

Protective and therapeutic effects of ethanolic extract of *Nasturtium officinale* (watercress) and vitamin E against bleomycin-induced pulmonary fibrosis in rats

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Abstract

Background and purpose: Pulmonary fibrosis is a chronic disease of the lungs caused by inflammation, species of reactive oxygen, and immune defects. Antioxidant properties of *Nasturtium officinale* has been reported in some studies. Therefore, the objective of the current study was to evaluate the effect of ethanolic extract of *Nasturtium officinale* (EENO) on bleomycin (BLM)-induced lung fibrosis in rats.

Experimental approach: Forty adult male Wistar rats (180-220 g) were randomly divided into 5 experimental groups. Normal control, BLM control received a single dose of BLM (6 IU/kg) intratracheally only on the first day, EENO + BLM group received EENO (500 mg/kg) one week before intratracheal BLM instillation and two weeks afterward, BLM + EENO group and BLM + vitamin E group received EENO (500 mg/kg) and vitamin E (500 mg/kg) half-hour after BLM installation, respectively. The animals were sacrificed on day 22. Change in body weight, lung index, serum level of malondialdehyde (MDA) and nitric oxide (NO) metabolite, lung tissue hydroxyproline content and lung pathology were assessed.

Findings/Results: Pre- or post-treatment with EENO attenuated pulmonary fibrosis as evidenced by normalized lung index, improved histological changes and inhibited collagen deposition (hydroxyproline) in the animal lung. EENO also decreased MDA and NO metabolite release in comparison to the BLM control. vitamin E (500 mg/kg) also significantly inhibited the BLM-induced lung toxicity.

Conclusions and implications: EENO can prevent BLM-induced lung fibrosis in rats *via* antioxidant activities. However, more studies are needed to elicit the exact mechanism of this effect.

Keywords: Bleomycin; Fibrosis; *Nasturtium officinale*; Vitamin E.

INTRODUCTION

Bleomycin (BLM) as an antibiotic with antitumor, antiviral, and antibacterial activity is commonly used in the treatment of different forms of cancer (1). Despite the development of new drugs in the treatment of cancer, BLM remains an important drug of chemotherapy regimens for the treatment of some tumors such as germinative tumors and Hodgkin's lymphoma (2). BLM hydrolase is mainly responsible for metabolizing BLM to safe

molecules. Interestingly, the lung and skin have the lowest concentration of this enzyme. Therefore, these organs are the most important sites of BLM toxicity (3). BLM-induced pulmonary fibrosis is a fatal adverse effect that has been occurred in up to ten percent of patients receiving the drug (4).

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DOI: 10.4103/1735-5362.305192

BLM-induced injury to endothelial cells and consequent infiltration of leukocytes weaken pulmonary protective barriers. There are various evidences that point out reactive oxygen species (ROS) and the inflammatory process plays an important role in the pathology and development of pulmonary fibrosis (5). Accumulated evidence proposed that several factors such as cell proliferation, collagen synthesis, and production and release of cytokines as well as leukotrienes are involved in the induction and progression of pulmonary fibrosis (6)

Emerging evidence has demonstrated that oxidant/antioxidant imbalance is also involved in the pathogenesis of pulmonary fibrosis (7). At this time, lung transplantation is the only real treatment for pulmonary fibrosis, however, gene therapy has become one of the choices for treatment in the future (8,9). Immunosuppressive medications like glucocorticoids are frequently administered in this situation although their impact on patients' survival and quality of life are uncertain (10). Therefore, the development of new drugs to prevent or control pulmonary fibrosis is still a problem.

In Iranian folk medicine *Nasturtium officinale* R. Br. (watercress), from the Brassicaceae family has been broadly used to treatment of a variety of diseases including hypertension, hyperglycemia, and abdominal pain (11). This is also used to treat other illnesses such as bronchitis, stomach ulcer, pneumonia, influenza, asthma, and diabetes (12). This plant grows mostly in districts near the river in the spring in Europe and some parts of Asia (13). This plant's leaves are commonly eaten in fresh form in salads, soups, and other recipes and are very rich in vitamins, particularly vitamin C (14). The anticancer, hepatoprotective, nephroprotective, and antihyperlipidemic properties of watercress have been demonstrated in both in vitro and in vivo conditions (15,16). Also, some studies have shown that watercress exhibits significant antioxidant and anti-inflammatory activities in experimental conditions (17).

