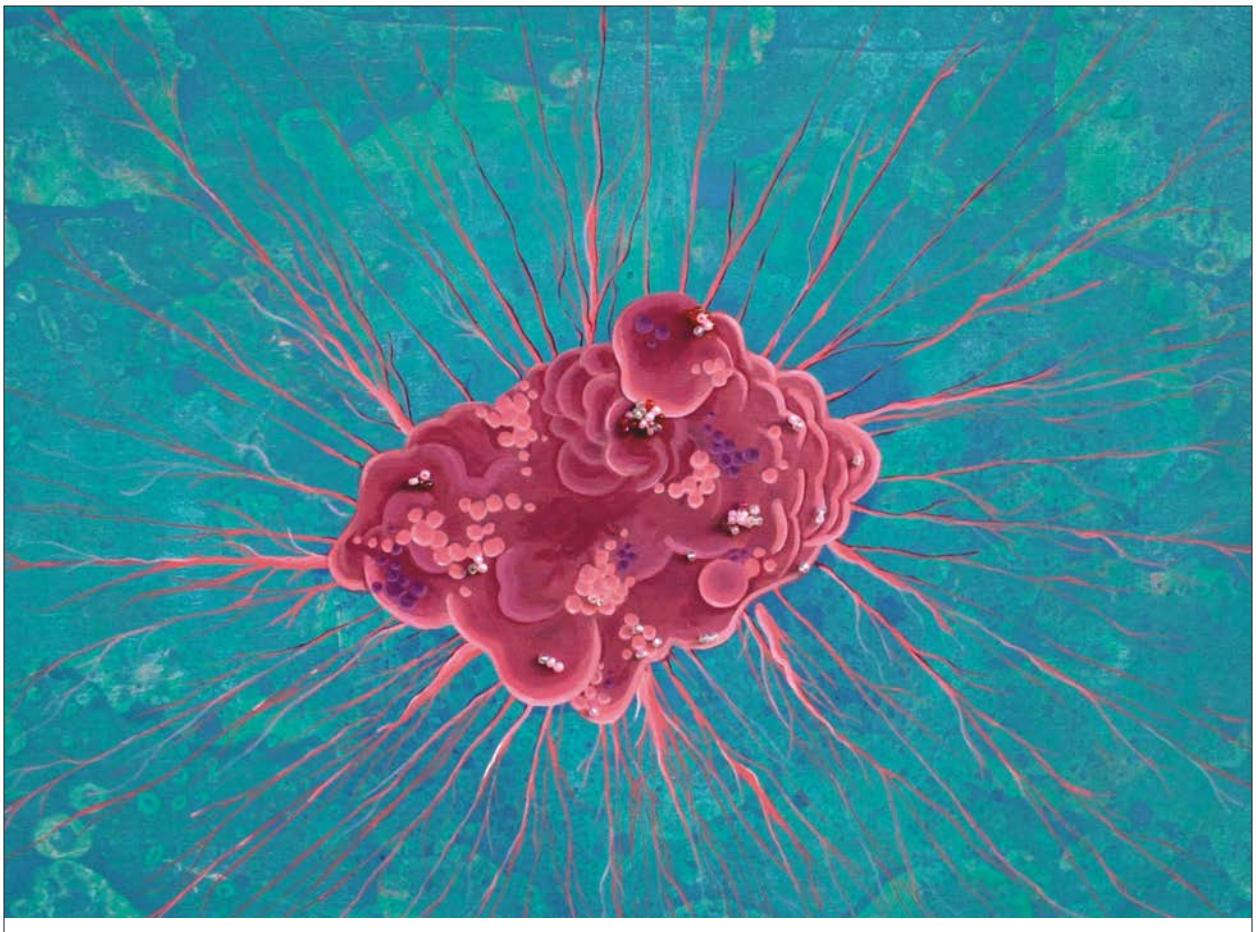

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Anti-Angiogenic Effectiveness of the Pomegranate Against Benzo(a)Pyrene Induced Lung Carcinoma in Mice

