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# Preparation and Evaluation of Preventive Effects of Inhalational and Intraperitoneal Injection of Myrtenol Loaded Nano-Niosomes on Lung Ischemia-Reperfusion Injury in Rats

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ABSTRACT

*Introduction:* Ischemia-reperfusion injury (IRI) is directly related to forming reactive oxygen species, endothelial cell injury, increased vascular permeability, and the activation of neutrophils and cytokines. Niosomes are nano-carriers and an essential part of drug delivery systems. We aimed to investigate the effects of myrtenol's inhaled and intraperitoneal niosomal form, compared to its simple form, on lung ischemia reperfusion injury (LIRI).
*Material and method:* Wistar rats were divided into ten groups. Simple and niosomal forms of myrtenol were inhaled or intraperitoneally injected daily for one week prior to LIRI. We evaluated oxidative stress, apoptotic, and inflammatory indices, nitric oxide, inducible nitric oxide synthase (iNOS), endothelial nitric oxide synthase (eNOS) and histopathological indices.
*Results:* Pretreatment with simple and niosomal forms of myrtenol significantly inhibited the indices of pulmonary edema, pro-inflammatory cytokines and proteins, oxidant agents, nitric oxide, iNOS, apoptotic proteins, congestion of capillaries, neutrophil infiltration, and bleeding in the alveoli. Furthermore, myrtenol increased anti-inflammatory cytokines, anti-oxidants agents, eNOS, anti-apoptotic proteins and the survival time of animals. The niosomal form of myrtenol showed a more ameliorative effect than its simple form.
*Conclusion:* The results showed the superior protective effect of the inhalation of myrtenol niosomal form against LIRI compared to its simple form and systemic use.


## Introduction

Lung ischemia-reperfusion injury (LIRI) is a common pathophysiological phenomenon that happens in clinical cases such as lung transplantation, cardiopulmonary bypass,[1] atherosclerosis, pulmonary embolism,[2] pulmonary thrombosis, various forms of acute vascular occlusion (stroke, myocardial infarction, and limb ischemia), trauma,[3] and shock.[4–6] LIRI is associated with increased microvascular permeability, pulmonary vascular resistance, sterile inflammation, endothelial cell dysfunction, pulmonary edema, pulmonary hypertension, and impaired oxygen exchange.[7,8] Lack of oxygen and nutrients during the ischemic period creates conditions in which the return of blood circulation leads to inflammation and oxidative damage through the induction of oxidative stress.[9] Some factors present in the endothelium cause the release and accumulation of reactive oxygen species (ROS), enzymes, and cytokines pro-inflammatory cytokines such as tumor necrosis factor-alpha ($TNF-\alpha$) and lead to further exacerbation of oxidative damage by attracting monocytes and macrophages.[10–12] ROS, $TNF-\alpha$, and iNOS activate the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-$\kappa$B) pathway and increase inflammation.[10,13,14] Finally, ROS and $TNF-\alpha$ activate internal and external apoptotic pathways, respectively.[10,15]

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Myrtenol is a monoterpene alcohol found in some aromatic plants such as Myrtus communis Linn, Tanacetum vulgare, and Aralia Cachemirica. Numerous studies have shown that myrtenol has anti-inflammatory, antioxidant, anti-apoptotic, anti-cancer, anti-aging, and anti-hypertensive properties and has protective effects against myocardial ischemia-reperfusion injury. Recently, we demonstrated the anti-inflammatory and anti-remodeling effects of simple myrtenol (intra-peritoneal) against allergic asthma in rats.[16]

Due to the increased interest in nanoscience in medicine and pharmaceutics, the use of nanocarriers is rising. Niosomes are a family of lipid-based nanocarriers consisting of non-ionic surfactants. Niosomes are a promising delivery system for a variety of drugs. They are more stable than liposomes, less likely to aggregate, and more biocompatible than other nanoparticles. Niosomes also effectively deliver a wide range of drugs, including hydrophobic, hydrophilic, and amphiphilic drugs. They are made of a mixture of cholesterol and non-ionic surfactants, which makes them a stable, biocompatible, and cost-effective option for drug delivery.[17,18]

Currently, there is no clinically specific therapeutic agent to prevent LIRI, and no report was found on the effect of myrtenol in either simple or nanoparticle (niosomal) forms on LIRI. Therefore, in this study, we investigated the possible protective effect of inhalation and intraperitoneal injection of niosomal myrtenol, compared to its simple form, against LIRI in rats.