In this context, the current study was designed to evaluate the effects of ethanolic extracts of *Nasturtium officinale* (EENO), according to the mentioned properties, in the

prevention of BLM-induced pulmonary fibrosis in rats compared to vitamin E.

MATERIAL AND METHODS

Thiobarbituric acid, trichloroacetic, hydrochloric acid, chloramine T, sodium acetate, hydroxyproline, sulfanilamide, and N-naphthyl ethylenediamine were purchased from Merck (Darmstadt, Germany). Bleomycin hydrochloride (Bleo-Cell[®], 15 mg/vials, Cell Pharm GmbH, Germany) was purchased from a pharmacy.

Animals

Forty healthy adult male Wistar rats (180-220 g) were purchased from the animal house of Yasuj University of Medical Sciences, Yasuj, I. R. Iran. All the animals were kept under standard laboratory conditions (12/12 h light/dark). The animals had open access to food and tap water and were divided into five groups at random. The experiment was carried out in accordance with the 'Guide for the Care and Use of Laboratory Animals' (Ethical code: 19720415942002).

Preparation of extract

Aerial parts of *Nasturtium officinale* were gathered from Kohgiluyeh and Boyer-Ahmad (Iran) province in May 2017 and authenticated by Dr. A. Jafari (Botany Department, Natural Resource and Animal Husbandry Research Centre, Yasuj University, Yasuj, Iran) and a voucher specimen (Herbarium No. HYU30230) was deposited there.

The air-dried powder of aerial parts (200 g) of the plant was extracted three times with an 1000 mL mixture of ethanol (7:3) at 37 °C for 72 h. The extract was filtered and concentrated by rotary evaporation and dried at 50 °C and then, it was dried at room temperature. The dried extract was weighed and kept at -20 °C for further studies (16).

Experimental design

All animals randomly were divided into five groups, eight each. (1) The control group was given a single dose of normal saline, then received everyday i.p. saline injection; (2) BLM-treated group was only treated

intratracheally BLM (6 IU/kg in 0.9% NaCl). Briefly, after anesthesia, the rats were fixed on a board, the tongue was pulled out with forceps, and the fluid was poured at the distal part of the mouth and teeth while the nose was gently closed (18). (3) EENO + BLM group was given EENO (500 mg/kg, p.o. once a day) before the induction of fibrosis and two weeks after the induction of fibrosis; (4) BLM + EENO group was given EENO (500 mg/kg, p.o., once daily) half an hour after BLM for three weeks; and (5) BLM + vitamin E group was given vitamin E (500 mg/kg, p.o., once a day) for 21 days after BLM challenge (19). The indicated doses were selected based on our previous studies (17)

Preparation of biochemical and histological samples

On day 22, the body weights of the animals were recorded, then they were euthanized by ether anesthesia and blood samples were collected by cardiac puncture for malondialdehyde (MDA) and nitric oxide (NO) metabolite levels assessment. Then, the lung was carefully removed and weighed. The pulmonary specimens were divided into two sections, one section was submerged in 10% formalin solution for histological analysis and one section was kept in liquid nitrogen for assessment of hydroxyproline level (20).

Assessment of lipid peroxidation

The serum level of MDA was measured according to our previous study (21). In brief, 375 mg of thiobarbituric acid was diluted in 2 mL of HCl (12 N), followed by 15 g of trichloroacetic acid for a total amount of 100 mL. A half milliliter of serum was then mixed with 2 mL of this solution and heated in a boiling bath of water for 15 min. Finally, the absorbance was read at 535 nm, and the amount of MDA was represented as $\mu\text{mol/mL}$.

Assessment of hydroxyproline content

The content of hydroxyproline as an index of lung fibrosis in the lung tissues was determined spectrophotometrically based on the previous study (22). The right lung tissues were weighed and homogenized. Briefly, for digestion, aliquots of 1 mL from the homogenized tissue were mixed with 6 M hydrochloric acid, at 150

$^{\circ}\text{C}$, for 4 h. After cooling, the solution was neutralized with 6 M sodium hydroxide and filtered. Then, the filtrate was mixed with the solution consisting of 1.4% chloramine T, 10% propanol, and 0.5 M sodium acetate (15), after 20 min Ehrlich's reagent was added at 65 $^{\circ}\text{C}$ for 15 min. After cooling, absorbance was read at 550 nm and the quantity of hydroxyproline was determined against a standard curve of known concentrations of hydroxyproline.