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ABSTRACT

Pomegranate, a constituent of flavonoids, ellagitannins and ellagic acid is exposed to exert compelling anti-carcinogenic effects. In the present study, we examined the anti-angiogenic and anti-tumorigenic potential of pomegranate juice on benzo(a)pyrene-induced mice lung carcinoma by analyzing the SOD and CAT anti-oxidants, MDA for oxidative stress and the marker of angiogenesis (CD34). Oral administration of pomegranate (10% w/v) to Swiss albino mice suppressed the development of lung carcinoma by disappointment MDA, histopathological lesions and the Micro-Vessels Density (MVD). The significant correlation ($p<0.05$) was present in an improvements the SOD and CAT anti-oxidants. The results obtained from the present study show that pomegranate inhibits the development of mice lung carcinogenesis through its ability to induce apoptosis and disappointment the formation of new vessels (angiogenesis). Our present findings were that the protective benefits of pomegranate juice may be due in part to its potent anti-oxidant properties and ability to reduce oxidative stress, histopathological injuries by suppressing the formation of reactive oxygen species and protecting the anti-oxidant mechanism and inhibition of MVD. So, this study suggested that the pomegranate is anti-angiogenic effectiveness in the benzo(a)pyrene induced lung carcinoma in mice.

Key words: Benzo(a)pyrene, lung carcinoma, pomegranate, chemoprevention, microvessels density, MVD

INTRODUCTION

Lung cancer is one of the most common malignant tumor in the world today, its incidence and mortality rank the highest among all malignant tumors. Deaths from lung cancer accounts for more than 17% of all cancers (Sikdar *et al*., 2014). According to the World Health organization, lung cancer is the most commonly diagnosed cancer with 1.4 million new cases reported every year and is the leading cause of cancer mortality worldwide exceeding the mortality rates of colorectal, breast and prostate cancers combined (Khan *et al*., 2012). It is usually detected at an advanced stage, which is not curable as it cannot be treated surgically or with radiation treatments. Smoking of tobacco is the main subject for lung carcinoma and is responsible for approximately 90% of all lung cancer cases (Ravenel, 2013). The occurrence of lung cancer drops very slowly after the termination of smoking, suggesting that ex-smokers are also at significant high risk for developing lung cancer (Bunn, 2012). Lung cancer can be divided into two major histopathological groups: Non-small-cell lung cancer (Van Zandwijk *et al*., 1995) and small-cell lung cancer (Schiller, 2000). About 80% of lung cancers are non-small-cell lung cancer and they are subdivided into squamous cell, large-cell adenocarcinoma and bronchioalveolar (Travis, 2011). Squamous cell carcinomas and adenocarcinomas are the most prominent. The remaining 20% of lung cancers show properties of neuroendocrine cells.

For lung cancer treatments, a thoracic surgeon is specially trained to perform lung carcinoma surgery. The target of surgery is the total removal of the lung tumor mass and the nearby lymph nodes in the chest (Colice *et al*., 2007). The 2nd type of treatments is radiation therapy which is the use of high energy X-rays or other particles to destroy cancer cells in the lung (Brooks *et al*., 1990). Like surgery, radiation therapy cannot be used to treat widespread cancer and followed by radiation and chemotherapy treatments. Radiation only destroys cancer cells directly in the path of the beam of radiation. It also injures the normal cells in its way, for this motive, the radiation cannot be used to treat large regions of the body. The chemotherapy use of drugs to destroy carcinoma cells, usually by blocking the ability cancer cells to divide and grow. It has been shown to improve both the quality and length of life for people with lung carcinoma of all stages. Cancer and its treatment often cause additional effects. In addition to treatment to stop, slow, or exclude the cancer, a significant part of cancer care is alleviating a person's symptoms and side effects (Bergman *et al*., 1994).

The importance of diet in the prevention of some diseases is well recognized (Yao *et al*., 1998). Natural antioxidant constituents are very significant in cosmetics or food businesses because of the capacities to decrease free radical mediated degradations of cells and tissues in organisms of human (Szyd*ł*owska-Czerniak *et al*., 2010). Therefore, attention has shifted to nutritious or non-nutritive phytochemicals present in natural plant-based diet as potential chemopreventive agents. It is now estimated that more than 1000 different phytochemicals possess chemopreventive activities (Surh, 2003).

Current scientific interest in the management of cancer is directed toward the utilization of naturally occurring compounds for chemotherapeutic (Aggarwal and Shishodia, 2006). In recent years, increasing attention has been focused on plant food-derived phytochemicals as potential anticancer drugs. Approximately, 70% of all drugs used nowadays for the treatment of cancer are derived from natural products (Ashokkumar and Sudhandiran, 2011). Fruits' polyphenols, spices and vegetables have illustrated anti-carcinogenic and anti-inflammatory activities *in vitro* and *in vivo* (Prakobwong *et al*., 2011).

