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Harmaline Improves Oxidative Stress and Inflammatory Markers in Human Lung Epithelial Cells Exposed to Elastase

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ABSTRACT

Background: Harmaline exhibits a diverse array of pharmacological properties, including antimicrobial, antidiabetic, osteogenic, immunomodulatory, emmenagogue, and antitumor activities. The current study aimed to investigating the effect of harmaline on oxidative stress factors in lung epithelial cells exposed to elastase.

Material and method: oxidative stress markers of lung epithelial cells were investigated in all cell groups including, control, H2O2, elastase and elastase plus harmaline (50, 100, 200 µm). lung epithelial cells (A549) were exposed to elastase with concentrations of 60 U/ml for 24 hours. In other groups, cells exposed to elastase were co-treated with three different doses of harmaline (50, 100 and 200 µm) for 24 hours at 37°C.

Results: the results show a significant effect of harmaline's protective effect on cell viability, free radical production (ROS), malondialdehyde (MDA) and total antioxidant capacity (TAC). harmaline significantly increased the viability and TAC level in the cells exposed to elastase. Also, harmaline significantly decreased the percentage of free radicals and the MDA level in the cells exposed to elastase.

Conclusion: The results obtained from this study showed a significant protective effect of harmaline on cell viability through increases in antioxidant defense system. Therefore, harmaline, can probably considered as a therapeutic strategy to prevent or treatment of lung diseases.

Keywords:
Lung Epithelial Cells, Respiratory System, Oxidative Stress, Harmaline.

INTRODUCTION

Chronic obstructive pulmonary diseases (COPD) include emphysema (destruction and enlargement of pulmonary alveoli), chronic bronchitis (chronic cough and increased mucus secretion) and small airways disease (increased resistance in small bronchioles). The pathophysiology of lung diseases includes a number of pathogenic processes such as inflammation, apoptosis, lack of cell regeneration, destruction of extracellular matrix and oxidative stress. Lungs are continuously exposed to oxidizing factors. Increased oxidative stress is one of the most important factors in causing pulmonary disorders (1). Elastase, as a protease, disrupts the balance between protease-antiproteases, and for this reason, it is used in animal models to develop chronic obstructive pulmonary diseases and emphysema (2). Previous studies have shown that elastase can lead to increased apoptosis in lung epithelial cells (3). Also, elastase leads to increased mucin secretion and increased expression of inflammatory factors such as interleukin-8 (IL-8) in bronchial epithelial cells. Inflammatory factors are a strong activator for the secretion of neutrophils, which themselves cause the secretion of more inflammatory factors (4-5). Also, elastase increases the expression of Cathepsin B and matrix metalloprotease-2 (MMP-2) in macrophages (6). Elastase causes the destruction of local defense mechanisms of the lung through the destruction of cell matrix molecules and the reduction of mucociliary clearance (7). Studies have shown that elastase increases the expression of Mucin 5AC gene through increasing the production of free radicals and causing oxidative stress, which is effective in the increased secretion of mucin and causing obstructive disorders in the lungs (8).

Oxidative stress is caused by an imbalance between the production of free radicals and

antioxidant system, which causes damage to a wide range of cells substances, including proteins and nucleic acids. Polyphenolic compounds are the largest group of phytochemicals, most of which are found in vegetable foods, and studies have shown the beneficial effects of polyphenols-rich substances on health.

Polyphenols have antioxidant properties that help the body's antioxidant defense against oxidative stress caused by free radicals, and the mechanism of this protective effect can be related to the intervention of these compounds in intracellular signaling pathways (10).

Pecan or harmala (P. harmala) belongs to the Zygophyllaceae family and is used as a therapeutic agent in traditional medicine in countries such as Pakistan, China, Morocco, Algeria and Spain to treat several chronic disorders. Pecan seeds are well known for their anti-inflammatory, anti-parasitic and anti-spasmodic effects. In addition, pecan is very effective for treating asthma and liver disorders, and reducing fever in chronic malaria (11-13).

