

Modulatory effects of green tea and aloe vera extracts on experimentally-induced lung fibrosis in rats: histological and immunohistochemical study

Mohamed Y. Salem, Nahla El-Eraky El-Azab and Eman M. Faruk*

*Correspondence: faruk_eman@yahoo.com



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Department of Histology and Cell Biology, Benha Faculty of Medicine, Benha University, Egypt.

Abstract

Background: Pulmonary fibrosis (PF) is a multifactorial disease in the human. The existing therapeutic agents display unfavorable adverse effects. Consequently, the discovery of novel therapeutic agents for the prevention and treatment of PF remains a challenge.

Aim of the work: Evaluation of the modulatory effect of green tea and aloe vera (AV) extracts on bleomycin induced pulmonary fibrosis in rats.

Material and methods: Fifty adult male rats were utilized and divided equally into 5 groups. The first was served as control group, the second was the bleomycin (BLM) group (daily I.P. dose 10 mg/kg body weight for 10 days), the third was the green tea (GTE) group which was given daily oral dose of GTE (150 mg/kg b.w.) for 14 days with concomitant IP administration of BLM for 10 consecutive days, the fourth was the Aloe vera (AV) group which was given daily oral dose of AV (300 mg/kg b.w.) for 14 days with concomitant IP administration of BLM for 10 consecutive days, the fifth was the GTE and AV group. Lung samples were taken 14 days after the treatment. Paraffin sections were prepared for histological; H&E and Masson's trichrome staining and immunohistochemical study.

Results: Green tea group revealed normal bronchioles, alveoli with apparently thin interalveolar septa, few cellular infiltration and extravasated RBCs and induced a significant decreased ($P \leq 0.05$) in collagen fibers accumulation and in caspase-3 expression compared with BLM group. Group IV (AV) and group V (GTE and AV) revealed alveoli with apparently thicker interalveolar septa, more cellular infiltration, congested blood vessels and extravasated RBCs in comparison with GTE group. Group IV showed insignificant decreased ($P \leq 0.05$) in collagen fibers accumulation compared with BLM group while the decrease was significant in caspase-3 expression. Group V showed insignificant decreased ($P \leq 0.05$) in collagen fibers accumulation and in caspase-3 expression compared with BLM group.

Conclusion: Green tea and aloe vera partially alleviate pulmonary fibrosis induced by bleomycin in rats. Supporting the potential benefits of using aloe vera and green tea as a potential novel therapeutic agents by diet.

Keywords: GTE, AV, blood vessels, pulmonary fibrosis

Introduction

There is a global resurgence of interest in herbal medicine. The importance of botanicals and herbals is becoming recognized by developed countries. The use of complementary or alternative medicine has increased tremendously in the West, with more

and more countries believing in its benefits, which is now regulating and licensing the sale of herbal products into their countries [1].

Green tea, a product of the dried leaves of *Camellia sinensis*, is the most widely consumed beverage in the world with no

known serious side effects [2]. Green tea is being widely studied for its beneficial effect in the treatment and prevention of human diseases. It is considered to be antiinflammatory, antioxidative, antimutagenic and anticarcinogenic and can prevent cardiac disorders [3]. Green tea polyphenols have demonstrated significant antioxidant, anticarcinogenic, anti-inflammatory, thermogenic and antimicrobial properties in numerous human, animal, and in vitro studies [4].

Aloe vera L. (Syn.: Aloe barbadensis Miller) is a cactus-like perennial plant belonging to family Liliaceae (sub-family of the Asphodelaceae), native to North Africa and cultivated in warm climatic areas [5]. Aloe vera (AV) consists of high content of phenolic compounds, glycosides (aloin), 1,8-dihydroxy-anthraquinone derivatives (aloe emodin), beta-1,4 acetylated mannan, mannose-phosphate and alprogen glucoprotein [6]. Recent scientific investigations on medicinal properties of AV made it worldwide novel valuable ingredient for food, cosmetic and pharmaceutical industry [7]. Leaf exudates and mucilaginous gel of Aloe possesses anti-inflammatory, antioxidant, antifungal, antibacterial, anti-arthritic, antirheumatoid [8], anticancer [9], cytoprotective, cardiac stimulatory and immunomodulatory activities [10]. It is used to protect against gastric ulceration, remedy against a variety of skin disorders, promotes wound healing [11].