## Material and Methods

### *Animal Selection and Ethical Approval*

Based on previous studies and a statistical consultant's opinion, we decided to use 160 adults male Wistar rats with an average weight of 180 g. The animals were purchased from the Kerman University of Medical Sciences animal farm. They were housed under standard conditions, including a 12 h/12 h light/dark cycle, ambient temperature (23 ± 2 °C), and free access to water and food. The experiments described in this work were conducted in accordance with the guidelines on ethical standards for investigation of animals. The protocol was reviewed and approved by the Ethic Committee of Kerman University of Medical Sciences, with the approval number IR.KMU.REC.1400.627. We followed established principles and practices to ensure the animals' welfare in this study and took all necessary steps to minimize their discomfort and suffering.

### *Study Design*

This study consisted of Two parts:

*Part One*

To achieve optimal encapsulation, controlled release of Myrtenol, and minimize pulmonary toxicity, a combination of cholesterol and various surfactants, including Spans (Sp40 and Sp60) and Tweens (Tw40 and Tw60) (Merck, Germany), was utilized to create niosomes. These surfactants were tested at varying concentrations (0.5 %, 1 %, 2.5 %, 5 %, 10 %, and 40 % mM) using two distinct methods: thin-layer hydration and heating. After careful evaluation, the best formulation was selected by heating method and using cholesterol, Sp40, and Tw40 with a concentration of 1 mM (final solution concentration of 1 mM).

*Niosomal myrtenol preparation.* To prepare niosomes, we used the heating method described in the source.[17] First, we dissolved myrtenol (Sigma Aldrich, USA) in a dimethyl sulfoxide solution (0.5 %). Then, we added glycerol (final concentration of 3 % by volume) and normal saline (21 mL) to the myrtenol solution. The mixture was heated to 60 °C and stirred (IKA® 104 C. IKA, Malaysia) at 1000 rpm for 5 min. Next, we added cholesterol (Merck, Germany) (30 % in total lipid concentration of 1 mM) to the mixture and stirred for 15 min at 120 °C and 1000 rpm. Finally, we added Sp40 and Tw 40 (equal molar ratios of 35) to the mixture and heated for 1 h at 60 °C and 1000 rpm. The prepared niosomes were kept overnight at room temperature for stabilization. The final concentration of niosomes was one mM. The molar ratio of cholesterol, Sp40, and Tw40 was 30:35:35. Light microscopy was used to confirm vesicle formation. Before administration to animals, the niosomes were sonicated and passed through a 0.2 $\mu$m filter. A transmission electron microscope (TEM, Philips EM208S 100KV) was used to determine the size, shape, and lamellarity of niosomes. The magnification of the images was 46.460 KX.

*Evaluation size analysis and zeta potential measurement of the myrtenol niosomes.* The particle size and size distribution of niosomes were determined using dynamic light scattering (DLS) (Particle size analyzer, Cordouan, VASCO II, France). The polydispersity index (PDI) was used to measure the size distribution. The zeta potential of vesicles was measured using Zetasizer Nano ZS (Zeta potential analyzer Cordouan, WALLIS, France).[18]

*Evaluation encapsulation efficiency of the myrtenol niosomes.* The encapsulation efficiency (EE) of myrtenol niosomes was determined by centrifugation (at 20,000 g for 45 min at four °C). The free and total myrtenol concentrations in the supernatant and pellet mixtures were assayed by spectrophotometry (at 247 nm).[19] The EE percentage was calculated as follows:

$$EE\% = \frac{Total\ content - Free\ content}{Total\ content} \times 100$$

*Evaluation* in vitro *release of myrtenol from niosomes.* The *in vitro* release of myrtenol from niosomes was determined by dialysis. Niosomes samples were centrifuged (at 20,000 g for 45 min at four °C), and the supernatant was discarded. An equivalent volume of normal saline was added to the pellet and placed on the stirrer. The product was poured into a closed dialysis bag (12–14 KDa). The exudate inside the beaker around the dialysis bag was sampled at different periods to check the myrtenol concentration by spectrophotometry (at 247 nm).

*Part Two*

A pilot dose-response study was performed to determine the optimum inhaled and intraperitoneal myrtenol dose. Seven days before LIRI, the rats received intraperitoneal myrtenol at 12.5, 25, 50, and 100 mg/kg or inhaled myrtenol at 4, 8, 16, and 32 mg/kg. The lung tissue was removed after LIRI under deep anesthesia to assess histopathological and molecular parameters. Based on the dose-response curve, 50 mg/kg for intraperitoneal injection and 32 mg/kg for inhalation were selected as the optimal doses for the rest of the experiments. The optimum dose of myrtenol was performed in the main study.