Assessment of NO metabolite level

Measurement of NO metabolite levels in serum was carried out spectrophotometrically with the Griess method according to our previous study (20). Briefly, 100 μL of serum was added to the same volume of Griess reagent to generate a purple azo dye. The Griess reagent (1.25% HCl, 5 mg/mL sulfanilamide with 0.25 mg/mL N-naphthyl ethylenediamine) was added at a rate of 0.1 mL/min. The absorbance of the dye output was measured at 540 nm with attention to a standard nitrite curve generated using NaNO_2 by Elisa reader.

Histological studies

The rats were euthanized and their lung was carefully removed and fixed in 10% buffered formaldehyde solution for 1 week. Then, the fixed biopsies were embedded in paraffin and cut into 3-4 μm slices (Automatic tissue processor, Auto Technique). The slices were mounted on glass slides and then stained with Hematoxylin-Eosin dye and examined for histopathological evaluation.

Statistical analysis

The collected results were statistically analyzed by SPSS software (Version 17). The results were presented as mean \pm SEM and evaluated by a one-way variance analysis (ANOVA) accompanied by Tukey's post-test. *P* values less than 0.05 were considered to show significant differences for all comparisons made.

RESULTS

The effect of EENO on bodyweight and lung index

As shown in Table 1, intratracheal instillation of BLM did not inhibit weight gain

but significantly increased lung weight when compared with the normal group ($P < 0.05$).

As illustrated in Fig.1, the lung index in the BLM-treated group was significantly higher than the control group ($P < 0.001$). Pre- or post-treatment with EENO (500 mg/kg) significantly normalized the increased lung index compared to the BLM-treated group ($P < 0.05$ and $P < 0.001$, respectively). Vitamin E (500 mg/kg) also considerably inhibited changes in lung index comparison to BLM-treated rats ($P < 0.01$).

The effect of EENO on hydroxyproline level

As illustrated in Fig. 2, BLM increased the hydroxyproline content in lung tissue in comparison with the control group ($P < 0.01$). Pre- and post-treatment with EENO (500 mg/kg) significantly decreased the hydroxyproline level when compared to BLM-treated group ($P < 0.001$ and $P < 0.01$, respectively). Vitamin E (500 mg/kg) also significantly reduced hydroxyproline content in treated animals compared to the BLM-treated group ($P < 0.05$).

Table 1. The effects of BLM, EENO, and Vit E on the body and lung weight. Data are presented as mean \pm SEM, n = 6. * $P < 0.05$ Indicate significant differences compared with the control group

Body weight	Groups				
	Control	BLM	EENO + BLM	BLM + EENO	BLM + Vit E
Initial body weight (g)	216.6 \pm 9.8	174.1 \pm 2	185.8 \pm 5	184 \pm 4.54	157 \pm 1.8
Final body weight (g)	274 \pm 14	212 \pm 10.7	222.5 \pm 9.9	235 \pm 9.2	230.5 \pm 10.7
Variation of weight (%)	26.7	22.2	19.75	27.7	31.4
Lung weight (g)	1.82 \pm 0.42	2.96 \pm 0.34*	2.2 \pm 0.43	1.85 \pm 0.32	2.08 \pm 0.40

BLM, Bleomycin; EENO, ethanolic extract of *Nasturtium officinale*; Vit E, Vitamin E.

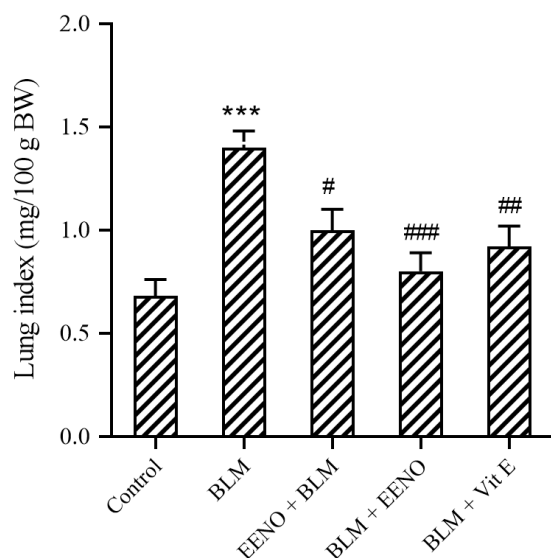


Fig. 1. Effect of EENO extract on lung index of BLM-induced pulmonary fibrosis in rats. Values are presented as mean \pm SEM of six independent observations in each group. *** $P < 0.001$ Indicate significant differences compared with the control group; # $P < 0.05$, ## $P < 0.01$, and ### $P < 0.001$ vs bleomycin-treated rats. BLM, Bleomycin; EENO, ethanolic extract of *Nasturtium officinale*; Vit E, vitamin E.