Pomegranate (*Punica granatum* L.) is polyphenols rich. The mainly and therapeutically felicitous compounds are flavonoids, ellagitannins, ellagic acid and 3-glucosides/3,5-diglucosides of the cyanidin, anthocyanins delphinidin and pelargonidin (Jurenka, 2008), that exert anti-carcinogenic, anti-inflammatory and anti-oxidant activities *in vitro* and *in vivo* (Ismail *et al*., 2012). Polyphenolics from pomegranate juice and peels inhibited aromatase activity relevant to the prevention of breast cancer (Sreeja *et al*., 2012), exhibited cytotoxic activities in hepatocellular carcinomas in rats (Bishayee *et al*., 2011) and suppressed chemical-induced colon cancer in rats (Kohno *et al*., 2004).

Thus, this study was designed to evaluate the effects of *Punica granatum* juice in mice models of lung cancer induced by benzo(a)pyrene.

MATERIALS AND METHODS

Materials: Benzo(a)pyrene and standard enzymes (SOD, CAT) were purchased from Sigma-Aldrich Corporation (St. Louis, MO), avidin-biotin kit and CD34 antibody were purchased from Zymed (Zymed Laboratories, USA). The *Punica granatum* was purchased from local market in Damanhour, Egypt.

Preparation of pomegranate extract: Fresh fruit of pomegranate procured from the local market was washed and the outer skins were hand-peeled. Following peeling out, the edible portion

(seed coat and juice) was squeezed in distilled water (1:10 by wt/vol.). The juice (extract) had a deep-red color. The red extract was filtered through filter paper (Whatman No. 1). Pomegranate extract was prepared freshly thrice a week to be used for oral feeding to animals.

Animals: Male albino mice (20-25 g), were supplied by Laboratory Animal Farm, Helwan, Egypt. The local committee approved the design of the experiments and the protocol conforms to the guidelines of the National Institutes of Health (NIH). The mice were housed in standard plastic cages (12 mice/cage) at an environmentally controlled room (constant temperature 25-27°C, with a 12 h light/dark cycle) before and during the experiments. The mice were fed a standard diet. Water was supplied *ad libitum*. Four groups were used, all treatments were given for 16 weeks were treated as follows:

Group I : Control group was corn oil received
Group II : Received pomegranate extract juice
Group III : Animals were treated with benzo(a)pyrene in corn oil (50 mg kg^{-1} b.wt. oral administration twice a week for 5 consecutive weeks, from week 1-6
Group IV : Animals were subjected to treatment with Pomegranate extract juice from the 1st to 16th week, twice a week as benzo(a)pyrene. Benzo(a)pyrene was managed to the animals simultaneously from the week 1-6 for induction of lung carcinoma

Anti-oxidants and oxidative stress (Malondialdehyde): At the end of the experimental time, the mice were sacrificed by cervical decapitation. Lung tissues were instantly excised, weighed and then homogenized in 0.1 M Tris-HCl buffer (pH 7.4). The lung tissue homogenate was taken for the SOD, CAT and MDA analysis as described. The activity of superoxide dismutase (SOD) was estimated by the method of Marklund and Marklund (1974). The enzyme activity is defined as units/mg protein. The activity of catalase (CAT) was estimated by the method of (Sinha, 1972) and expressed as nmol of H_2O_2 consumed/min/mg protein. The extent of the peroxidative reactions was determined by measuring MDA in tissue homogenates. The MDA was measured by the thiobarbituric acid method (Buege and Aust, 1978).

Histopathological observations: For histological observations, the formalin-fixed tissue samples were paraffin embedded, thin-sectioned and then mounted on microscopic slides using the standard histopathological techniques. The tissue sections were stained with hematoxylin and eosin (H and E) and examined by light microscopy.

CD34 expression analysis: Immunohistochemical analysis of formalin-fixed, paraffin-embedded tissue was accomplished using the avidin-biotin complex immunoperoxidase method. Slides were deparanised in xylene, hydrated in graded alcohol and endogenous peroxidase activity was blocked by 30 min treatment with 3% hydrogen peroxide in absolute methanol at room temperature. Sections were then incubated in Phosphate-Buffered Saline (PBS, pH 7.4) and treated with the primary monoclonal antibody specific for CD34 (1:200). The section was rinsed in PBS and incubated for 30 min with biotinylated secondary antibody. Streptavidin-conjugated peroxidase was applied and finally sections were rinsed with PBS, developed with di-aminobenzidine tetrahydrochloride substrate (DAB, Chromogen) for 3 min and counter stained with hematoxylin.