Pulmonary fibrosis is the end-stage of a group of chronic diseases categorized as idiopathic interstitial pneumonias [12]. An increasing number of people are affected by pulmonary fibrosis worldwide, with increasing morbidity and mortality rates [13]. Multiple factors, including transforming growth factor (TGF)- β 1, connective tissue growth factor (CTGF), platelet-derived growth factor (PDGF), inflammatory cytokines, chemokine (C-C motif) ligand 2/monocyte chemo attractant protein-1 (CCL2), have been implicated in the pathogenesis of pulmonary fibrosis [4]. Currently, strong evidence proves that cellular redox state and oxidant/antioxidant balance play important roles in the pathogenesis of pulmonary fibrosis [15]. Oxidative stress induces structural cell apoptosis and up regulates proinflammatory cytokine synthesis. Dysregulations in the balance of some growth factors induced by oxidative stress possibly play major roles in distinguishing between normal and pathologic tissue repair [13]. Lung fibrosis develops in a number of clinical diseases, including interstitial lung diseases and idiopathic interstitial pneumonias, as part of several systemic connective tissue diseases and childhood interstitial lung disease syndromes, and in response to many types of lung injury, including radiation and some chemotherapeutic drugs. Initial therapies focused on aggressive anti-inflammatory treatment; however, this approach has not improved loss of lung function or survival. Currently, there are no approved medical antifibrotic therapies for pulmonary fibrosis [16].

The aim of the present study was to evaluate, the modulatory effect of green tea and AV extracts on bleomycin induced pulmonary fibrosis in rats.

Materials and methods

In this study, 50 adult male albino rats of average weight 150–200 g were used. The animals obtained from the animal house, Moshtohor faculty of Veterinary Medicine, Benha University Moshtohor city Egypt. Strict care and cleaning measures were utilized to keep the animal in a normal healthy state; the animals were kept in animal cages under the prevailing atmospheric conditions. They were kept on normal balanced diet and tap water. All ethical protocols for animal treatment were followed and were supervised by the animal facilities. All animal experiments received approval from the Institutional Animal Care Committee.

Bleomycin sulfate (Merck Millipore, MA, United States) was purchased from NOOR SCIENTIFIC & TRADE (8 El Noor St, Shoubra El Khaema, Kaliobyeya, Egypt). Bleomycin (BLM) dissolved in normal saline and was given intraperitoneal (IP) in a dose of 10 mg/kg/day/10 days) to induce lung fibrosis [17]. Green tea extract (GET) was obtained from Technomade Group (Nasr City, Cairo, Egypt) as 350 gm powdered extract. It was freshly dissolved in distilled water before administration by gastric tube at a dose of 150 mg/kg b.w. [18].

Preparation of Aloe vera extract

AV solid gel in the center of the leaf was collected and homogenized resulting mucilaginous, thick and straw colored homogenate was obtained and lyophilized. Then the lyophilized sample was extracted using 95% ethanol. The filtrate was collected and evaporated to dryness under reduced pressure in a rotary evaporator at 60°C. The residue was stored in dry sterilized small containers at 4°C until further use. The drug solutions were prepared freshly each time. The dosing schedule used was once per day. The extracts were administered orally daily to rats by gastric tube at a dose of 300 mg/kg b.w. [19].

Experimental procedure

The rats were divided into four groups of 10 animals in each group (n=10) as follows.

Group I

normal control rats.

Group II (BLM group)

BLM induced pulmonary fibrosis rats.

Group III (green tea group)

Rats were given daily oral dose of GTE for 14 days with concomitant IP administration of BLM for 10 consecutive days.

Group IV (AV group)

Rats were given daily oral dose AV leaf gel extract for 14 days with concomitant IP administration of BLM for 10 consecutive days.

Group V (green tea and AV group)

Rats were given daily oral dose of GTE and AV leaf gel extract for 14 days with concomitant IP administration of BLM for 10 consecutive days.

After 14 days of the treatment, the fasted rats were sacrificed. A small portion of lung was fixed in 10% formalin for H&E and Masson's trichrome staining [20] and immunohistochemical detection of caspase-3 using a standard avidin-biotin peroxidase complex system according to the kit used (Neomarkers) followed by diaminobenzidine (DAB) visualization [21]. Sections were counterstained with hematoxylin [22].