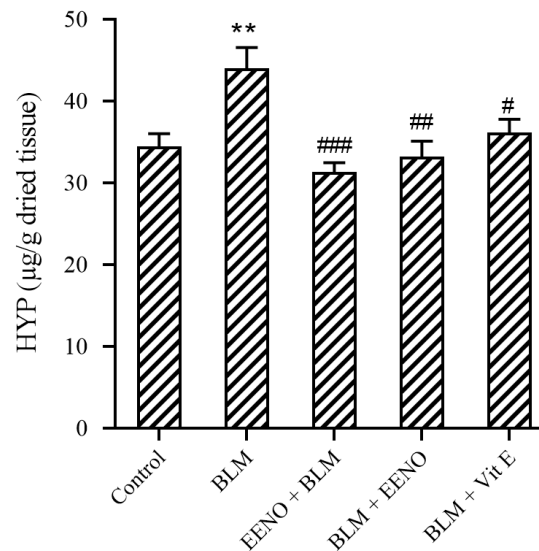


Fig. 2. Effect of EENO extract on HYP content of BLM-induced pulmonary fibrosis in rats. Values are presented as mean \pm SEM of six independent observations in each group. ** $P < 0.01$ Indicate significant differences compared with the control group; # $P < 0.05$, ## $P < 0.01$, and ### $P < 0.001$ vs bleomycin-treated rats. BLM, Bleomycin; EENO, ethanolic extract of *Nasturtium officinale*; Vit E, vitamin E; HYP, hydroxyproline.

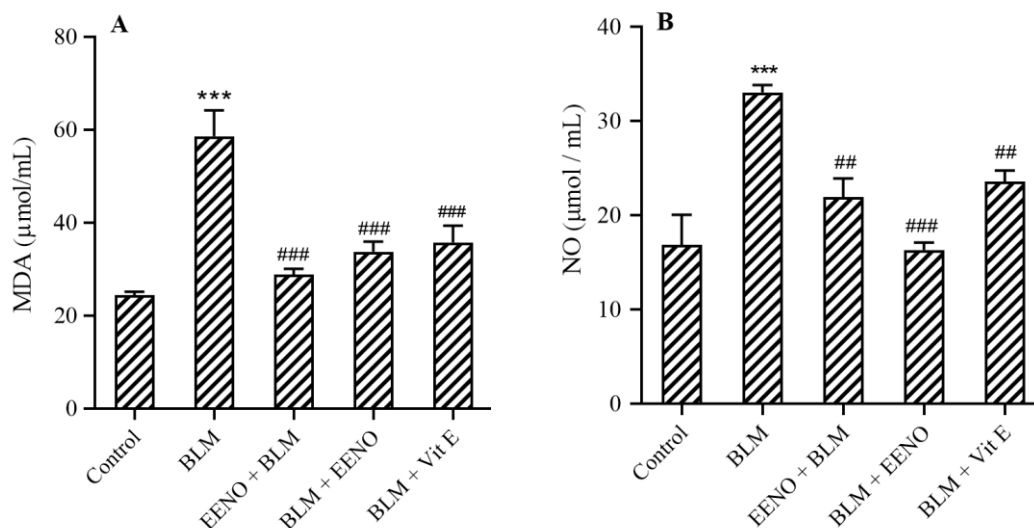


Fig. 3. Effect of EENO extract on serum levels of MDA and NO of BLM-induced pulmonary fibrosis in rats. Values are presented as mean \pm SEM of six independent observations in each group. *** $P < 0.001$ Indicate significant differences compared with the control group; # $P < 0.01$ and ### $P < 0.001$ vs bleomycin-treated rats. BLM, Bleomycin; EENO, ethanolic extract of *Nasturtium officinale*; Vit E, Vitamin E; MDA, malondialdehyde; NO, nitric oxide.