Harmaline is one of the alkaloids derived from the pecan plant, which has anti-inflammatory and anti-oxidative stress properties. The sweeping effects of this substance have been proven in in vivo and in vitro studies. Harmaline has also shown several biological activities in various studies, including anti-inflammatory, antibacterial, antifungal, antiviral, antioxidant, analgesic, cardioprotective, antitumor, antidiabetic, and protection against brain degeneration (14-16).

Harmaline is also reported to contain flavonoid compounds. Harmaline has a wide range of pharmacological effects, including inhibition of platelet aggregation, inhibition of monoamine oxidase, anti-anxiety and behavioral effects. There are immune system modulating effects as well as reports on the ameliorating effects of harmaline in heart disease (17-18).

According to the mentioned data and considering the lack of investigation of the effect of this antioxidant on human respiratory cells in the in vitro condition, the present study aims to investigate the protective role of harmaline as a natural antioxidant agent in improving the oxidative stress caused by elastase in human lung epithelial cells (A549 cells).

MATERIAL AND METHODS

The A549 cells were purchased from Pasture Institute of Iran. after culturing, the grouping was done as follows:

1. Negative control group
2. Positive control group receiving H2O2 (a well-known oxidant) 100 μM for 24 h at 37°C (19).
3. The group receiving elastase (ELS), 60 U/ml for 24 hours at 37°C (20).
4. The group receiving elastase 60 U/ml along with harmaline 50 μm for 24 hours at 37°C (ELS+HR50).
5. The group receiving elastase 60 U/ml along with harmaline 100 μm for 24 hours at 37 degrees Celsius (ELS+HR100).
6. The group receiving elastase 60 U/ml along with harmaline 200 μm for 24 hours at 37°C (21) (ELS+HR200).
7. The group receiving the most effective harmaline dose for 24 hours at 37°C (HR200).

Lung epithelial cell culture

A549 cell line cultured with a density of $5x10^6$ cells in 100 mm cell culture dishes containing RPMI-1640 medium plus 10% FBS. The final volume of 10 ml in an incubator at 37 °C and CO2 were set at 5% and the medium was changed once every two days. When the flask was filled (Confluency 80-90%), passage was given and after reaching the appropriate number of flasks and cells, the groups were determined and an equal number of cells were added to each group in the flask and the treatment period was started. Then, the cells were removed from the incubator and the desired assays were performed according to the required protocols (19).

MTT analysis

In this method, after the incubation time, the culture medium was discarded. 200 microliters of culture medium containing half mg/ml MTT solution was added to each well and placed again for 2 to 4 hours in a carbon dioxide incubator at 37 degrees Celsius. During the incubation time, MTT was regenerated by the succinate dehydrogenase system, which is one of the enzymes of the mitochondrial respiratory cycle. The regeneration and decomposition of this ring resulted in the production of purple formazon crystals, which were easily recognized under the microscope. The amount of color produced is directly related to the number of cells that are metabolically active (viable cells). Considering

that formazon crystals are insoluble in water, they must be converted to a soluble state with a solvent such as dimethyl sulfoxide (DMSO) before colorimetry (19). In case of significant decrease in cell life in the treated cells, it can be concluded that the test substance has cytotoxicity. Finally, the average absorbance is divided by the absorbance of the control and multiplied by 100. The resulting numbers show the percentage of viability. Then the survival rate was drawn in the form of a graph and the results were interpreted and analyzed based on the graph.

Free radicals (ROS) detection in lung epithelial cells

The amount of ROS release was determined by flow cytometry and using H2DCFDA, which is an oxidant-sensitive probe (19).

Measurement of TAC and MDA

For this purpose, after the end of the study period, the cell supernatant was removed and applied to measure the TAC and MDA concentration level using the special assay kits and via spectrophotometric method.

Measurement of inflammatory biomarkers

At the end of the study, the cell supernatant was removed and applied to measure the IL-6 and IL-10 concentration levels using the special ELISA kits and via spectrophotometric method.