Morphometric study

The mean area percentage of collagen fibers accumulation and of caspase-3 expression were quantified in 10 images for each group using Image-Pro Plus program version 6.0 (Media Cybernetics Inc., Bethesda, Maryland, USA). Collagen fibers accumulation and caspase-3 expression in group III, IV and V compared with group II (affected group) using the t-test, with $P < 0.05$ as the level of statistical significance. Statistical analyses were carried out using IBM SPSS Statistics software for Windows, Version 20 (IBM Corp., Armonk, NY, USA).

Results

H&E

The control group showed normal histological architecture of lung with many alveoli, alveolar sac, alveolar ducts, bronchioles, blood vessels (Figure 1A) and thin interalveolar septum (Figure 1B). The group II (BLM group) showed lung bronchioles, alveoli with thick interalveolar septa encroaching on alveoli (collapsed alveoli), cellular infiltration, dilated congested blood vessels and extravasated red blood cells (RBCs) (Figure 1C). Sections of group III (green tea group) revealed bronchioles, alveoli with apparently thin interalveolar septa, few cellular infiltration and extravasated RBCs (Figure 1D). In sections of Group IV (AV group) revealed alveoli with apparently thicker interalveolar septa, more cellular infiltration, congested blood vessels and extravasated RBCs in comparison with green tea group (Figure 1E). In sections of Group V (green tea and AV group) lung architecture, alveoli, thick interalveolar septa, cellular infiltration and congested blood vessels appeared near to those of the BLM group (Figure 1F).

Masson trichrome

The control group showed very thin collagen fibers around alveoli or within the interalveolar septa (Figure 2A). The BLM group showed extensive accumulation of collagen fibers around alveoli or within the interalveolar septa (Figure 2B). The green tea group showed minimal accumulation of collagen fibers in interalveolar septa (Figure 2C) while collagen fibers accumulation was moderate in AV group (Figure 2D) Group V (green tea and AV group) showed marked accumulation of collagen fibers around alveoli or within the interalveolar septa (Figure 2E).

Immunohistochemical study

Positive immunohisto-chemical staining of caspase-3 demonstrated as brown cytoplasmic staining (index for the degree