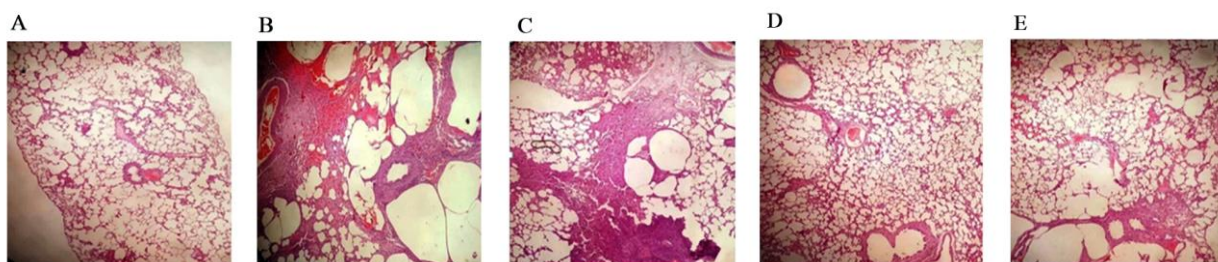


Fig. 4. The Influence of EENO on histopathological changes of BLM-induced pulmonary fibrosis in rats. (A) Normal lung histology in the saline control; (B) inflammatory cell infiltration and fibrosis in the BLM group; (C) weaker fibrosis in the BLM + vitamin E group (500 mg/kg); (D) reduced lung fibrosis in the EENO + BLM group (500 mg/kg); and (E) marked prevention of fibrosis in the BLM + EENO group (500 mg/kg). BLM, Bleomycin; EENO, ethanolic extract of *Nasturtium officinale*.

The effect of EENO on NO metabolite and MDA level

As shown in Fig. 3A and B, BLM caused a significant rise in the serum NO metabolite and MDA level compared with the control group ($P < 0.001$). Treatment with EENO (500 mg/kg) before and after the BLM challenge inhibited significantly the increase in the serum NO metabolite and MDA level when compared to the BLM-treated group ($P < 0.01$). Vitamin E (500 mg/kg) also, considerably decreased the serum NO level in treated animals compared to BLM-treated rats ($P < 0.01$).

Histopathological assessment

As presented in Fig. 4A, pathological evaluation of the lung tissue in the normal group showed the well-organized pathological

construction of lung tissue without any inflammatory infiltration and interstitial fibrosis. However, the BLM group lung examination revealed that BLM induced inflammatory infiltration, emphysema, and bleeding in the lung that represented the damage of the alveoli (Fig. 4B). EENO (500 mg/kg) treatment considerably reduced inflammatory influx and interstitial fibrosis as compared to the BLM-treated group (Fig. 4D and E). Treatment with vitamin E is also moderately was able to prevent BLM-induced alterations (Fig. 4C).

DISCUSSION

Pulmonary fibrosis is a chronic progressive interstitial lung disease as a result of the

excessive accumulation of collagen, epithelial cell damage, and disruption of the integrity of alveolar leading to decreasing lung function (23). BLM-induced pulmonary fibrosis in animals has been developed to better recognize the underlying pathophysiology and examine new components to the treatment of the disorders (24). It has been well-known that BLM-endotracheal instillation decreases body weight whereas increasing lung weight. These alterations can reduce to some extent with appropriate medication such as anti-inflammatory and antioxidants agents (7).

The findings of the current study showed that BLM endotracheal instillation did not prevent the gain of weight but increased lung index in the group treated with BLM compared to the normal group. This finding was consistent with Abidi *et al.* study (7). Treatment with EENO and vitamin E considerably reduced the indicated changes induced by BLM. In this line, a previous study reported EENO was able to normalize the ratio of kidney weight/100 mg BW in the vancomycin-induced kidney toxicity in the rat (16).

An essential pathological hallmark of pulmonary fibrosis is extreme deposition collagens, and hydroxyproline is a specific component of collagen hydrolysis. Therefore, lung tissue hydroxyproline content regards as a pulmonary fibrosis mark (25). The results of the current study indicated that the hydroxyproline level in the lung of BLM-treated animals was considerably higher than that in the control group, whereas the lung hydroxyproline level in the EENO-treated group was considerably lower than that in the BLM group. The subsequent confirmatory pathological study showed significant alveolar space structure alteration in BLM-induced animals with collapsed alveoli, interalveolar inflammation, inflammatory infiltration, and emphysema. Similar histopathological changes have been established by other investigations (25). This finding proposes that the protection activities of EENO on BLM-induced pulmonary fibrosis were highly associated with reduced lung hydroxyproline levels. In this line, it has been reported that EENO displays potential anti-fibrotic properties in animal models of liver fibrosis (15,26).