Detection of microvessel density (MVD): The MVD was assessed through immunohistochemical analysis with antibody to the endothelial marker CD34 and determined according to the method of Weidner *et al*. (1993). Briefly, the immunostaining sections was initially screened at low magnifications (10× and 40×) to identify hot spots (areas with the highest microvessel numbers), which are the regions of highest neo-vascularization. Any brown color stained endothelial cell or endothelial cell cluster that was clearly separated from adjacent microvessels. Within the hot spot region, the stained microvessels were counted in a single high-power (400×) field and the average vessels count in three hot locations was considered the value of MVD.

Statistical analysis: All results are expressed as the Mean±SD. Data was analyzed with a one-way ANOVA. The differences among the group was considered significant when $p<0.05$.

RESULTS

The tumor formation was shown in the benzo(a)pyrene treated group. In group 3 treated with benzo(a)pyrene plus pomegranate extract juice revealed inhibition of lung tumor growth in 9 of 12 mice.

Anti-oxidants and oxidative stress observations: In the Table 1, the SOD and CAT were highly significant decreasing in the benzo(a)pyrene injected group but the animals treated pomegranate juice were no differences when compared with control one. The superoxide dismutase and catalase were significant increasing in benzo(a)pyrene treated with pomegranate juice. The MDA was highly significant increasing in benzo(a)pyrene injected animal group when compared with the control group. While the MDA was significant decreasing in benzo(a)pyrene treated with pomegranate juice group.

The histolopathological results of lung tissues were focused on proliferative, inflammation necrosis lesions. The histolopathological evidences of premalignant or malignant neoplasms were not found within the control group or pomegranate juice treated group (Fig. 1a-b). On the other hand, the tissues of mice treated with benzo(a)pyrene and treated with pomegranate juice showed the signs of protection in lung tissue by the reduction of cell proliferation, appearance of inflammatory cells and hyperplasia in addition to normal histology similar to that of the control group except some lesions (Fig. 1f). Conversely, mice that injected with benzo(a)pyrene alone produces 100% lung carcinogenesis (Fig. 1c-e).

Table 2 and Fig. 2a-d show the immunohistochemical expression of the CD34 angiogenetic factor evaluated. The MVD was determined by counting the microvessels number/high-power field (400×) in the lung tissues stained with CD34 antibody. Mice in group 3 that injected with benzo(a)pyrene alone revealed increasingly higher MVD counts in CD34 positive stain in lung tissue than those of mice from the control, pomegranate juice and benzo(a)pyrene treated with pomegranate juice groups.

Table 1: Antioxidants (SOD and CAT) and MDA in control, Pomegranate, Benzo(a)pyrene and Benzo(a)pyrene treated with Pomegranate juice groups

	Antioxidants (U mg^{-1})		
Groups	SOD	CAT	MDA (nmol g^{-1})
Control	610.09±18.70	208.22±22.88	10.32±1.83
Pomegranate juice	640.13±20.70	245.04±17.67	8.34±0.99
Benzo(a)pyrene	498.18±20.80*	111.10±11.12*	23.45±3.09*
Benzo (a)pyrene+pomegranate juice	585.93±17.12	198.56±38.89	11.97±1.98

*$p<0.05$ (significant)

