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Evaluation the Inhibitory Effect of Atorvastatin and the Olibanum Extract to Reduce Bleomycin-induced Pulmonary Fibrosis

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ABSTRACT: Bleomycin is an effective antibiotic with anti-tumor activity that has a minimum toxic effect on hematopoietic tissues and the immune system and can be widely used with the other chemotherapeutic agents. Lung injury is the most devastating side-effect of the anti-cancer drug Bleomycin which often progresses to pulmonary fibrosis. The objective of the present study is to evaluate antioxidant and anti-inflammatory effects of Atorvastatin and the extract of Olibanum on Bleomycin-induced pulmonary fibrosis. Male Wistar rats were randomly divided into four groups. The positive control group received Bleomycin Sulfate (1 U/100 gBW) intra-tracheal. In the negative control group, normal saline and propylene glycol (PG) and in the combination group, 10 mg/kgAtorvastatin and simultaneously 120 mg/kg hydro-alcoholic extract of Olibanum were injected intraperitoneally a week before Bleomycin administration and treatment was continued until 14 days afterwards. The levels of Malondialdehyde (MDA) and Hydroxyproline (OH-Proline) were evaluated in the lung which showed 138 and 90 percent increases in Bleomycin group compared to the control group, respectively (P<0.05). An increase in the above factors in the combination group have been equal to 11 and 24 percent (P<0.05). Histological studies showed reduced fibrosis in the combination group. according to the obtained results, it can be said that the combined group could control the pulmonary fibrosis and play an effective role in its reduction.

Keywords: Bleomycin, pulmonary fibrosis, Atorvastatin, Olibanum

INTRODUCTION

Bleomycin is an effective anti-tumor drug with a broad anti-cancer spectrum which is present in the most of multi-drug chemotherapy regimens due to its minimum toxic effects on the bone marrow and hematopoietic system and also poor immunosuppressive properties (Giri *et al.,* 1980). The most dangerous side effect of Bleomycin is pulmonary fibrosis that its prevalence depending on the risk factors is reported as 2-4% in patients under treatment and its mortality is reported in 1-5% of the patients (Sato *et al.,* 1999). Pulmonary fibrosis is one of the chronic interstitial diseases and has the lowest chance of improvement among all interstitial pulmonary diseases. The mechanism of Bleomycin-induced pulmonary fibrosis includes the participation of neutrophils and releasing oxygen radicals, involvement of pulmonary macrophages and releasing inflammatory mediators, involvement of cytokines and growth factors and increased collagen production, the participation of reactive oxygen species and therefore cutting of DNA and lipid peroxidation (Nadery, 2004). On the other hand, it is suggested that the immune system of the body is responsible for a major part of pulmonary toxicity. Studies in patients with germ cell tumors showed that Bleomycin cannot be completely eliminated from the combination of chemotherapy protocol as it is effective following treatment (Loehrer, 1997).

Atorvastatin is one of the most important components of statin family and has fewer side effects and more effectiveness compared to the other members of this family (Wierzbicki, 2001). Statins in addition to cholesterol-lowering effects and preventing cardiovascular diseases have some known pleotropic properties such as anti-inflammatory, anti-oxidant and neuroprotective effects. This drug class through multiple mechanisms such as reduced cholesterol, reducing the stability of the structure of lipid rafts, effect on the activity and regulation of the immune cells as well as reducing cytokines and chemokines and their anti-fibrotic effects and by having scavengering properties on oxygen free radicals impose their antioxidant effects (Malekinejad *et al.,* 2013). Atorvastatin can reduce the increase severity of inflammatory cells, hydroxyl proline and collagen content in lung tissue and reduce early damages of lung tissue when exposed to cytotoxic agents through inhibiting free radicals (Nacar *et al.,* 2014). Some studies show that the mortality is reduced among Atorvastatin's consumers in pneumonia (Thomsen *et al.,* 2008).