An imbalance between antioxidant agents and free radicals causes oxidative stress, which plays an important role in the pathophysiology of various diseases, including liver and kidney toxicity, inflammation and pulmonary fibrosis (27,28). Several data are pointing to the important role of oxidative stress in lung fibrosis pathophysiology (29). It has been reported that ROSs and reactive nitrogen species rise in animal models of pulmonary fibrosis (30). NO, an endogenous short-term free radical, is generated by three NO synthase (NOS) isoforms, including neuronal, endothelial, and inducible NOS isoforms (31). Recent studies have shown that an excessive generation of NO, nitrogen dioxide, and peroxynitrite plays a significant role in the development of pulmonary fibrosis in both human and animal models pulmonary fibrosis (32). Our results indicated that the serum NO metabolite concentration in the pulmonary fibrosis caused by the BLM was significantly higher than that in the normal group. The rise in nitrite concentration of BLM-treated rats is in accordance with the result of El-Khouly *et al.* (29). Kalayarasan *et al.* reported that BLM augmented iNOS expression in pulmonary fibrosis (33). The serum level of NO metabolite in the EENO-treated group was significantly lower than that in the BLM-treated group, proposing that EENO could attenuate lung fibrosis *via* suppressing the generation of RNAs. In this regard, watercress was observed to possess a NO scavenging property *in vitro* condition, an ability that might be because of its phenolic/flavonoid content (34). It also reported that watercress produced a noticeable effect on the elevated NO concentration in the other animal models of toxicology (17).

It has been established that ROS generation is associated with the Fe (II) to Fe (III) oxidation. This cycle promotes oxygen reduction to free radicals that participate in BLM-antitumor activity (29). Intracellular ROSs attack the cell membrane *via* polyunsaturated fatty acids disintegration, which clarifies in consequence MDA rise subsequent BLM challenge. MDA is a reactive carbon agent that is used as a marker of lipid peroxidation (35). In line with the previous studies, our data showed that

BLM-endotracheal instillation induced a significant increase in the serum levels of MDA in BLM-treated animals. Pre- and post-treatment with EENO similar to vitamin E significantly reduced the increased levels of MDA serum due to BLM endotracheal instillation. These findings indicated that EENO could decrease lung fibrosis by inhibiting oxidative stress pathways.

It is important to mention, that phytochemical evaluation of the *Nasturtium officinale* extract in our previous study revealed that benzenepropanenitrile (48.3%), phytol (10.1%), α -cadinene (9.5%), and linolenic acid (8.0%) are its main constituents (27). Furthermore, the existence of phenolic and flavonoid compounds in the total extract of *Nasturtium officinale* has been quantified. The presence of rutin, chlorogenic, and caffeic acids were also reported in the plant (36). *Nasturtium officinale* was identified by some studies for its antioxidant and anti-inflammatory benefits (15,17,37). In this context, these phenolic components in the plant *via* the antioxidant and anti-inflammatory activities could have a significant role in the attenuating of pulmonary fibrosis.

At last not at least, pre- or post-treatment with EENO lessened the BLM-induced pulmonary, proposing the potential value of EENO as a component to avoid and manage pulmonary fibrosis. Furthermore, the data of the present study indicated the efficacy of EENO on pulmonary fibrosis were similar to those of vitamin E, indicating strong antioxidant activity of the extract.

CONCLUSION

This study confirmed the anti-fibrotic efficacy of EENO against BLM-induced pulmonary fibrosis in rats. EENO seemed to cause a pneumoprotective effect through the improvement of antioxidant status and reduction in collagen accumulation in lung tissue. These results propose the potential therapeutic properties of *Nasturtium officinale* for preventing or treatment of pulmonary fibrosis. However, further studies will be needed to clarify the exact mechanisms, finding

human dosage, and possible toxicity of EENO in the management of pulmonary fibrosis.

Acknowledgments

We would like to appreciate the staffs of the Medicinal Plants Research Center, Yasuj University of Medical Sciences, Yasuj, Iran for their technical support.

Conflict of interest statement

The authors declare no conflict of interest in this study.

Authors' contribution

The idea was developed by H. Sadeghi and I. Javadi. H. Sadeghi, J. Nikbakht and A.H. Doustimotlagh supervised this work. *in vivo* investigations were done by E. Panahi Kokhdan, N. Omidifar, and S. Ramezani. Data collection and analysis were performed by E. Panahi Kokhdan, N. Omidifar, S. Ramezani and A.H. Doustimotlagh. H. Sadeghi, N. Danaei. J. Nikbakht and R. Abbasi contributed to manuscript preparation and revision.

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