar patternas shown in Figure 7a, also, sections taken from groups received the protector only indicated no pathological abnormalities [Figure 7b]. Pulmonary lesions induced after 28 weeks in Group III_1 showed squamous metaplasia where glandular epithelium transformed to squamous flat scale-like cells [Figure 7c], comparing to control group. Treatment with the protector in Group IV_1 showed similar histology to control group in 20% of samples while 80% of samples showed only moderate inflammation [Figure 7d]. But, Group V_1 showed moderate inflammation and mucosal thickening [Figure 7e]. In experiment (2) lung lesions induced after 16 weeks in Group III_2 were variable cellular pleiomorphism, coarse uneven chromatin, little cell maturation, and well-differentiated columnar cells with pleomorphic nuclei expanding within the lung parenchyma as finger-like projections various grades of dysplasia and papillary hyperplasia [Figure 7f]. Treatment with the protector in Group IV_2 improved the pulmonary lesions where only inflammation ranging from bronchitis to interstitial pneumonitis appeared [Figure 7g]. Whereas in Group V_2 moderate grade dysplasia was seen in Figure 7h.

DISCUSSION

The result of this study demonstrated that the combination of lysine, proline, vitamin C, EGCG and zinc nutrients in addition to alpha-1-antitrypsin protein shows great potential to inhibit B(a)P induced lung injuries via their synergy, and has the ability to modulate lung tumorigenesis in male Swiss albino mice using a safe and multi-targeted approach.

It is found that sialic acid, a sensitive marker for the progression of tumor growth[28] was significantly elevated in experiment (1) and (2) in the groups exposed to B(a)P as compared to control animals. It is noticeably to mention that serum TSA reached its highest level in groups received B(a)P only. According to Anandakumar *et al*.[29] B(a)P can induce

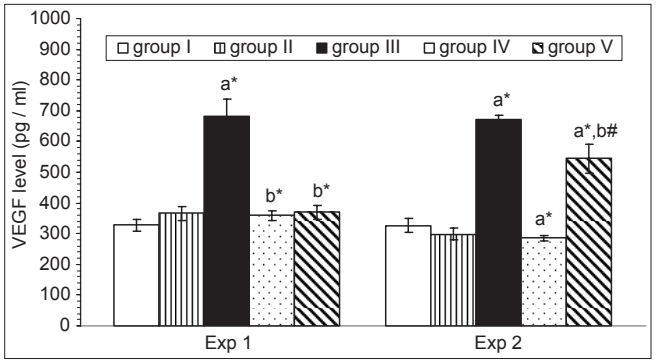

Figure 3: Effect of the protector mixture on the serum (VEGF) levels of various studied groups of experiment (1) and (2). Data are presented as Mean ± SE for each group, [a]significant difference from control group (I), [b]significant difference from B(a)P treated group (III), *and # are statistical significance at *$P < 0.001$, #$P < 0.05$

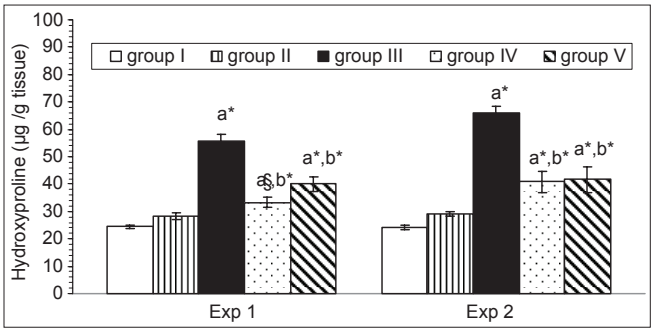

Figure 4: Effect of the protector mixture on hydroxyproline levels in the lung tissue of various groups of experiment (1) and (2). Data are presented as Mean ± SE for each group, [a]significant difference from control group (I), [b]significant difference from B(a)P treated group (III), *and § are statistical significance at $P < 0.001$ and $P < 0.01$ respectively