Olibanum is a plant from maples family and is obtained from certain genus of Boswellia. The medicine of the plant is a resin which is leaked when the bark is cut. The main ingredient of resin which is available in free form or in combination with other ingredients is Boswellic acid that is a group of Penacillic terpenoids (Poeckel and Werz 2006). alpha-Boswellic acid (alpha-BA), beta-Boswellic acid (beta-BA) and 11-keto-Boswellic acid (KBA) are the main ingredients of Boswellic acids (Rall *et al.,* 1996). Anti-inflammatory effects of Boswellia are specially related to KBA which is the specific inhibitor of 5-lipooxygenase enzyme and inhibits leukotriene biosynthesis as a dose-dependent manner (James *et al.,* 2006). These compounds are the pre-inflammatory products of 5-lipoxygenase including 5-HETE and B4-leukotriene biosynthesis (LTB4) which are active chemotactic factors (Ammon *et al.,* 1991), as well as reduces C4-leukotriene (LTC4) production (Wildfeuer **et al.,** 1998), but they do not have any effect on the activity of 12-lipoxygenase and cyclooxygenase (COX) (Ali and Mansour 2011). Boswellic acids destroy a wide range of free radicals with their scavengering properties. Extracellular superoxide dismutase and glutathione peroxidases are the inhibitors of oxidative activities. The performed tests show the increased level of these enzymes in the blood and lung of fibrotic rats when they were under treatment by Olibanum (Ali and Mansour 2011). Boswellic acid use was successful in preventing damages due to activating leukocytes that resulted in release of active oxygen species and nitrogen and thus also influence on inflammatory process and is considered as an effective factor against oxidants (Kaplan *et al.,* 2006). TGF-β1 regulation can be also responsible for improving a part of antioxidant status of Boswellic acid (Ali and Mansour 2011). The analysis result of compounds has been shown flavonoids and more polyphenols in aqueous extract of Olibanum and higher triterpenoids in hydro-alcoholic extract (Moussaieff *et al.,* 2008). According to the conducted research, ethanol is best solvent for the extraction of Olibanum and the most triterpenoidcan be extracted (Seyyed *et al.,* 2015).

Given the special place of Bleomycin in chemotherapy of cancer and according to histological and biochemical similarities between pulmonary fibrosis of Bleomycin and pulmonary fibrosis disease in human, in addition to that its inhibition is an effective step in improving cancer treatment, opens a new window to treat the pulmonary fibrosis due to the drugs and also idiopathic pulmonary fibrosis. The existing evidences suggest that concentration inhibition of reactive oxygen species and preventing development of collagen fibers play the main role in control of pulmonary fibrosis. It is expected that antioxidant and anti-inflammatory effect of Atorvastatin can be an effective treatment on Bleomycin-induced pulmonary fibrosis.

MATERIALS AND METHODS

A. Laboratory animals

Male Wistar rats weighing approximately 200±25 g were purchased from the Laboratory Research Center of University of Isfahan (Iran) and put in the special cages. Then, they were kept for a week and with standard laboratory conditions at 22±2ºC, relative humidity of 45-55% and light-dark cycle of 12:12 hours. In all phases, laboratory animals had free access to water and food and working with mice was carried out in accordance with the rules of ethics committee and support the animals' rights.

B. Test material

Atorvastatin was purchased from the pharmaceutical manufacturing company of Aria (Tehran). The plant sample of Olibanum (B.serrata species) was prepared from the pharmaceutical company of Goldaroo (Isfahan) and was extracted there. Percolation was performed as hydro-alcoholic for extraction. Bleomycin was prepared from KAYAHU Company (Japan) and other chemicals and reagents using in the test with analytical grades were prepared from Merck Company (Germany).

C. Test protocol

At the beginning of the experiment, rats of each group were weighed and numbered, so evaluating the condition of each one individually would be possible during the trial period. The rats were divided into 4 groups of 5 rats including control of sterile saline, control of Propylene Glycol (PG), Bleomycin (BLM) and a combination group includes Atorvastatin 10 mg/kg (Khodayar *et al.,* 2014). and hydro-alcoholic extract of Olibanum 120 mg/kg (Ziyaurrahman and Patel (2012). (AVA+BA). Length of test period was totally 21 days and was performed in two periods of pre-treatment and post-treatment. Pre-treatment period was 6 days and after Bleomycin administration in the seventh day, treatment continued for 14 days.

To create pulmonary fibrosis, intra-trachea administration of Bleomycin was used (Giri *et al.,* 1980), as it creates fibrotic changes in shorter time in the lung. For this purpose, in the seventh day and after animals were anesthetized by Ketamine (50 mg/kg), Bleomycin was administrated with installation rout and with the amount of 1U/100 g of body weight of each animal which had been solved in 0.3 ml of normal saline. The control groups were received only 0.3 ml sterile saline by intra-trachea rout.