*Animal grouping.* The animals were randomly divided into ten main groups ($n$ = 16) (see Table 1). Group One (sham): Animals were tracheostomized and connected to a ventilator. Group Two (ShNVI:): the sham group that inhaled niosomes containing DMSO 0.05 %. Group Three (ShNVP): a sham group that received niosomes containing DMSO 0.05 % intraperitoneally. Group Four (Control Lung Ischemia-Reperfusion): The left lung's artery, vein, and bronchus were obstructed for 60 min, and then blood flow and ventilation were restored for 120 min.[20] Group Five (NVI): animals that inhaled niosomes containing DMSO 0.05 % for one week before LIRI. Group Six (NVP): animals that received niosomes containing vehicle intraperitoneally for one week before LIRI. Group Seven (SMI): Animals were

**Table 1**
Animal grouping.

| Group of animals | Symbol used in figures | IR | Pretreatment | Dose (mg.kg) | Route (In/Ip) |
|---|---|---|---|---|---|
| Group 1 | Sham | No | No pretreatment | – | – |
| Group 2 | ShNVI | No | 7 days pre-treated with Niosomes containing DMSO | 0.05 % | In |
| Group 3 | ShNVP | No | 7 days pre-treated with Niosomes containing DMSO | 0.05 % | I.p |
| Group 4 | NVI | Yes | 7 days pre-treated with Niosomes containing DMSO | 0.05 % | In |
| Group 5 | NVP | Yes | 7 days pre-treated with Niosomes containing DMSO | 0.05 % | I.p |
| Group 6 | CLIR | Yes | No pretreatment | – | – |
| Group 7 | SMI | Yes | 7 days pre-treated with simple form of myrtenol | 35 mg/kg | In |
| Group 8 | NMI | Yes | 7 days pre-treated with niosomal form of myrtenol | 35 mg/kg | In |
| Group 9 | SMP | Yes | 7 days pre-treated with simple form of myrtenol | 50 mg/kg | I.p |
| Group 10 | NMP | Yes | 7 days pre-treated with niosomal form of myrtenol | 50 mg/kg | I.p |

IR: Ischemia Reperfusion, In: Inhalation, Ip: Intraperitoneal.

pre-treated with the inhaled simple form of myrtenol for 30 min daily for one week prior to LIRI. Group Eight (NMI): Animals were pre-treated with inhaled niosomal form of myrtenol for 30 min daily for one week prior to LIRI. Group Nine (SMP): Animals were pre-treated with an intraperitoneal injection of the simple form of myrtenol for one week prior to LIRI. Group Ten (NMP): Animals were pre-treated with an intraperitoneal injection of the niosomal form of myrtenol for one week prior to LIRI.

We did not observe significant difference between sham, ShNVI, or ShNVP groups or between the control lung ischemia-reperfusion, NVI, and NVP groups. Therefore, in the result section, we did not report the data of the last four groups to prevent overcrowded figures.

*Tracheostomy and control of animal respiration during surgery.* The animals had to remain anesthetized for 4 h. We used ketamine (80 mg/kg) and xylazine (10 mg/kg) for induction, halothane 2 %, and nitrous oxide ($N_2O$) for maintenance of anesthesia during the surgical procedure. After ensuring deep anesthesia, an incision was made in the shaved skin of the neck, from the sternum to the level of the hyoid bone. The trachea was exposed, and a transverse incision was made on a tracheal cartilage to insert a suitable-sized cannula into the trachea. The cannula was fixed, and animals were ventilated at a tidal volume of 6 ml/kg and a rate of 70 breaths per minute.[20]

*Lung ischemia reperfusion procedure.* The rat was then placed with the left side of the chest facing up. Heparin (50 IU) was injected through the jugular vein, and the animal's chest was cut in the fifth intercostal space until the left lung was accessible.[20] The left lung hilum (including the airway, the pulmonary artery, and the vein) was blocked with a microclamp to cut off ventilation and blood flow to the left lung. Following 60 min of occlusion, the clamp was removed to restore blood flow and ventilation for 120 min.[20]