Data analysis

All experiments were performed 3 independent repeats. SPSS software was used to analyze the data. ANOVA statistical test and appropriate post hoc test were used to compare between groups if normal. Using the K_s test, the normality of the data was checked, and the equality of variances test was also reported. Data were calculated as Mean±SEM and $P<0.05$ was considered as a significant level.

RESULTS

Cell viability in response to harmaline

In order to investigate the protective effect of harmaline on cell viability, as the results shown in figure. 1, there is a significant difference between cell viability in the studied groups ($P<0.05$). the highest average cell viability was in the negative control group and HR200 and the lowest average cell viability was in the positive control group. As shown in figure. 1, exposure to 100 µM of H2O2 and also 60 U/ml elastase for 24 hours significantly decreased cell viability compared to the control group. Also, the data shows that in the treatment groups with different doses of harmaline has significantly increased cell viability compared to the ELS group, and among the groups treated with harmaline, the ELS+HR200 group has increased cell viability more than the other two groups, which means that the dose of 200 µM is most effective concentration.

Free radical production in response to harmaline

According to the results of figure 2, there is a significant difference between the production of free radicals in the studied groups ($p<0.05$). the highest average production of free radicals was observed in the positive control group and ELS, and the lowest production rate of free radicals was observed in the negative control group and HR200. As shown in Figure 2, exposure to 100 µM H2O2 and 60U/ml elastase for 24 hours has significantly increased the production of free radicals compared to the control group. Also, the results shows that the elastase treated groups with different doses of harmaline has significantly reduced the amount of free radicals compared to the ELS group. Statistical analysis showed that in the ELS+HR200 and ELS+HR100 groups the ROS production was significantly lower compared to the ELS+HR50 group.

Oxidative stress marker in response to harmaline

As the results shown in figure 3, there is a significant difference between the MDA level in the studied groups ($p<0.05$). the highest average of MDA was observed in the positive control group and ELS, and the lowest amount of oxidative stress was shown in the negative control group and HR200. as shown in Figure 3, exposure to 100 µM of H2O2 and 60 U/ml of elastase for 24 hours significantly increased MDA compared to the control group. Also, the data shows that the treatment of the elastase-group with different doses of harmaline significantly reduced MDA compared to the ELS group. the effective dose of harmaline in reducing oxidative stress marker was 200 µm.

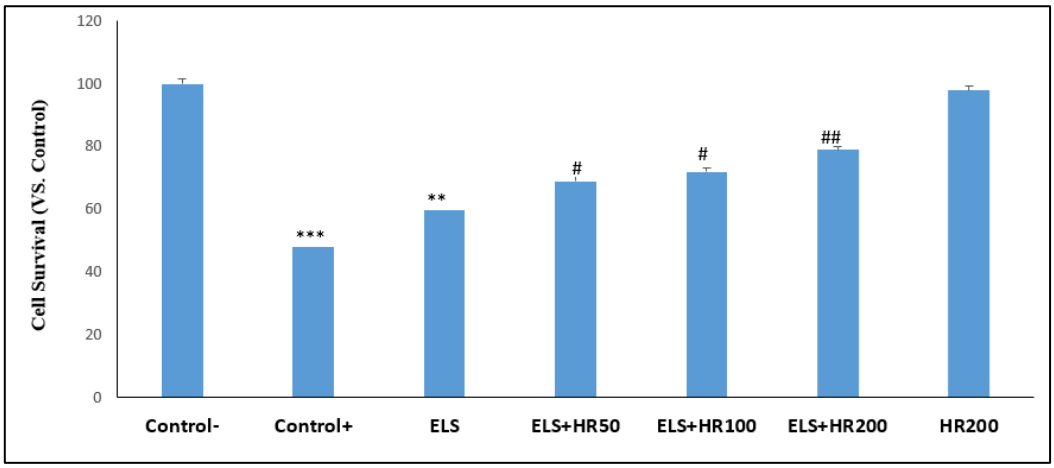

Figure 1: Comparison of cell viability in all groups including: negative control group, positive control, elastase receiving group (ELS), elastase receiving group with 50 µm harmaline (ELS+HR50), elastase receiving group with with 100 µm harmaline (ELS+HR100), the group receiving elastase with 200 µm harmaline (ELS+HR200) and the group receiving the most effective dose of harmaline (HR200). * Comparison with negative control group and # comparison with ELS group. Data are Mean±SEM, One-Way ANOVA statistical test and HSD post hoc test.