Negative control groups received Bleomycin solvent means sterile saline intraperitoneally during pre-treatment. On the seventh day, they received 0.3 ml sterile saline with intra-tracheal rout and in the days after it, they were treated by the solvent of tested drug means normal saline and propylene glycol intraperitoneally.

Bleomycin group that had received sterile saline intraperitoneally during post-treatment, also received a dose of intra-trachea Bleomycin and in the later days, it was practiced like pre-treatment period. Treatment group which was included Atorvastatin and the hydro-alcoholic of Olibanum received 0.3 ml normal saline as Atorvastatin solvent and 0.3 ml propylene glycol as Olibanum solvent in separated administrations intraperitoneally in the first 7 days as the pre-treatment associated with treatment recommended doses.

Since the weight of animal is an important criterion, body weight changes during the tests and specially till the fifth day and after this time, once in every 4-5 days was noted. Also, animals should be considered in terms of general situation particularly to the twelfth day that there is the possibility of respiratory problems.

Tested animals were euthanized in day 21 and the lung was removed for further testing. After weighing, lung was divided into two parts and kept at the temperature -15ºC for biochemical and histological studies inside a specific container.

D. Biochemical and histological tests

Concentrations of lipid peroxidation was determined by measuring Malondialdehyde (MDA) which is considered as an index for oxidative stress with Tio Barbituric acid (TBARS) and the reagent TBA (Take *et al.*, 2012). To have a standard curve from the concentrations of 0.39, 0.78, 1.56, 3.125, 6.25, 12.5, 25 and 50 were used. Sample absorption was read by spectrophotometer at 532 nm. The result was stated as mol/L.

Hydroxy proline amino acid was measured as an index for collagen amount of the lung using Woessner method (Woessner, 1961). Hydroxy proline in the protein using the oxidant is oxidized to pyrrole and then produced a colorful complex with para-Dimethylaminobenzaldehyde that its absorption was read with spectrophotometer at 557 nm. The result was calculated based on µg of hydroxy proline on g of lung tissue by the standard curve of hydroxy proline. To prepare the standard curve, the amounts of 0.1, 0.2, 0.4, 0.6, 0.8 and 1 ml of standard solution of hydroxy proline were used.

Histological tests were summarized in three slices (dehydration, paraffin entry to tissue, embedding, sectioning), H&E staining (to review cells generally) and Mason Terry Chrome (to distinguish collagen fibers).

Statistical analysis: Averages comparison by analysis of variance (ANOVA), average of variable level as mean±SEM and significant results through Duncan's complementary test were evaluated. P<0.05 was considered as the significant difference in all tests.

RESULTS

Lung indices: morphological changes in the lung of animals showed that Bleomycin injection causes weight gain of lung fibrosis compared to a normal lung and hemorrhage spots and emphysema lesions are observed due to tissue destruction. The lung weight gain in the treatment group is less than positive control group and do not have a significant difference with the negative control group. The average weight of lung in BL, PG, saline and AVA+BA groups at the end of test period were 1.32±0.14, 1.30±0.057, 2.55±0.75, 1.37±0.062 respectively that there was a significant difference between Bleomycin group and other groups (P<0.05) (Chart 1).

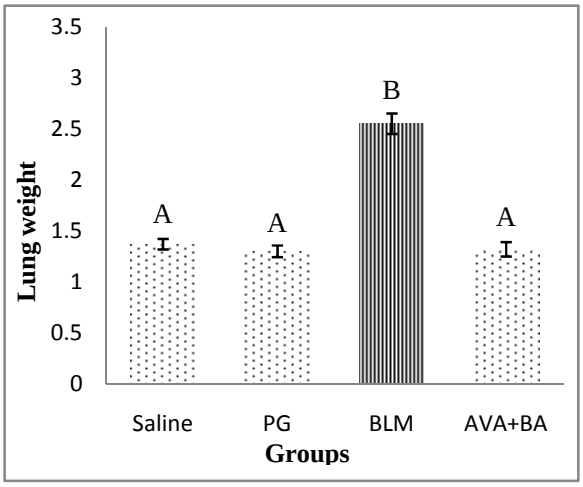

Chart 1.