*Aerosol drug delivery.* We employed a delivery system for administering Aerosol to the animals, which comprised a Plexiglas Chamber and a jet nebulizer manufactured by Omron in Germany (Omron Nebulizer Model NE-C900-E, Classification; Class II). The nebulizer consisted of two main parts: a compressor and a medication tank. The compressor supplied compressed air to the medication tank, which converted the drug into an aerosol mist. This mist was then introduced into the animal compartment through the outlet of the medication tank. The animals were exposed to the aerosol mist for 30 min. In order to ensure that a sufficient amount of the drug reached the rats during inhalation, the dosage of the drug administered to the rats was estimated based on a formula described by Wattenberg and colleagues, who had employed a similar approach in their studies.[21]

$$Dl = \frac{C \times MV \times T}{BW}$$

DL: Dose Level C: Concentration, T: Duration of Exposure (minute), MV: Minute Volume (L/Min), BW: Body Weight.

*Evaluation of lung edema.* At the end of the reperfusion period, the whole lung was removed and weighed immediately. The ratio of the lung weight (LW) to body weight (BW) and the ratio of the wet weight to dry weight of the lung were used as indices of pulmonary edema. For drying, the left lung's lower lobe was weighed and was then incubated at 60 °C for 48 h.[22] Protein concentration in bronchoalveolar fluid (BALF) was measured using a bicinchoninic acid (BCA) protein assay kit.[23]

*Animal survival time and arterial blood gas analysis.* To assess the survival time, at the end of ischemia-reperfusion, the right hilum was

closed to allow gas exchange for 120 min only by the left lung. The survival duration was then computed as the time the animal endured during this 120 min interval.[24] To assess the efficiency of the lung function, arterial blood oxygen was measured. Animals were first ventilated at a tidal volume of 6 ml/kg and a rate of 70/min, and a positive end-expiratory pressure of 2 cm $H_2O$ and 100 % $FiO_2$ for 5 min. Blood was taken from the ascending aorta, and a blood gases measured by blood gas analyzer.

*Bronchoalveolar fluid collection.* After sacrificing the animal and removing the heart and lung blocks, 2 mL of phosphate-buffered saline (PBS) was gavaged into the trachea by a syringe connected to a cannula. After 2 min, the liquid was aspirated and stored at -80 °C for later evaluation.[24]

*Histopathological evaluation of the lung tissue.* Part of the left lung was fixed in 10 % formaldehyde, and four 4 micron sections were prepared. A pathologist used hematoxylin-eosin (H&E) staining to evaluate edema, intra-alveolar hemorrhage, capillary congestion, and neutrophil infiltration blindly. The lung injury was scored using a 5-point scoring system: 0: without, 1: 0 to 25 %, 2: 25 to 50 %, 3: 50 to 75 %, and 4: 75 to 100 % change in the measured indices.

*Evaluation of cleaved caspase-3 by Immunohistochemistry (IHC).* For this purpose, the samples were fixed in formalin overnight and then dehydrated by scored ethanol concentrations. The samples were molded into molten paraffin and randomly cut into 5 micron-thick sections. The lamellae were placed in the oven at 74 °C to remove paraffin for 15 min. In order to block the non-specific site, the cells were incubated with the blocking solution (Peroxidase Block, UMR1000PD, USA) at room temperature for 40 min. Tissue incubation was performed with a primary monoclonal antibody against the cleaved caspase-3 antibody (ZYTOMED, Berlin, Germany) overnight at four °C. The cells were washed with PBS buffer and incubated for one hour in a dark environment at room temperature with the corresponding secondary antibody (ZYTOMED, Berlin, Germany). The samples were washed thrice with PBS buffer and stained at room temperature with H-Hoc (5 g/ml) for 15 min. The slides were imaged by reverse fluorescence microscopy.[25]

*Evaluation proteins expression by western blot technique.* The western blotting technique evaluated the expression of apoptotic, anti-apoptotic, iNOS, eNOS, and NF-kB proteins. The technique consisted of three general steps: 1- Protein to gel electrophoresis, 2- Protein transfer to PVDF membranes, and 3- protein staining with antibodies. The images were recorded by an enhanced chemiluminescence (ECL) system and a gel docking device. After digitizing the images, the band density was measured using Image J software (National Institutes of Health).[26]

*Evaluation of Myeloperoxidase (MPO).* The tissue MPO content was used to quantify the infiltration of neutrophils in the lung tissue. The amount of MPO was measured by a commercial kit (Navand Salamat Co, Iran).[24]