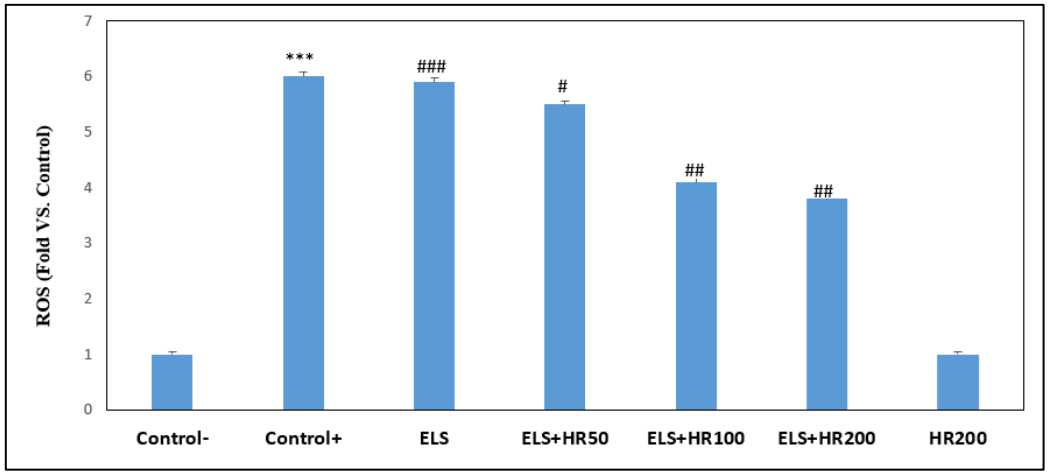

Figure 2: Comparison of free radicals in all groups including: negative control group, positive control, elastase receiving group (ELS), elastase receiving group with 50 µm harmaline (ELS+HR50), elastase receiving group along with 100 µm harmaline (ELS+HR100), the group receiving elastase along with 200 µm harmaline (ELS+HR200) and the group receiving the most effective dose of harmaline (HR200). * Comparison with negative control group and # comparison with ELS group. Data are Mean±SEM, One-Way ANOVA statistical test and HSD post hoc test.