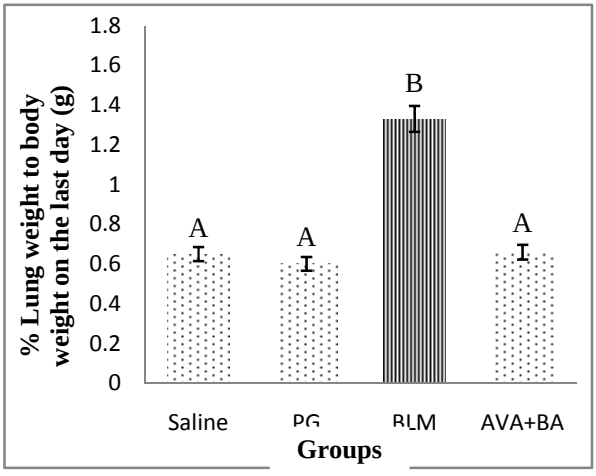

Chart 2.

Lung weight ratio to body weight of the animals was calculated due to the simultaneous changes of these factors. The results in this case showed that this ratio in PG, saline, BL and AVA+BA were 0.65±0.036, 0.60±0.045, 1.33±0.32 and 0.66±0.077 respectively that there was a statistical significant difference between Bleomycin group and other groups (P<0.05) (Chart 2). The total amount of pulmonary Malondialdehyde has increased 138.02 percent in Bleomycin group compared to the normal saline group which is considered as a significant difference in variance analysis and Duncan's test (P<0.05). Treatment group had an 11.92% increase compared to the negative control group. On the other hand, pulmonary MDA in combination group, 55.73 percent was decreased compared to Bleomycin group which was considered as a significant difference statistically (P<0.05). The calculated descriptive indices show that there is not a significant difference between combination group and negative control group (Chart 3).

The total amount of pulmonary hydroxy proline has increased 90.12 percent in the Bleomycin group compared to the control group and is considered as a significant difference compared to the control group (P<0.05). In the combination group 24.68 percent of increase was observed compared to the normal saline group. Also, OH-Proline in the combination group showed a 35.29 percent decrease compared to the Bleomycin group which is considered as a significant difference statistically (P<0.05). The calculated descriptive indices showed that there is a significant difference between the combination group with negative control and Bleomycin groups (Chart 4).