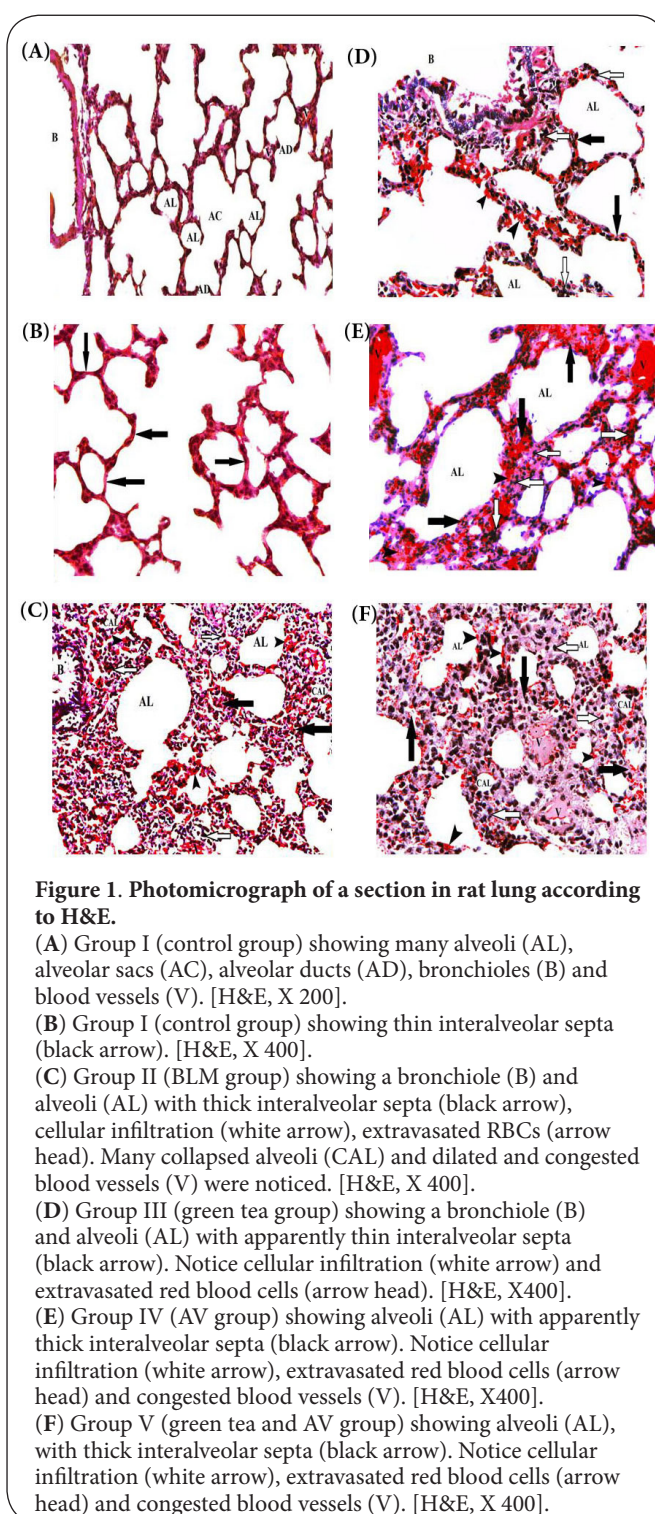


Figure 1. Photomicrograph of a section in rat lung according to H&E.

(A) Group I (control group) showing many alveoli (AL), alveolar sacs (AC), alveolar ducts (AD), bronchioles (B) and blood vessels (V). [H&E, X 200].
(B) Group I (control group) showing thin interalveolar septa (black arrow). [H&E, X 400].
(C) Group II (BLM group) showing a bronchiole (B) and alveoli (AL) with thick interalveolar septa (black arrow), cellular infiltration (white arrow), extravasated RBCs (arrow head). Many collapsed alveoli (CAL) and dilated and congested blood vessels (V) were noticed. [H&E, X 400].
(D) Group III (green tea group) showing a bronchiole (B) and alveoli (AL) with apparently thin interalveolar septa (black arrow). Notice cellular infiltration (white arrow) and extravasated red blood cells (arrow head). [H&E, X400].
(E) Group IV (AV group) showing alveoli (AL) with apparently thick interalveolar septa (black arrow). Notice cellular infiltration (white arrow), extravasated red blood cells (arrow head) and congested blood vessels (V). [H&E, X400].
(F) Group V (green tea and AV group) showing alveoli (AL), with thick interalveolar septa (black arrow). Notice cellular infiltration (white arrow), extravasated red blood cells (arrow head) and congested blood vessels (V). [H&E, X 400].

of nuclear apoptosis). Negative cytoplasmic staining of caspase-3 was found in cytoplasm of bronchial cells, alveolar and interalveolar septal and along the endothelial lining of blood vessels of control group (Figure 3A). In BLM group, caspase-3 was highly expressed (Figure 3B), in green tea

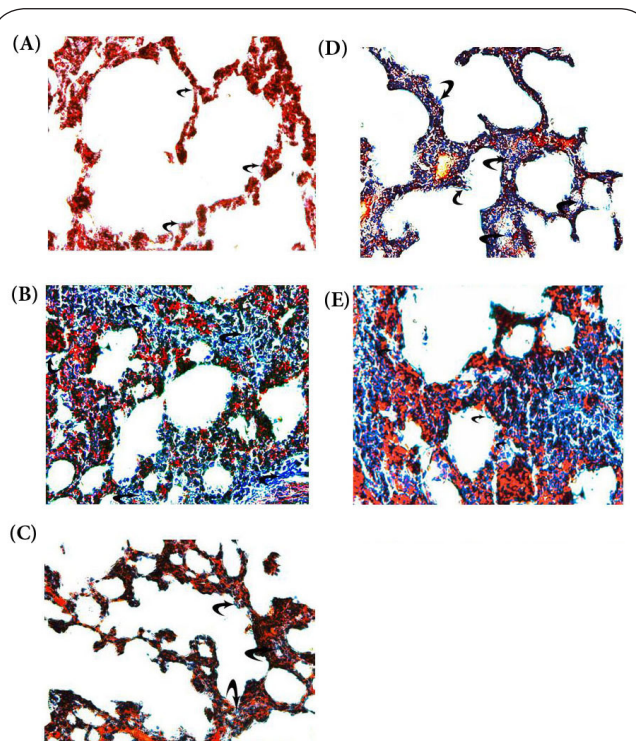


Figure 2. Photomicrograph of a section in rat lung according to masson trichrome.