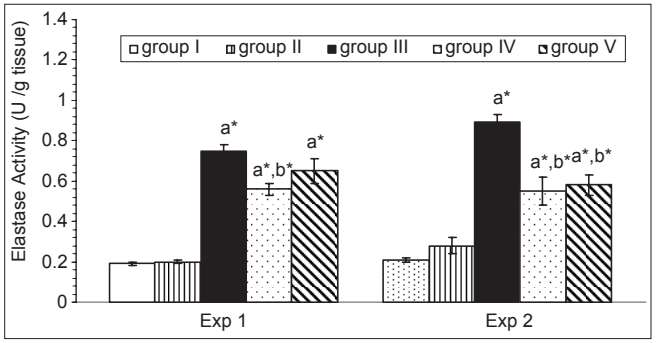

Figure 5: Effect of the protector mixture on elastase activity in the lung tissue of various groups of experiment (1) and (2). Data are presented as Mean ± SE for each group, [a]significant difference from control group (I), [b]significant difference from B(a)P treated group (III), *statistical significance at $P < 0.001$

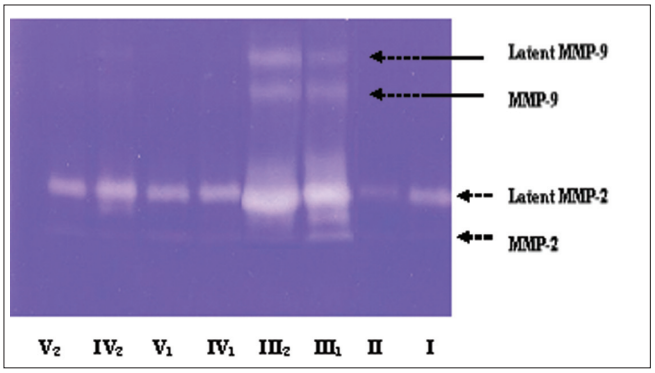

Figure 6: Gelatin zymogram showing four transparent bands in each lane corresponding to MMP-9 and MMP-2 expression, either latent or active forms in different groups