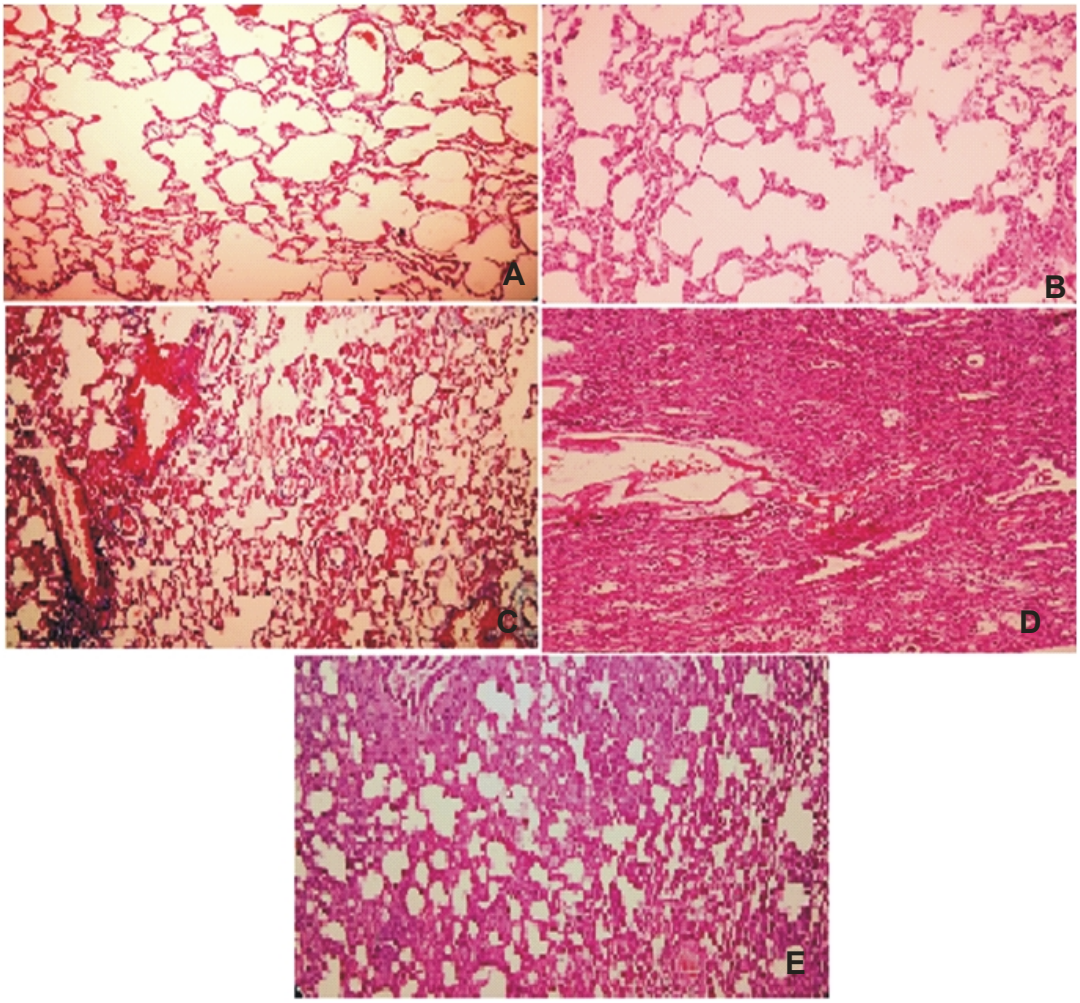

Plate I. A- Open section of the rat's lung tissue, three weeks after the intraperitoneal injection of normal saline, B- Open section of the rat's lung tissue, three weeks after the intraperitoneal injection of Propline glycol, C- a sample of normal lung that a dose of 0.3 ml of sterile saline was administrated to it through endotracheal and also it has just received sterile saline through intraperitoneal injection during 21 days of the experiment, it is stained by Masson's trichrome strain, D- Open section of the rat's lung tissue, two weeks after the endotracheal injection of a dose of Bleomycin, E- Open section of the rat's lung tissue, one week before and two weeks after the endotracheal injection of a dose of Bleomycin, the treatment group of Atorvastatine + Boswellic extract.