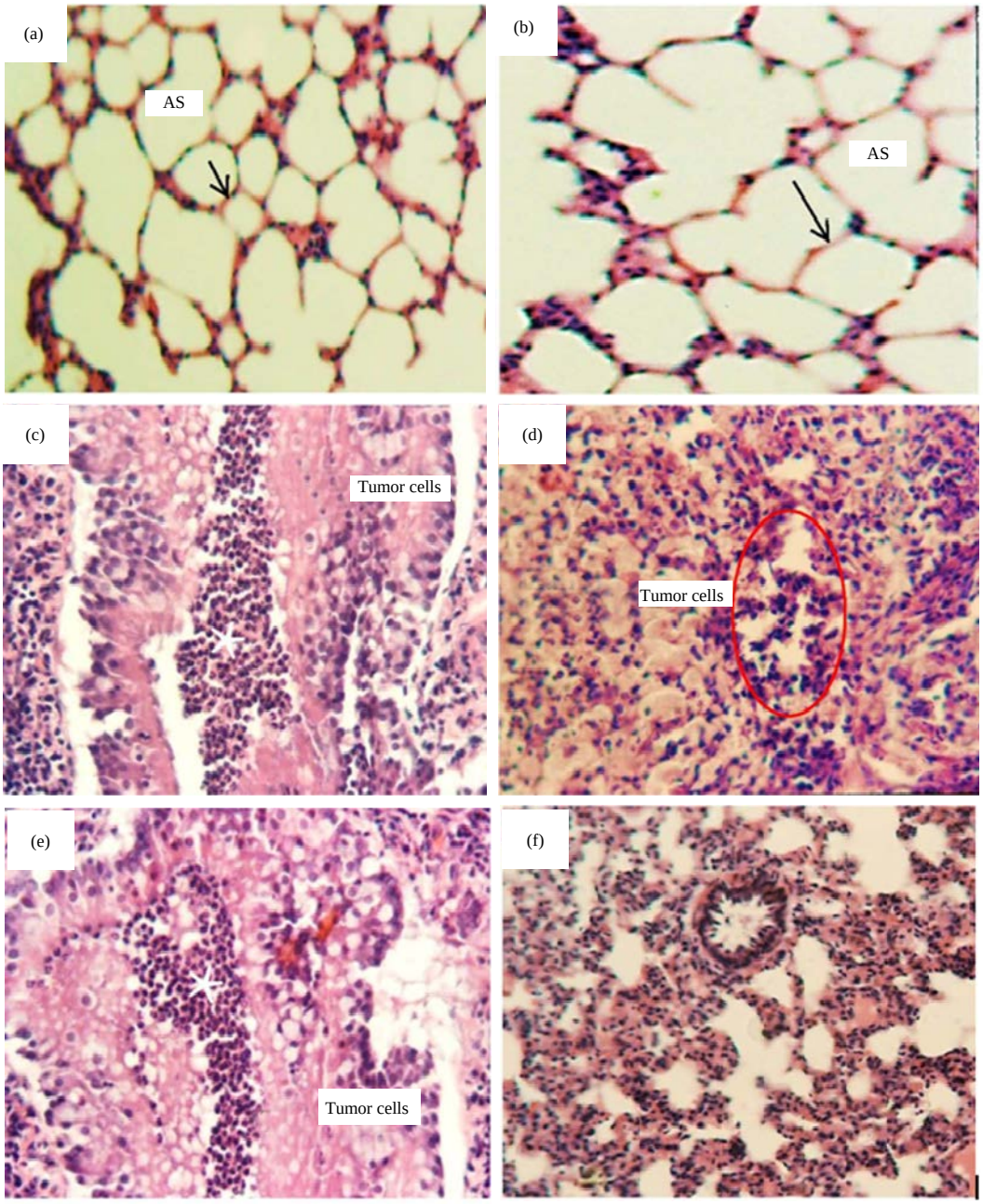

Fig. 1(a-f): Photomicrographs of hematoxylin and eosin stained lung sections of the control and pomegranate groups (original magnification, ×200), (a-b) Showing no obvious lesion in the lung tissues, alveolar sac AS: Alveolar septum (arrow), (c-e) H-E staining of lung cancer sections (benzo(a)pyrene group), Severely reduced pulmonary alveolus, interstitial edema, inflammatory cells (*), necrotic area (red circle), hyperplasia lesions, hemorrhage and (f) H-E staining of lung sections of animals' groups 4 (benzo(a)pyrene plus pomegranate treatment group) showing mild histopathological changes. The reduced severity of lung injury and improve the lung histopathology was shown