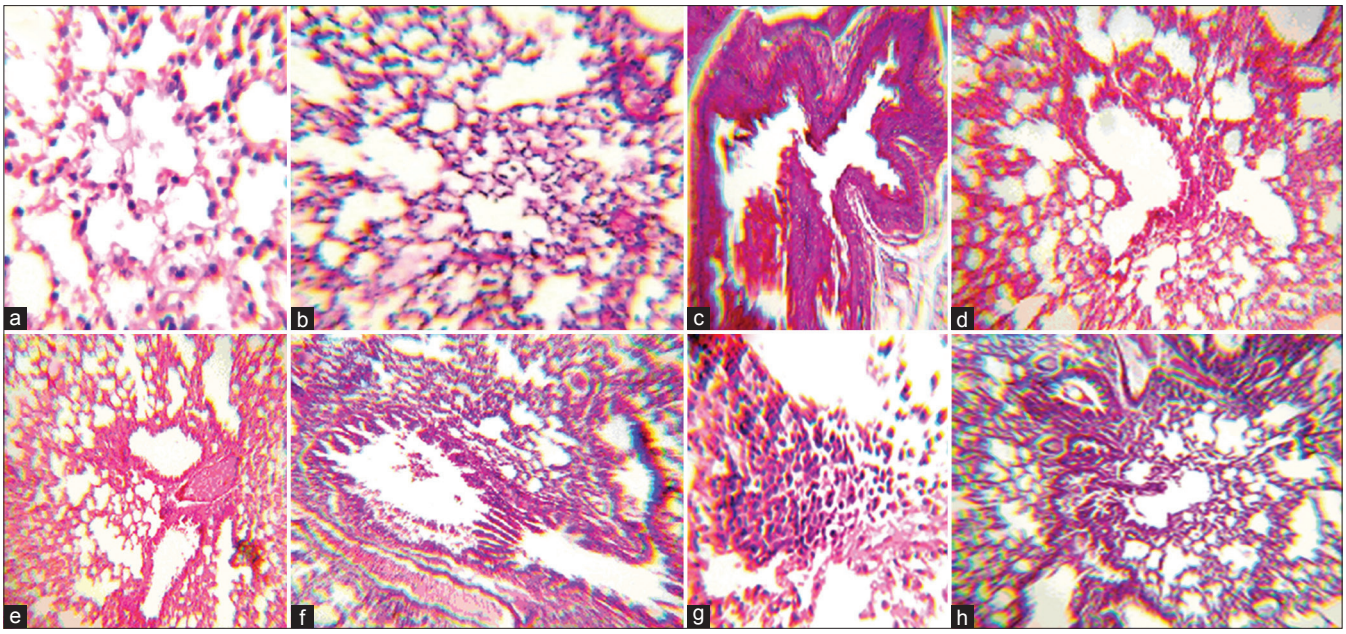

Figure 7: Represents the photomicrographs of mice lung sections in all studied groups. Sections were stained with hematoxylin and eosin (H and E, original magnification x100). (a) Lung of control mice revealed normal architecture (Group I); (b) lung of mice treated with protector mixture alone showed no pathological changes (Group II); (c) lung of mice treated once with 100 mg B(a)P/kg (Group III_1); (d) lung of mice treated by 100 mg B(a)P/kg body weight simultaneously with protector mixture (Group IV_1); (e) lung of mice treated with 100 mg B(a)P then after 7 weeks received the protector mixture (Group V_1); (f) lung of mice treated with 8 doses each of 50 mg B(a)P (Group III_2); (g) animals were gavaged with B(a)P and treated I.P with the protector mixture after the first carcinogen dose (Group IV_1); (h) animals were gavaged with 50 mg B(a)P then treated I.P with the protector mixture after the last carcinogen dose (Group V_2)

deleterious alterations in protein-bound carbohydrate components including sialic acid in Swiss albino mice. Suresh *et al*.[30] also mentioned that the cause of sialic acid elevation during tumorigenesis is through the shedding of sialic acid from the tumor cell surface or possibly as a product of the tumor itself. However, administration of the protector mixture in Group IV and Group V causes significant decrease in TSA levels when compared with Group III of the two experiments. This reduction in sialic acid level indicates that the present protector mixture has the ability to suppress neoplastic alteration by maintaining TSA status.

In the present study, we have observed significant increased levels of lipid peroxidation, assayed as TBARS, in the groups exposed to B(a)P, when compared with control groups. Such elevation in TBARS in these groups is due to the genotoxic property of B(a)P, which is a very effective carcinogen enhancing oxidative stress and consequently inducing free radical formation, which in turn react with lipids in the cell membrane causing lipid peroxidation.[31] On the other hand, the administration of the protector mixture in groups (IV) and (V) significantly reduces lipid peroxidation comparing with Group III in both experiments. This demonstrates that such protector mixture can modulate lipid peroxidation due to the strong antioxidant effects of vitamin C, EGCG and zinc in this protector mixture. According to Kato *et al*.[32]; Weyers *et al*.[33] vitamin C is a potential scavenger of superoxide radical and singlet oxygen and can intercept free radical induced chain reaction and prevent further oxidation. Moreover, vitamin C is in a unique position to scavenge destructive peroxyl radicals before they have a chance to damage lipids. Also, EGCG has been shown to have strong antioxidant activity as it is capable of protecting cells against oxidative stress by direct scavenging of reactive oxygen species and inhibiting lipid peroxidation.[34] EGCG may also function indirectly by inhibiting both redox-sensitive transcription factors and pro-oxidant enzymes.[35] In addition, it is reported that zinc is required for the activity of some antioxidant enzymes and results in induction of some other ultimate antioxidants proteins. Also, its presence in zinc-containing enzymes was found to protect specific regions of these enzymes from free radical attack.[36]

VEGF as reliable marker of angiogenic activity sustaining tumor growth was evaluated in the present work.[37] In both experiments (1) and (2) VEGF level was found to be significantly increased in Group III when compared with control groups. According to Bergers and Benjamin[38] inhibiting tumor angiogenesis, can block one of the fundamental requirements for tumor growth. In this study, it was found that protector administration improves VEGF level, in both experiments. VEGF level decreased significantly in Group IV and Group V when compared with Group III. This reduction in VEGF value after the protector administration could be related to the antiangiogenic actions of the protector mixture.