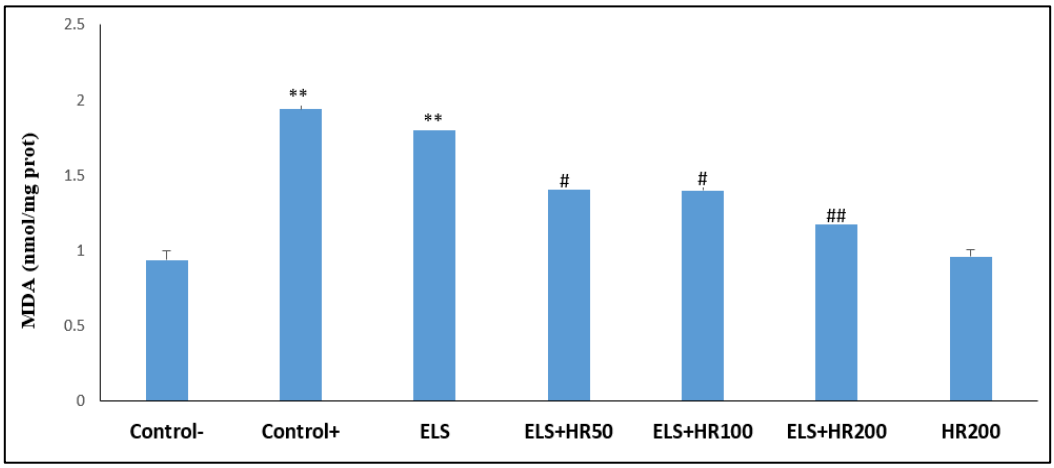

Figure 3: Comparison of the level of malondialdehyde (MDA) in all studied groups including: negative control group, positive control, elastase receiving group (ELS), elastase receiving group with 50 µm harmaline (ELS+HR50), The group receiving elastase with 100 µm harmaline (ELS+HR100), the group receiving elastase with 200 µm harmaline (ELS+HR200) and the group receiving the most effective dose of harmaline (HR200). * Compared with the negative control group and # compared with the ELS group. Data are Mean±SEM, One-Way ANOVA statistical test and HSD post hoc test.

Antioxidant marker in response to harmaline

As shown in figure 4, there is a significant difference between TAC level in the studied groups ($P<0.05$). the highest average amount of TAC was in the negative control group and HR200, and the lowest average TAC was related to the ELS group. The cell group exposure to 100 µM of H_2O_2 and 60 U/ml of elastase for 24 hours significantly reduced the TAC level compared to the control group. Also, the figure 4, shows that the treatment with elastase with different doses of harmaline has significantly increased the TAC level compared to the ELS group. In this regard, the ELS+HR200 group shows the highest increase in TAC level compared to other treatment group.

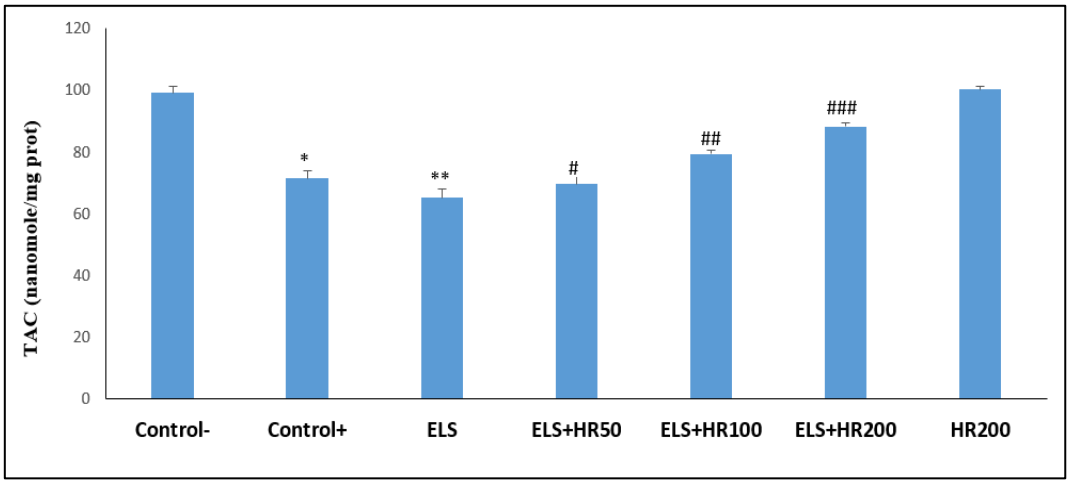

Figure 4: Comparison of total antioxidant capacity (TAC) in all studied groups including: negative control group, positive control, elastase receiving group (ELS), elastase receiving group with 50 µm harmaline (ELS+HR50), the group receiving elastase with 100 µm harmaline (ELS+HR100), the group receiving elastase with 200 µm harmaline (ELS+HR200) and the group receiving the most effective dose of harmaline (HR200). * Compared with the negative control group and # compared with the ELS group. Data are Mean±SEM, One-Way ANOVA statistical test and HSD post hoc test.

Inflammatory biomarkers in response to harmaline

As shown in figure 5, there is a significant increase in IL-6 as pro-inflammatory marker and significant decreases in IL-10 as anti-inflammatory cytokine concentration levels in the elastase exposure cells ($P<0.001$) compare to the control. The elastase exposure cell groups treated with harmaline showed significant decreases in IL-6 and increases in IL-10 concentration levels compared to the ELS group. In this regard, the ELS+HR200 group shows the highest increases in IL-10 level and lowest IL-6 level compared to other treatment groups.