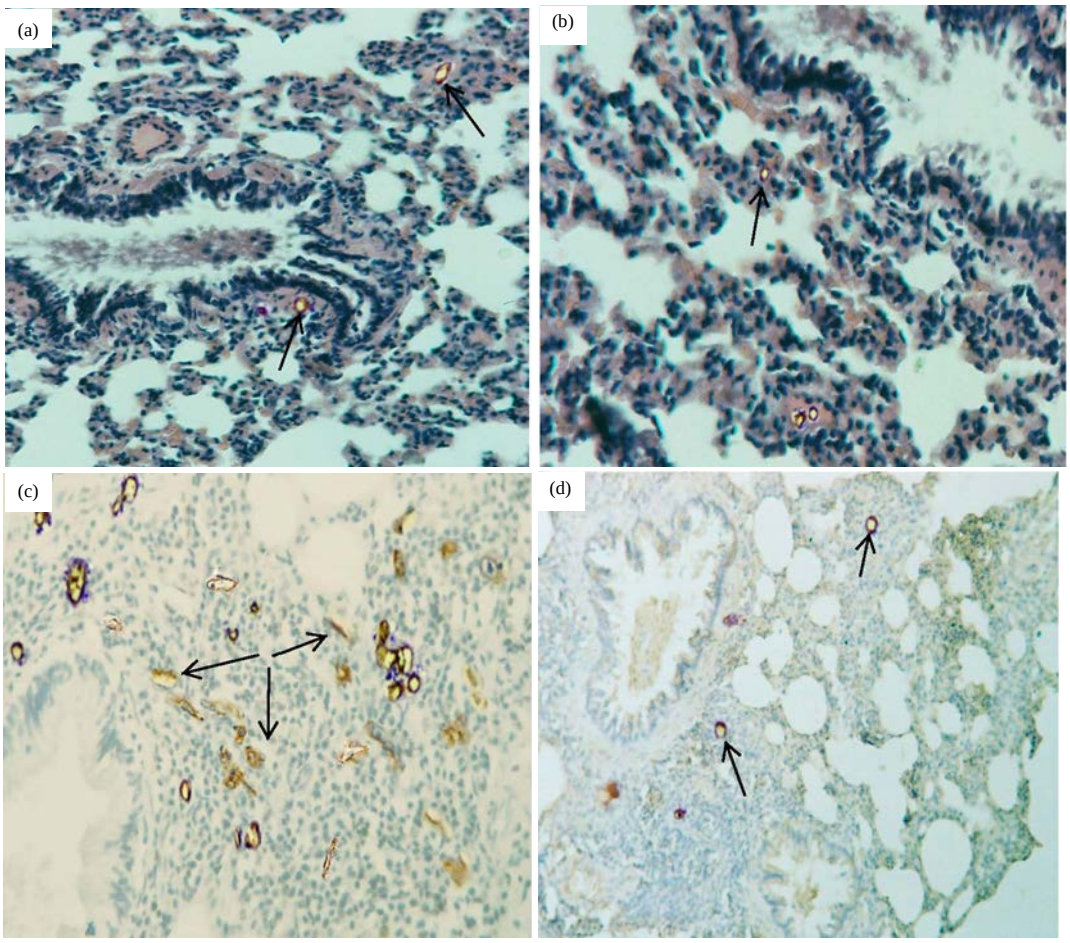

Fig. 2(a-d): Immunostain of anti-CD34 antibody in the experimental animals' groups, positive reaction (arrows). Immunoperoxidase X200

Table 2: Micro-vessels density in control, pomegranate, benzo(a)pyrene and benzo(a)pyrene treated with pomegranate juice groups

Groups	No. of mice	MVD (%)	p-value
Control	12	8.1±2.1	-
Pomegranate juice	12	7.3±3.5	-
Benzo(a)pyrene	12	30.0±3.5	0.028*
Benzo(a)pyrene+pomegranate juice	12	10.5±3.5	0.045*

*Significant at $p<0.05$, MVD: Micro-vessels density

DISCUSSION

Benzo(a)pyrene, a prototypical and well characterized member of the polycyclic aromatic hydrocarbon group (Srivastava *et al*., 2000), is a procarcinogen agent in the process of incomplete combustion of organic materials (Gelboin, 1980). The adverse effects of benzo(a)pyrene, including immunotoxicity, neurotoxicity, teratogenicity and carcinogenicity, on various species of experimental animals have been described previously (Wolterbeek *et al*., 1995; Davila *et al*., 1996; Mendola *et al*., 2002; Min *et al*., 2011).

Because pomegranate juice has been shown to possess remarkable antioxidant effect (Afaq *et al*., 2005) and because some antioxidants are showing promise in prevention and therapy of cancer (Malik *et al*., 2005). Pomegranate fruit is a rich source of two types of polyphenolic

compounds: Anthocyanins (such as cyanidin, pelargonidin and delphinidin), which give the fruit and juice its red color and hydrolyzable tannins (such as pedunculagin, punicalin, gallagic, punicalagin and glucose ellagic acid esters), which account for 92% of the antioxidant activity of the whole fruit. It has been shown that the antioxidant activity of pomegranate juice is higher than that of red wine and green tea (Aviram and Dornfeld, 2001).