*Evaluation of oxidative stress and inflammation.* Superoxide dismutase (SOD), glutathione peroxidase (GPX), malondialdehyde (MDA), total oxidant status (TOS), reactive oxygen species (ROS), and NO metabolites were measured by their specific kits according to the manufacturer's instructions (Navand Salamat Co, Iran and Behboud Tahghigh Co, Kerman, Iran).[27] Interleukin (IL)-6, 10, 17, and TNF-$\alpha$ in the lung tissue were measured according to the instructions of the associated ELISA kits (Karmania Pars Gen Co, Iran).[16]

*Statistical Analysis*

GraphPad Prism v.6 (Academic institutions) software was used for statistical analysis. First, the distribution of data was examined by the Shapiro–Wilk test. One-way ANOVA and Tukey post hoc tests were used to compare the quantitative data normally distributed. *P* values less than 0.05 were considered as significant.

## Results

*Physical Properties of the Myrtenol Niosomes*

The size was 430 ± 10 nm for niosomes loaded with myrtenol and 415 ± 03 nm for empty niosomes that were insignificant (Fig. 1A and B). DLS measurement showed that the niosomes were almost uniform in size (PDI for 0.624 ± 0.09 for niosomes loaded with myrtenol and 0.610 ± 0.06 nm for empty niosomes). The zeta potential values for empty and loaded niosomes did not show a statistically significant difference (−26.7 ± 2.7 mv vs. −21.6 ± 4.2 mv, respectively). These zeta potential values are acceptable and indicate stable formulations.

The encapsulation efficiency of myrtenol was 83.67 ± 0.42 %. Fig. 1C shows that the release rate from the niosomal myrtenol was much slower than the simple form. In simple form, almost all myrtenol was released in about 3 h.

*The Effects of Myrtenol on Lung Edema and BALF Protein*

The data showed a significant increase in the wet/dry weight and lung/body weight ratios in the ischemia-reperfusion group compared to the sham group. Inhalation and intraperitoneal injection of both simple and niosomal forms of myrtenol significantly prevented this increase in edema indices (Fig. 1D and E). The amount of total protein in the BALF also increased in the CLIR group compared to the sham group. Inhalation and intraperitoneal administration of simple and niosomal forms of myrtenol significantly prevented the increase in total protein (Fig. 1F).

*The Effects of Myrtenol on Arterial Blood pH, $PaCO_2$, and $PaO_2$*

Arterial blood pH did not differ among the groups, while $PaO_2$ significantly decreased and $PaCO_2$ significantly increased in the CLIR group compared to the sham group. Inhalation and intraperitoneal administration of simple and niosomal forms of myrtenol significantly prevented these alterations (Table 2).

*The Effect of Myrtenol on Lung Histopathology*

Histological micrographs from the sham group showed almost normal results (Fig. 2A). Severe edema, capillary congestion, neutrophil infiltration, and bleeding in the alveolar region were observed in the CLIR group (Fig. 2B). Inhalation and intraperitoneal administration of simple and niosomal forms of myrtenol reduced these disturbances (Fig. 2C–F). The niosomal form of myrtenol was found to be more potent than the simple form. The results of the IHC for caspase-3 showed that the number of apoptotic cells in the CLIR group increased (Fig. 2K). Inhalation and intraperitoneal administration of simple and niosomal forms of myrtenol prevented these increases (Fig. 2L–O). The effect of administering a simple and niosomal form of myrtenol supported the results of Bax and $Bcl_2$ measurements (Fig. 6B and C).