(A) Control group showing very thin collagen fibers around alveoli and within the interalveolar septa (curved arrow).
 (B) BLM group showing extensive accumulation of collagen fibers around alveoli and within the interalveolar septa (curved arrow).
 (C) Green tea group showing minimal accumulation of collagen fibers in interalveolar septa (curved arrow).
 (D) AV group showing moderate accumulation of collagen fibers in interalveolar septa (curved arrow).
 (E) Green tea and AV group showing marked accumulation of collagen fibers around alveoli and bronchiole and within the interalveolar septa (curved arrow). [Masson's trichrome, $\times 400$].

group, it was mildly expressed (**Figure 3C**) while caspase-3 expression was moderate in AV group (**Figure 3D**). Group V (green tea and AV group) showed high caspase-3 expression in cytoplasm of alveolar cells (**Figure 3E**).

Morphometric results

The mean area % of collagen fibers accumulation and of caspase-3 expression for all groups was represented in **Tables 1 and 2** and (**Histograms 1 and 2**). There was a significant decreased ($P \leq 0.05$) in collagen fibers accumulation and in caspase-3 expression in group III compared with group II (BLM group) while this decrease was insignificant in group V. Group IV showed insignificant decreased ($P \leq 0.05$) in collagen fibers accumulation compared with group II while the decrease was significant in caspase-3 expression.

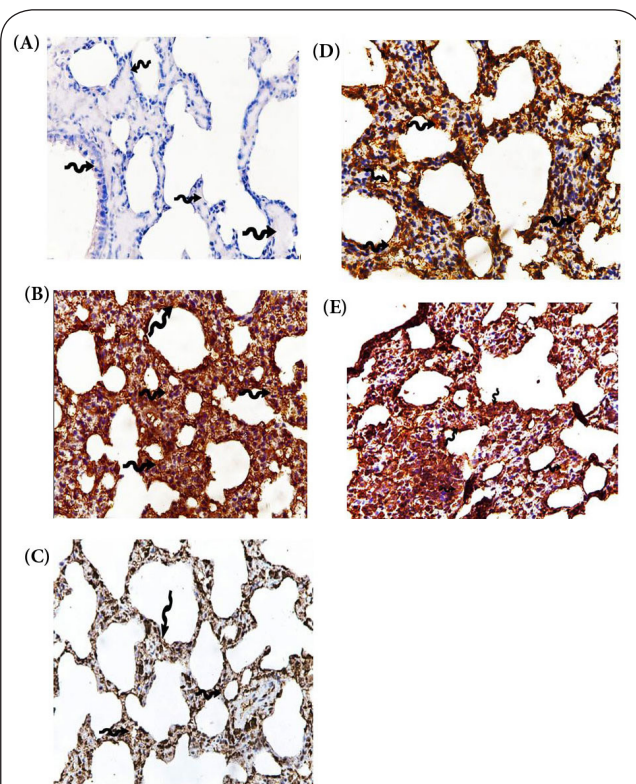


Figure 3. Photomicrograph of a section in rat lung according to immunohistochemical study.

(A) Control group showing negative caspase-3 immunostaining of cytoplasm of bronchial cells, alveolar and interalveolar septal cells and along the endothelial lining of blood vessels (zigzag arrow).
 (B) BLM group showing highly caspase-3 expression in cytoplasm of alveolar cells, septal cells and along the endothelial lining of some blood vessels (zigzag arrow).
 (C) Green tea group showing mild caspase-3 expression in cytoplasm of alveolar cells, septal cells and along the endothelial lining of some blood vessels (zigzag arrow).
 (D) AV group showing moderate caspase-3 expression in cytoplasm of alveolar cells, septal cells and along the endothelial lining of some blood vessels (zigzag arrow).
 (E) Green tea and AV group showing high caspase-3 expression in cytoplasm of alveolar cells, septal cells and along the endothelial lining of some blood vessels (zigzag arrow). [caspase-3 immunostaining $\times 400$].