The present results show that levels of SOD and CAT activities were significantly lower in mice of benzo[a]pyrene injected group than in control and pomegranate juice groups. Also, a significant increase in the MDA level was observed only in the mice treated with benzo(a)pyrene. Our results suggest that ROS generated during the redox cycling of benzo(a)pyrene may interact with membrane lipids and consequently induce lipid peroxidation. Gupta *et al*. (1988) demonstrated that benzo(a)pyrene significantly increased lipid peroxidation in the lung 12 h after administration. Moreover, the lipid peroxidation remained significantly greater than the control value for up to 7 days. This finding was entirely consistent with our results. Generally, free radicals damage lipids and proteins and modify antioxidant enzymes, such as SOD and CAT. Recently, Leadon *et al*. (1988) demonstrated that SOD could prevent benzo(a)pyrene-induced toxicity.

In the present study, the anti-oxidant activity of pomegranate juice was evaluated, where the data demonstrate that pomegranate juice reduced lipid peroxidation in tissue homogenate of the lung. The pomegranate ability to reduce the oxidant molecules seems likely by scavenging the Reactive Oxygen Species (ROS). Because of its rich concentration of diverse, free-radical-scavenging bioflavonoid (Moneim *et al*., 2011). Another study in rats with carbon tetrachloride-induced liver damage demonstrated that pretreatment with pomegranate juice resulted in the reduction of MDA while the free-radical scavenging activity of CAT and SOD were significantly enhanced (Murthy *et al*., 2002).

In the present study, we reported for the first time a rodent model of cancer weakness caused by the development of lung adenocarcinoma and the protective effect of pomegranate against this inexorable condition. Pomegranate inhibited the induction of inflammatory infiltration, mitigated the reduction of cell proliferation and other cancer lesions in animals that injected with benzo(a)pyrene and treated with pomegranate juice. The effects of pomegranate on lung tumorigenesis were examined by authors both in vitro and in vivo (Khan *et al*., 2006, 2008). Tumors from the benzo(a)pyrene injected animals and treated with pomegranate were examined for effects on cell proliferation and various signaling ways. Tumors had low proliferative indices as examined by PCNA and ki-67 staining (Adhami *et al*., 2009).

The protection against cancer may arise from various mechanisms, including inhibition of the chemical carcinogen formation, decreased of activation and/or increase of detoxification of carcinogens (Schempp *et al*., 2002), induction of cell cycle arrest (Meruelo *et al*., 1988) and apoptosis (Krusekopf and Roots, 2005).

For cancer progression, angiogenesis is essential to the development, growth and support the blood supply for tumor growth and metastasis (Folkman, 1990; Weis and Cheresh, 2011). It has been experimentally revealed that sold tumors cannot grow beyond 1-2 mm in diameter without angiogenesis (Folkman, 1990). Tumor growth is dependent on the balance between increasing tumor-cell numbers through the proliferation of cells and decreasing numbers through an apoptotic process (Metodieva, 2008). If tumor angiogenesis is not sufficient, apoptotic cell death is accelerated the tumor growth is inhibited (Holmgren *et al*., 1995). The present results confirmed that the oral administration of pomegranate juice significantly reduced CD34 expressions in lung tissue of benzo(a)pyrene group, when treated with pomegranate juice, thus, indicating that

neo-vascularization is entirely inhibited and this may be attributable to its activity in prevention of metastasis (Dana *et al*., 2015). According to the present data of CD 34 staining, it was observed that the MVD in the pomegranate plus benzo(a)pyrene group obviously reduce when compared with the benzo(a)pyrene group. This study assumed that pomegranate administration inhibited tumor neovascularization either by limiting early VEGF production and/or by targeting the expression of other proangiogenic mediators (Dona *et al*., 2004).

CONCLUSION

The protective benefits of pomegranate juice may be due in part to its potent antioxidant properties and ability to reduce oxidative stress, histopathological injuries by suppressing the formation of reactive oxygen species and protecting the anti-oxidant mechanism and inhibition of MVD. So, this study suggested that the pomegranate is anti-Angiogenic effectiveness against benzo(a)pyrene induced lung carcinoma in mice.

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