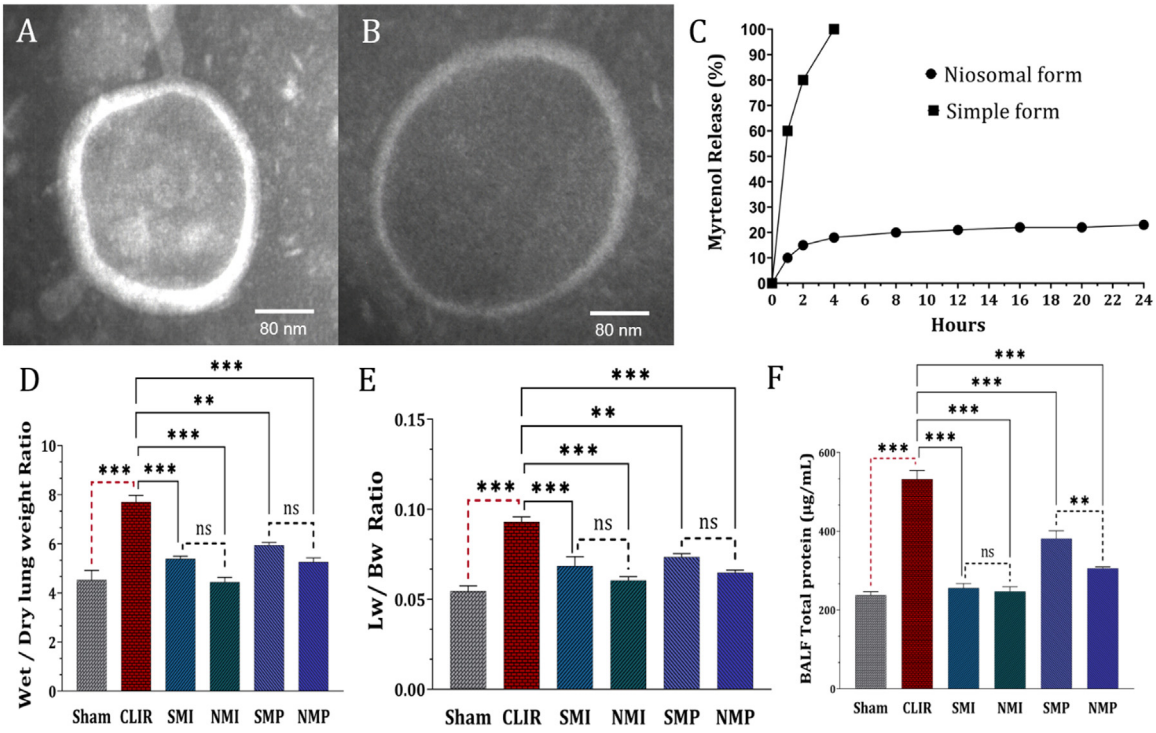


**Figure 1.** Transmission electron microscopy image of (A) unloaded niosomes and (B) myrtenol loaded niosomes, original magnification: 46.460 x. (C) release curve for simple and niosomal myrtenol. (D–F) Effects of inhalation and intraperitoneal (IP) administration of simple and niosomal forms of myrtenol on (D) wet/dry lung weight ratio, (E) lung to body weight ratio, and (F) total protein of BALF. Data are mean ± SEM. $n = 7$ in each group. Sham: sham group, CLIR: control lung ischemia reperfusion injury group, SMI: inhalation of simple form myrtenol, NMI: inhalation of niosomal form of myrtenol, SMP: IP injection of simple form of myrtenol, MNP: IP injection of niosomal form of myrtenol. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

### *The Effect of Myrtenol on Oxidative Stress*

Our findings showed that the level of oxidizing agents MDA and TOS in the lung tissue and BALF significantly increased in the CLIR group compared to the sham group. Inhalation and intraperitoneal administration of myrtenol's simple and niosomal form significantly prevented these increases (Fig. 3A and B). Also, the antioxidants CAT, SOD, GPX, and TAC in the CLIR group decreased significantly compared to the sham group. Inhalation and intraperitoneal administration of simple and niosomal forms of myrtenol significantly prevented these decreases. Notably, the effect of myrtenol's niosomal form was more potent than the effect of the simple form (Fig. 3C–F).

**Table 2**
The effects of inhalation and intraperitoneal administration of simple and niosomal forms of myrtenol on arterial blood gas (ABG) and pH parameters.

| Group | pH | $PaO_2$ (mmHg) | $PaCO_2$ (mmHg) |
|---|---|---|---|
| Sham | 7.26 ± 0.14 | 143.71 ± 0.21 | 24.85 ± 0.08 |
| LIR | 7.22 ± 0.17 | 115.14 ± 0.09 *** | 33.71 ± 0.06 *** |
| SMI | 7.27 ± 0.13 | 132.57 ± 0.13 † | 26.28 ± 0.03 †† |
| NMI | 7.31 ± 0.09 | 139.71 ± 0.11 †† | 25.01 ± 0.02 †† |
| SMP | 7.28 ± 0.18 | 130.25 ± 0.21 † | 28.13 ± 0.14 † |
| NMP | 7.26 ± 0.23 | 138.06 ± 0.19 †† | 26.14 ± 0.07 †† |

Data are presented as the mean ± standard deviation of at least 7 rats in each group. ABG: arterial blood gas, Sham: sham group, CLIR: control lung ischemia reperfusion injury group, SMI: inhalation of simple form myrtenol, NMI: inhalation of niosomal form of myrtenol, SMP: IP injection of simple form of myrtenol, MNP: IP injection of niosomal form of myrtenol.