Discussion

Pulmonary fibrosis is a chronic, progressive and lethal diffuse interstitial lung disease. It has re-emerged as a focus of scientific study, due to its increasing incidence [23] and there has been no effective medication for this progressive disease up to now [24]. The BLM induced pulmonary fibrosis group (group II) in the present study showed thick interalveolar septa encroaching on alveoli (collapsed alveoli), cellular infiltration, dilated congested blood vessels with extravasated RBCs, extensive accumulation of collagen fibers around alveoli or within the

Table 1. Showing the mean area %, SD of collagen fibers accumulation in groups I, II, III, IV and V. Group III, IV and V compared with group II.

	Group I	Group II	Group III	Group IV	Group V
Mean area %	0.17%	14.28%	1.77%	9.67%	12.52%
SD±	0.0437	3.1858	0.6003	3.7466	1.7967
P value	--	--	0.000	0.063	0.206
Significance	--	--	S	NS	NS

SD=Standard deviation; S=Significant at P<0.05;
 NS=Non Significant

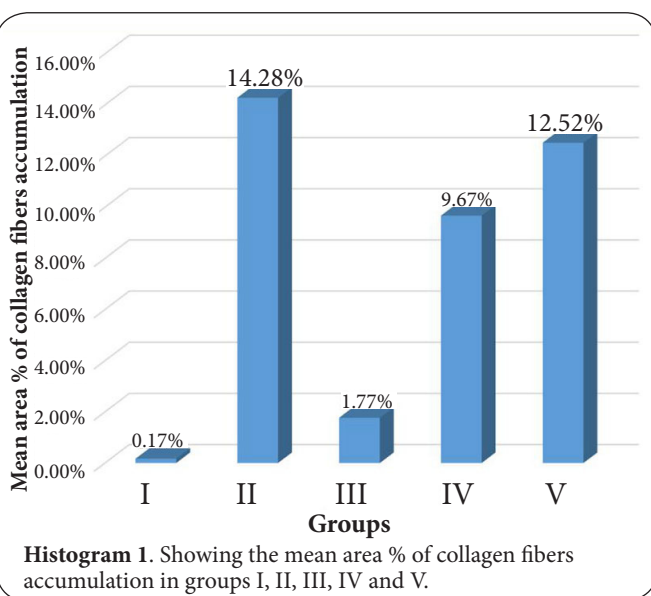
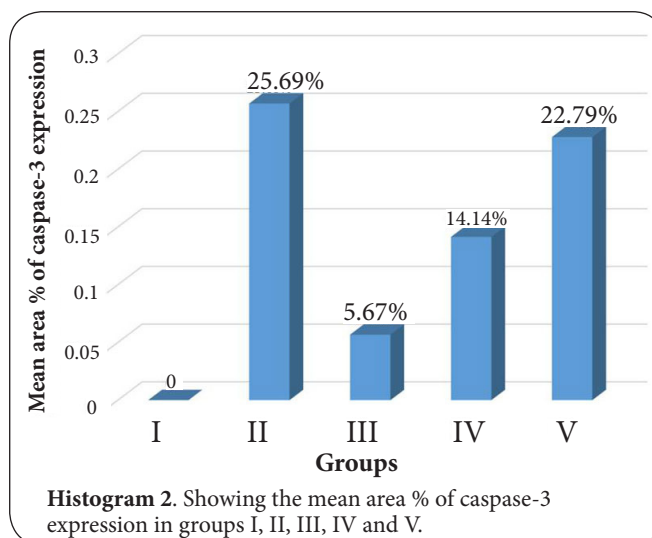


Table 2. Showing the mean area % of caspase-3 expression and SD in groups I, II, III, IV and V. Group III, IV and V compared with group II.

	Group I	Group II	Group III	Group IV	Group V
Mean area %	0	25.69	5.67	14.14	22.79
SD±	0	4.1012	0.7010	5.0297	1.0006
P value	--	--	0.000	0.002	0.093
Significance	--	--	S	S	NS

SD=Standard deviation; S=Significant at P<0.05;
 NS=Non Significant;

interalveolar septa and highly expressed caspase-3 reaction. In agreement with these findings, some researchers reported that in BLM-induced models of pulmonary fibrosis many inflammatory cells including neutrophils and eosinophils were observed in the alveoli and interstitium, alveolar cell damage, fibroblast proliferation and subsequent collagen deposition and obliteration of the alveolar space [25-27]. Other researcher reported that bleomycin increases lung epithelial cell apoptosis



in a reactive oxygen species (ROS)-dependent manner [28]. BLM-induced pulmonary fibrosis was explained by some authors who reported that the chemotherapeutic mechanism of BLM results from the chelation of iron ions with oxygen, which leads to production of DNA-cleaving superoxide, and also hydroxide free radicals [29]. It is the increased production of ROS that lead to bleomycin's pulmonary toxicity, and may eventually lead to lung fibrosis. Also previous researches reported that reactive oxygen species have been shown to directly activate profibrotic transforming growth factor- β (TGF- β) in the lung. TGF- β promotes the development of inflammation, and increases activity at sites of inflammation, and induces the proliferation of the fibroblasts, leading to severe pulmonary fibrosis [30,31].