DISCUSSION

According to our knowledge this is the first experiment investigated the efficacy of harmaline on oxidative stress factors in lung epithelial cells exposed to elastase. In this study, the antioxidant protective effects of harmaline on A549 lung epithelial cells under elastase-induced toxicity were documented.

COPD is a major global epidemic that is increasing worldwide as the population ages. COPD mainly occurs in smokers and people over 40 years of age. The prevalence increases with age and is currently the third leading cause of death worldwide. In 2015, the prevalence of chronic pulmonary disorders was 174 million, and there were approximately 3.2 million COPD-related deaths worldwide. However, due to misdiagnosis of COPD, its prevalence is likely to be underestimated (22). COPD is a progressive and irreversible disease and is associated with airway restriction. Neutrophils play an important role in the pathophysiology of COPD by causing chronic inflammation and tissue destruction. Neutrophils that have migrated from the wall of pulmonary vessels destroy the walls of alveoli by releasing reactive oxygen species and proteases such as elastase (1).

Oxidative stress refers to the imbalance of the redox state of the body and disruption of the antioxidant system, during which the increase of free radicals has a major effect on the physiological and pathophysiological mechanisms of the lung (23). The main cause of oxidative damage is reactive oxygen species, which play an important role in the creation of

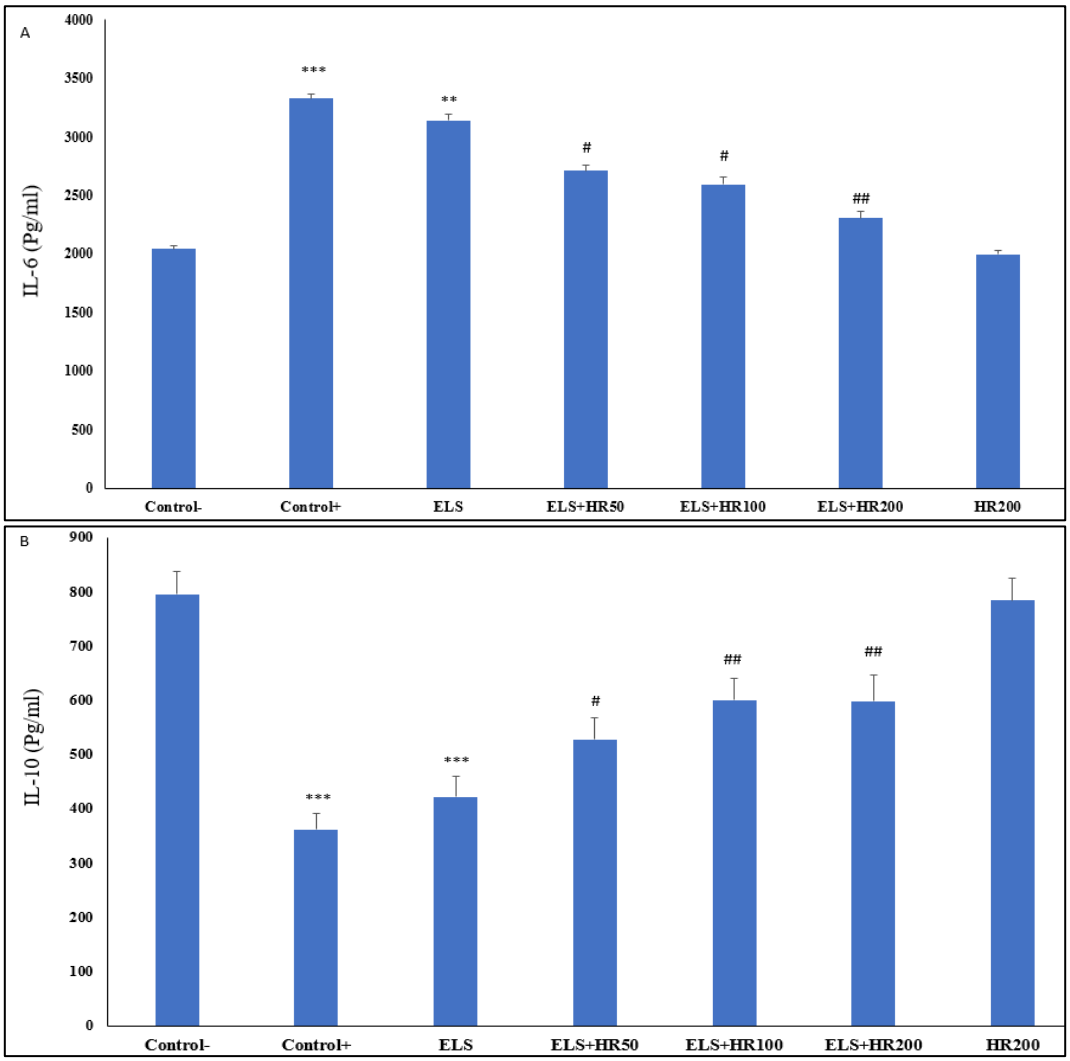

Figure 5: Comparison of IL-6 (A) and IL-10 (B) in all studied groups including: negative control group, positive control, elastase receiving group (ELS), elastase receiving group with 50 µm harmaline (ELS+HR50), the group receiving elastase with 100 µm harmaline (ELS+HR100), the group receiving elastase with 200 µm harmaline (ELS+HR200) and the group receiving the most effective dose of harmaline (HR200). * Compared with the negative control group and # compared with the ELS group. Data are Mean±SEM, One-Way ANOVA statistical test and HSD post hoc test.