*** $P < 0.001$ *vs.* Sham.

† $P < 0.05$.

†† $P < 0.01$ *vs.* CLIR.

### *The Effects of Myrtenol on Inflammatory and Anti-Inflammatory Cytokines*

The results showed that the level of pro-inflammatory cytokines TNF-$\alpha$, IL-6, and IL-17 in the lung tissue (Fig. 4A–C) and BALF (Fig. 4E–G) increased significantly in the CLIR group compared to the sham group. Inhalation and intraperitoneal administration of simple and niosomal forms of myrtenol significantly prevented the increase in these cytokines. A reverse effect was found on the anti-inflammatory cytokine IL-10 (Fig. 4D and H).

### *The Effects of Myrtenol on MPO, ROS, and NO Metabolites*

Lung tissue MPO, ROS, and NO levels increased significantly in the CLIR group. Administration of different forms of myrtenol significantly prevented the increase in MPO, ROS, and NO levels. This preventive effect was more potent in the niosomes form compared to the Simple form (Fig. 5A–D).

### *The Effects of Myrtenol on Bax and $Bcl_2$*

Western blot analysis showed that the expression of Bax pro-apoptotic protein increased, and $Bcl_2$ anti-apoptotic protein decreased in the lung tissue of the CLIR group compared to the sham group (Fig. 6A–C). The $Bcl_2$/Bax ratio also decreased significantly in the CLIR group (Fig. 6D). Pretreatment with inhalation and intraperitoneal administration of simple and niosomal forms of myrtenol significantly prevented the changes in the expression of these proteins.