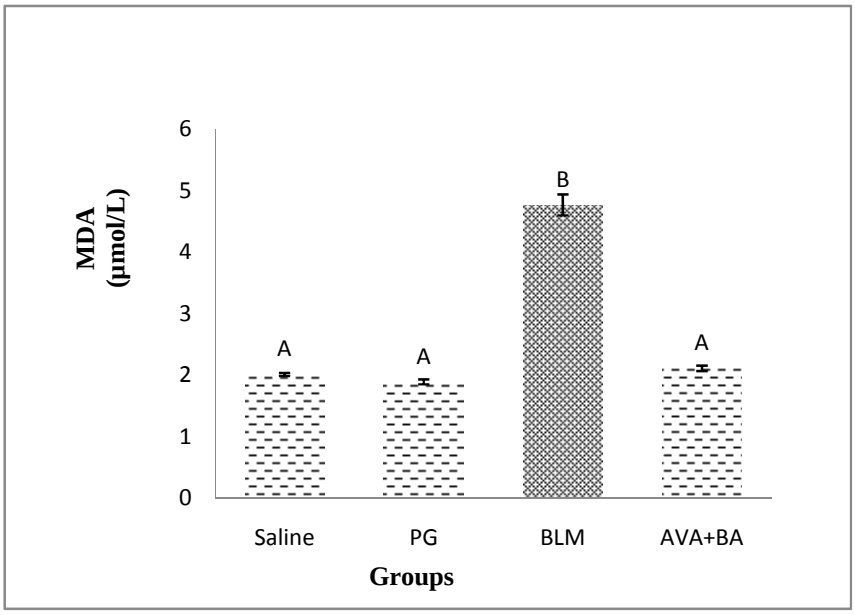

Chart 3

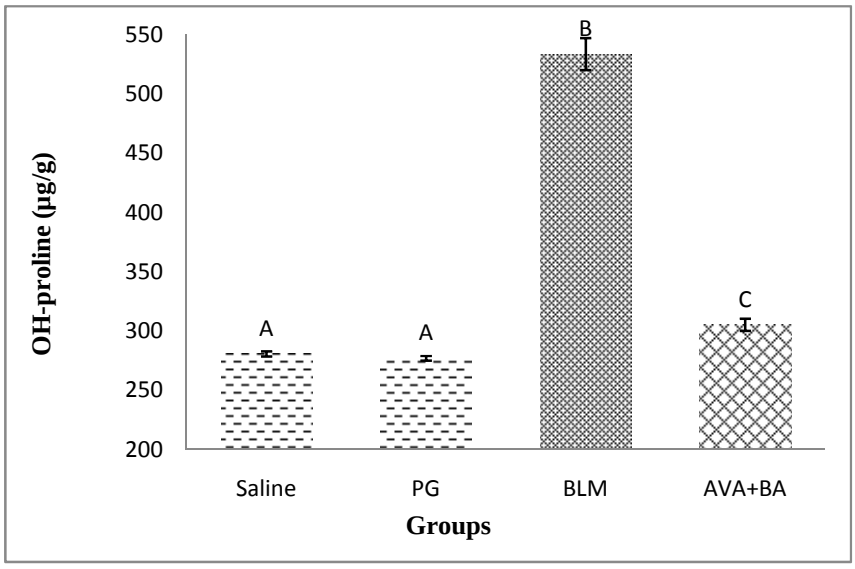

Chart 4

The effect of the combination of Atorvastatin and Boswellic extract on the lung weight (chart 1), the ratio of the lung weight to the body weight on the last day (%)(chart2), the amount of Malon dialdehyde (chart 3), the amount of pulmonary Hydroxyproline (chart4) for the studied groups: using One Way ANOVA and Duncan's test, according to Duncan's test (P 0.05), the groups with similar color have the same effects and there are no significant differences between them.

CONCLUSION

The surveys show that combination of two mentioned materials can reduce the progress of pulmonary fibrosis. Bleomycin injection causes an increase in the lung weight of mice compared to the normal lung.

This increased weight can be due to edema and fluid accumulation in the lung tissue which an appropriate space is provided because of the damage and destruction of alveoli and also duo to collagen accumulation which is the characteristic of Bleomycin-induced pulmonary toxicity (Selman *et al.,* 2001). The lung weight ratio to the total body weight of the animal showed the most amount in Bleomycin group which is probably because of edema and pulmonary infiltration. On the other hand, weight loss due to animal's anorexia and its destructive effect has caused a rise in this index.

In the pulmonary fibrosis disease, Malondialdehyde is produced because of free radicals production by Bleomycin and its effect on membrane lipids of pulmonary cells (Gao *et al.,* 2012). Increase the amount of this compound is a sign to show the extent of lipid peroxidation. In the Atorvastatin group and Olibanum extract, a relative decrease in MDA was seen which is probably related to antioxidant effects and removing free radicals in the treatment groups. These effects showed their best in the combination group and were placed in the limit of negative control group.

Fibrosis occurs due to a large accumulation of extracellular matrix proteins specially collagen in connective tissue (Behnamrasuli *et al.,* 2001). Evaluation of hydroxy proline amounts showed that the treatment group has a significant difference with Bleomycin group and effect aggregation can be understood as the reason of it. However, this amount is not at the range of negative control group.

The obtained results from the microscopic study of lung showed that the combination group could reduce the thickness of alveolar wall and progress of fibrosis in the mice that received Bleomycin. Comparison of rating in these groups is quite significant.

Overall results indicate that the combination factor has had a positive effect in improving of Bleomycin-induced pulmonary fibrosis and although it could not prevent it, but has avoided the progress of fibrosis a lot and delayed the process which is probably because of the antioxidant and anti-inflammatory mechanisms together. On the other hand due to the fact that one of the possible mechanism involved in Bleomycin-induced pulmonary fibrosis is intervention in the immune system, one of the reasons of positive results obtained from treatment can be the ability to influence of Atorvastatin and Olibanum on the immune system.

It is recommended that in case of an increase in treatment dose of drugs and persistence in their administration, better results can be achieved. More complete results provide the possibility of Atorvastatin and Olibanum extract together in treatment of fatal pulmonary fibrosis in humans.

FURTHER STUDY

This study can contribute to future research on effects of antioxidant and anti-inflammatory properties and reduce the Bleomycin-induced pulmonary fibrosis for helping to Patients with cancer.

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ABBREVIATIONS: AVA: Atorvastatin, BA extract: Boswellic acid extract, BLM: Bleomycin, PG: Propylene glycol, OH-Proline: Hydroxyproline, MDA: Malonedialdehyde

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