other reactive species, the development of aging disorders and degenerative diseases. Malondialdehyde as a lipid peroxidation factor, adenosine triphosphate and cytochrome-C as a cell life factor and antioxidant prooxidant balance as a cell regeneration oxidation balance factor are usually used to measure oxidative stress in the body. Malondialdehyde is one of the major products of the degradation of unsaturated fatty acids by hydroxyl radicals (24). On the other hand, cytochrome C, which controls redox signaling in mitochondrial oxidative phosphorylation, affects cellular energy under the influence of poisoning. Therefore, in the human body, the balance between the production and removal of reactive oxygen species is vital under the title of prooxidant-antioxidant balance (25).

Oxidative stress is one of the effective factors in the occurrence of many diseases, which can include diseases such as cardiovascular diseases, stroke, diabetes, diabetic nephropathy, Parkinson's, Huntington's, Alzheimer's, autism, cancer and other phenomena such as aging. pointed out (26). One of the types of diseases caused by oxidative stress is the effect of oxidative stress on lung epithelial cells.

Some of the medicinal plants and their active ingredients have had significant effects on oxidative stress factors. One of the plants that seems to be effective in this field is the pecan plant (27). Due to its phenolic and antioxidant compounds, pecan extract can be effective in the healing process of lung diseases (28). Among the main compounds of pecan root and seed, we can mention the family of beta-carbolines, which are alkaloids, and include Harman, harmine, harmaline, and harmalol (29). including. It has been shown that pecan seed extract has

antioxidant, antimicrobial, antitumor and vasodilating effects (30). Harmin and Harmalan have a high capacity in removing free radicals. The effect of harmaline in removing free radicals is three times more than harmain (31).

A549 cells are type 2 lung epithelial cells. This cell line was developed for the first time in 1972 by Giard et al. through harvesting and culture of cancerous lung tissue from a tumor sample from a 58-year-old Caucasian man. In laboratory culture conditions, A549 cells grow as monolayer cells, adherent and attached to the culture flask. Another characteristic of these cells is that they are able to synthesize lecithin and contain a high level of unsaturated fatty acids, which are important for maintaining the phospholipid membrane in the cell. The A549 cell line is widely used as a laboratory model for epithelial type II pleurisy, drug metabolism, and as a transfection host. Since cell lines are cells with stable and stable genetic and phenotypic characteristics, they are useful tools for investigating the effects of substances and drugs in primary studies (6, 7).