The green tea group (group III) in the present study showed thin interalveolar septa, few cellular infiltration and extravasated RBCs and significant decreased ($P \leq 0.05$) in collagen fibers accumulation and in caspase-3 expression compared with BLM group. In agreement with these findings, previous studies [18] the patchy fibrosis; also quantitative determination of hydroxyproline confirmed the reduction of collagen deposition [18]. Other studies reported that GTE constitutes an important source of antioxidants. Besides polyphenols, GTE contains additional antioxidants such as carotenoids, tocopherols (vitamin E derivatives) and vitamin C. Tea contains also minerals that function as co-factors in antioxidant enzymes: zinc, selenium and manganese. Polyphenols have additional mechanisms in which they reduce oxidation level besides direct role as antioxidants [32,33]. The antifibrotic effect of GTE explained by some authors who reported that it might be through the inhibition exerted by GTE on gelatinases involved in the damage to lung extracellular matrix, matrix metalloproteinases [18] and others have demonstrated that GTE both inhibits the signal transduction of TGF- β by binding to transforming growth factor receptor II (TGFR) and attenuates the expression

of α -smooth muscle actin (α -SMA) in MRC-5 cells (human lung fibroblasts), which is a myofibroblast cell line, when it is stimulated by TGF- β . Myofibroblasts play crucial roles in the pathogenesis of tissue fibrosis [34].

Some scientists reported that glutathione peroxidase activity, superoxide dismutase enzymes, and a phenolic antioxidant were found to be present in Aloe vera gel, which may be responsible for its antioxidant effects [35,36]. In spite of this, the AV group (group IV) in the present study showed alveoli with apparently thick interalveolar septa, cellular infiltration, congested blood vessels and extravasated RBCs and insignificant decreased ($P \leq 0.05$) in collagen fibers accumulation while the decrease was significant in caspase-3 expression compared with BLM group. These results may be explained by previous researchers who reported that high molecular weight polypeptide constituent from the AV gel as glucomannan, a mannose-rich polysaccharide, and gibberellin, a growth hormone, interact with growth factor receptor on the fibroblast, thereby stimulating its activity and proliferation, which in turn increases collagen synthesis after topical and oral application [35,37,38].

Some researchers reported that administration of multiple antioxidants could be a more effective mode used in the treatment of obstructive lung diseases [39] and others stated that herb pair which consists of two single herbs presents significantly better pharmacological efficacy than individual herbs because the synergistic interactions between the components of herbals greatly contribute to the enhancement of their therapeutic efficacy [40]. Contrary to this, group V (green tea and AV group) of the present study revealed thick inter-alveolar, collapsed alveoli, cellular infiltration, dilated congested blood vessels with extravasated RBCs and insignificant decreased ($P \leq 0.05$) in collagen fibers accumulation and in caspase-3 expression compared with BLM group. These results may be explained by previous researches who demonstrated that there was not synergistic anti-oxidative capacity when the flavonoid compounds were used in combination and the combination of two strong antioxidants may not necessarily engender stronger efficacy, it may even produce antagonistic interaction. In contrast, when a weaker antioxidant was combined with a stronger one, the former may regenerate the latter, thus improving the overall free radical quenching capacity of the combination [41].

Conclusion

Green tea and aloe vera partially alleviate pulmonary fibrosis induced by bleomycin in rats. Supporting the potential benefits of using aloe vera and green tea as a potential novel therapeutic agents by diet.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

Authors' contributions	MYS	NEE	EMF
Research concept and design	✓	--	--
Collection and/or assembly of data	--	✓	--
Data analysis and interpretation	✓	✓	✓
Writing the article	✓	--	✓
Critical revision of the article	✓	✓	✓
Final approval of article	✓	✓	✓
Statistical analysis	✓	--	--

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