Several studies also showed that vitamin C has antiangiogenic properties. Ashino *et al*.[39] mentioned that vitamin C affects angiogenesis through its antioxidant activity and through stimulating collagen synthesis where angiogenesis involves degradation of the extracellular matrix proteins surrounding the pre-existing vessels. Also, Mikirova *et al*.[40] hypothesized that vitamin C may exert antiangiogenic effects through its inhibition of nitric oxide, the major stimulus of tumor angiogenesis. Shankar *et al*.[41] reported that EGCG also has anti-angiogenic property and reduces tumor growth through suppression of a variety of VEGF-induced angiogenic processes such as the inhibition of protein kinases B and vascular endothelial-cadherin phosphorylation. Also, EGCG could inhibit angiogenesis by suppressing cellular production of VEGF itself and inhibits formation of VEGF-receptor complex.[42] According to Uzzo *et al*.[43] zinc supplementation may have important implications for inhibiting the angiogenic potentials of malignant cells, predominantly through suppression of NF-B signaling, the transcription modulator of VEGF.

Tumor growth and invasion essentially requires the degradation of basement membrane components and extracellular matrix macromolecules, which considered the physical protective barriers in restraining the expanding growth of tumor cells.[44] Proteinases are vital proteolytic enzymes that play a central role in facilitating neovascularization and tumor growth by degrading or remodeling such extracellular matrix components that constitute the pericellular connective tissue, therefore the status of the pulmonary connective tissue was monitored. The data of our study revealed that all animals received B(a)P in the two experiments had significantly altered proteolytic enzymes comparing to the control animals, which is represented by highly expressed gelatinase-A and gelatinase-B, with elevation of both elastase activity and hydroxyproline content the marker of collagen breakdown. However, these parameters in all groups received the mixture together with B(a)P showed a significant reduction when compared with groups received the carcinogen only.

From these findings we postulated that this mixture could be a powerful natural protector against matrix degradation and could strengthen the connective tissue. This can be explained by the fact that nutrients such as lysine, proline and vitamin C acid could act as natural inhibitors of matrix proteolysis through increased connective tissue strength and stability surrounding tumor cells and, as such, they have the potential to modulate tumor growth, contributing to encapsulation of the tumor.[15] Furthermore, the presence of alpha-1-antitrypsin (the major circulating inhibitor of the proteases) in the mixture, could reverse the biochemical abnormalities caused by excessive collagenolytic activity and could interact with elastase enzyme to shield the lungs from its effect by neutralizing its action on increased lysis of extracellular matrix macromolecules.[45,46] Also, vitamin C has a critical role in stability of extracellular matrix structure and prevents degradation action of the ground substance surrounding the tumor.[15] As well, it is an effective biocatalyst that modifies lysine and proline needed for collagen support. Moreover, vitamin C itself stimulates the production of new collagen and strengthens connective tissue.[47] Furthermore, Maeda-Yamamoto *et al*.[48]; Hazgui *et al*.[49] demonstrated that EGCG is a direct and potent inhibitor of gelatinases due to its ability to form a reversible complex with them. At the same time, it can cause suppression of extracellular signal-regulated kinase phosphorylation, which results in the inhibition of gelatinases expression, leading to the reduction of their enzyme activities in cancer cells. Moreover, EGCG is a potent natural inhibitor of elastase enzyme thus reducing elastase-mediated progression to tumor invasion.[50,51]

Histopathological findings confirmed that groups received B(a)P only in the two experiments have lung lesions that were distributed in the lung alveoli and bronchi. Such abnormalities were basal cell hyperplasia and squamous metaplasia and various grades of dysplasia, which was considered to be important precancerous lesions. On the other hand, treatment of groups with the protector mixture was shown to markedly reduce the lesions resulted from B(a)P and restored the abnormalities in lung tissues towards normal with minimal histological changes.

In conclusion, the results of the present study demonstrate that the dosage of B(a)P carcinogen highly affects on many parameters estimated in the present study such as TSA, elastase enzyme, and hydroxyproline. Where such parameters were highly altered in experiment (2), which received higher B(a)P dose than experiment (1) which received lower dose of the same carcinogen. Furthermore, the actions of such mixture on almost all parameters are more effective in groups received the mixture simultaneously with B(a) P than in groups received the mixture consecutively after the carcinogen dose indicating that, the present mixture has more prophylactic action rather than therapeutic action and it can be used as a safe protective agent because it has no adverse effects on liver and kidney since there is no change in both liver and kidney function tests (data not shown).

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