The findings of this research showed that elastase and H2O2 significantly decrease the viability of healthy cells and harmaline treatment increases the viability. Among the groups treated with different doses of harmaline, the group treated with 200 micromoles of harmaline had the highest cell viability.

According to the study of Snider et al. in 1986, elastase is the creator of the experimental model of emphysema, and the injection of one dose of it quickly causes morphological and histopathological changes in the lungs (32). The study of Aoshiba et al. in 2001 showed that serine proteases cause cytotoxicity through increased production of ROS and oxidative stress, and in this way lead to widespread tissue destruction and lung damage (33). The results of both studies are consistent with the results of the current research.

In the present study, after the proximity of A549 lung epithelial cells with elastase and H2O2 for 24 hours, the results showed that treating the lung epithelial cells with elastase leads to an increase in free radicals in these cells, and the results also show that the treatment of diseased groups with elastase different doses of harmaline significantly remove free radicals. In a study conducted by Lin et al. in 2016, the use of natural antioxidants can be effective in reducing oxidative stress and combating oxygen free radicals by increasing the expression of the Nrf2 antioxidant defense system in chronic lung damage (34).

Comparison of oxidative stress indices including MDA in different groups showed that the exposure of lung epithelial cells to elastase and H2O2 significantly increases the oxidative stress index and the treatment of groups treated with different doses of harmaline reduces the amount of oxidative stress and the dose of 200 Harmaline has the greatest effect in reducing oxidative stress. The study by Radan et al. in 2019 also showed that natural antioxidants are effective on the signaling pathway that regulates antioxidant genes through increasing the biosynthesis levels and activity of antioxidants such as GSH, causing resistance to the factors that cause damage caused by oxidative stress in the lung. They go around (60). The results of the mentioned studies in this field are in line with the results of the present research.

Another goal of this research was to investigate the effect of harmaline on antioxidant indices in cells exposed to elastase, and the results showed that treating cells with elastase and H2O2 decreases antioxidant indices including TAC and treatment of treated cells with different doses Harmaline increases TAC. The study by Mughadam et al. in 2021 on the effect of the active ingredient of pecan plant (harmaline) on the serum levels of liver index enzymes in rats with non-alcoholic fatty liver showed that the serum levels of ALT, AST, ALP, cholesterol and LDL significantly In the groups treated with harmaline, the levels of the antioxidant enzymes catalase, glutathione peroxidase, and SOD increased significantly in the treated groups (35), and the results of this study are in line with the findings of the current study.

In summary, the results of the current experiment a significant effect of the protective effect of harmaline on cell viability indices, free radical production rate, oxidative stress indices including MDA and oxidative stress indices including TAC, and harmaline significantly increases viability and TAC levels in cells exposed to elastase. Also, harmaline has significantly reduced the amount of free radicals and the amount of MDA in the cells exposed to elastase.

Harmaline is one of the alkaloids found in

pecan plant extract, which has antioxidant activity in very low concentrations (37). Harmaline alkaloid reduces oxygen free radicals due to its antioxidant activity and increases the activity of antioxidant enzymes such as catalase and superoxide dismutase. Increasing the activity of antioxidant enzymes causes the reduction of oxygen free radicals and as a result, the reduction of lipid peroxidation and the reduction of MDA. Oxygen free radicals can affect lipid, protein and DNA, but the sensitivity of lipids is more than other biological molecules. Also, determination of MDA concentration is one of the indicators of oxidative stress which is evaluated in many studies. Antioxidants neutralize active free oxygen species by different mechanisms. Harmaline, with its antioxidant properties, can also cause the reduction of active free oxygen species and, as a result, the reduction of MDA levels (37).

CONCLUSION

The results of the study show a significant effect of the protective effect of harmaline on cell viability indices, free radical production, oxidative stress and antioxidants capacity. Therefore, according to the results of the present study and due to the prevalence of lung diseases that affect the health and lifestyle of the patient, harmaline is suggested as a supplement for prevent or treatment of respiratory system disorders.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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