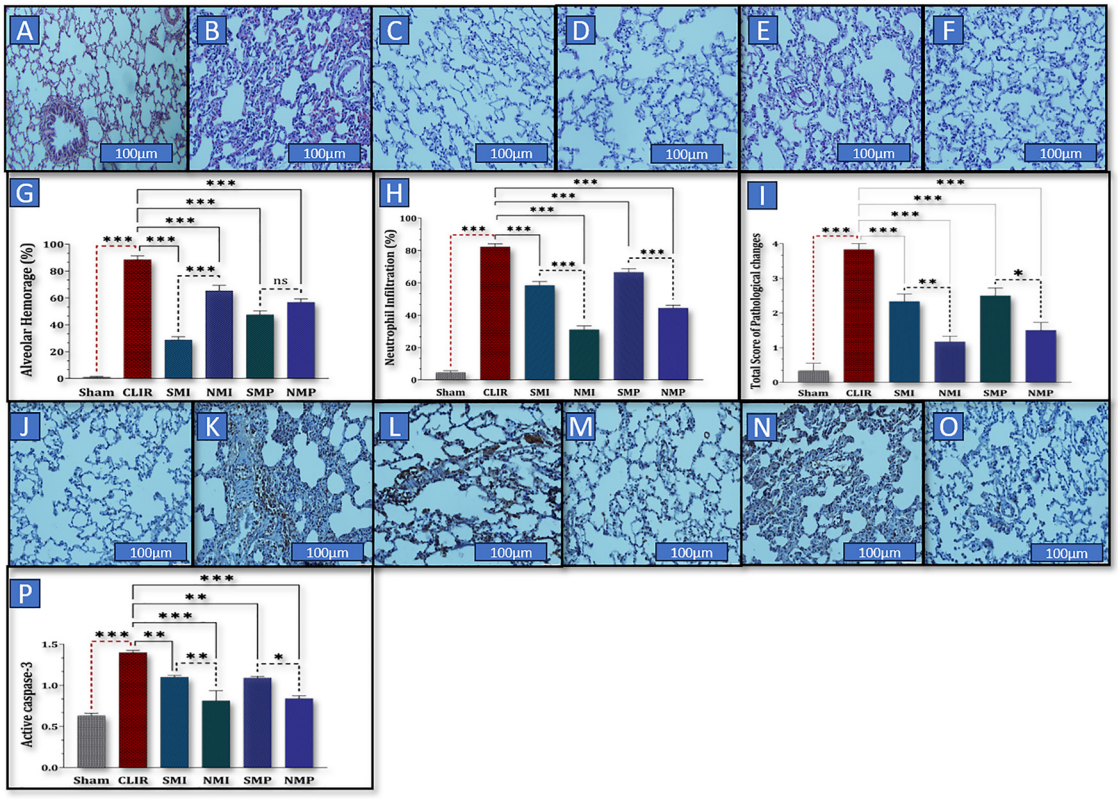


**Figure 2.** The effects of inhalation and intraperitoneal administration of simple and niosomal forms of myrtenol on pathological changes (A–I) and caspase-3 expression (J–P) in lung tissue of rats. The lung was stained with hematoxylin & eosin and IHC (light microscopy, 10 X). (A and J) sham group, (B and K) CLIR group, (C and L) SMI group, (D and M) NMI group, (E and N) SMP group, (F and O) NMP group, (G) alveolar hemorrhage, (H) neutrophil infiltration, (I) total score of pathological changes and (P) quantification of active caspase-3 in lung tissue. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

*The Effect of Myrtenol on eNOS, iNOS, and NF-kB Parameters*

The expression of eNOS, iNOS, and NF-$\kappa$B proteins in the lung tissue increased significantly in the CLIR group compared to the sham group. Administration of simple and niosomal forms of myrtenol significantly prevented the increase in the expression of iNOS and NF-$\kappa$B (Fig. 6F and G). In contrast, the expression of eNOS increased in these groups (Fig. 6E). The effect of the niosomal form of myrtenol was significantly more significant than the effect of the simple form.

*The Effects of Myrtenol on Survival Time*

Our results showed that the survival time in the inhaling and intraperitoneal groups of simple and niosomal myrtenol increased significantly compared to the CLIR group (Fig. 6H).

## Discussion

Considering the positive effects observed in past research and our previous studies regarding the effects of myrtenol and the importance of Niosomes as a vehicle for the delivery of therapeutic molecules, these materials can lead to improved drug stability, controlled drug release, prevention of drug degradation, and increased the duration of the effectiveness of the drug in the desired tissue. These measures can ultimately lead to the best treatment results. For this reason, we loaded myrtenol into Niosomes and then used them using two methods of inhalation and intraperitoneal administration to prevent ischemia-reperfusion-induced lung injury in rats and compared the results.

In the present study, niosomal (and simple) myrtenol decreased the level of pro-inflammatory cytokines TNF-$\alpha$, IL-6, and IL-17 and increased the anti-inflammatory cytokine IL-10. The reduction of NF-$\kappa$B expression was also more pronounced by inhalation of niosomal myrtenol. These findings were consistent with our previous studies, in which systemic use of myrtenol decreased the level of the pro-inflammatory cytokines IL-1$\beta$ and TNF-$\alpha$ and increased the level of the anti-inflammatory cytokines INF-$\gamma$ and IL-10 in asthmatic rats.[16,28] Similarly, in another study, myrtenol treatment significantly reduced the level of IL-1$\beta$ and TNF-$\alpha$ in the liver of diabetic rats.[29] Due to the known importance of leukocytes, neutrophils, and the NF-$\kappa$B pathway in the inflammatory process, it can be suggested that one of the possible explanations for the anti-inflammatory activity of myrtenol is that it weakens the uptake of leukocytes and neutrophils at the site of inflammation and inhibits NF-$\kappa$B.

We observed that myrtenol, especially its niosomal form, significantly decreased the MDA, TOS, ROS, iNOS, and nitric oxide, increasing the activity or the level of CAT, SOD, GPx, TAC, and eNOS. The increase in eNOS upon treatment with myrtenol could be due to several factors. Myrtenol may directly activate eNOS or indirectly activate eNOS by increasing the levels of nitric oxide precursors. Myrtenol may also protect eNOS from degradation, or it may increase the expression of eNOS. The increase in eNOS could also be because myrtenol has anti-inflammatory effects. Inflammation can lead to the suppression of eNOS activity, so the anti-inflammatory effects of myrtenol could lead to an increase in eNOS activity. Our previous study on asthmatic rats found that myrtenol can decrease the MDA level and increase the SOD and GPx in the BALF and lung tissue.[28] Myrtenol probably decreases ROS production by reducing neutrophil recruitment to the lung tissue. This is supported by myrtenol's reduction of MPO in the lung tissue.

On the other hand, increasing tissue ROS scavengers leads to tissue cleansing from ROS. SOD, which fights the production of free

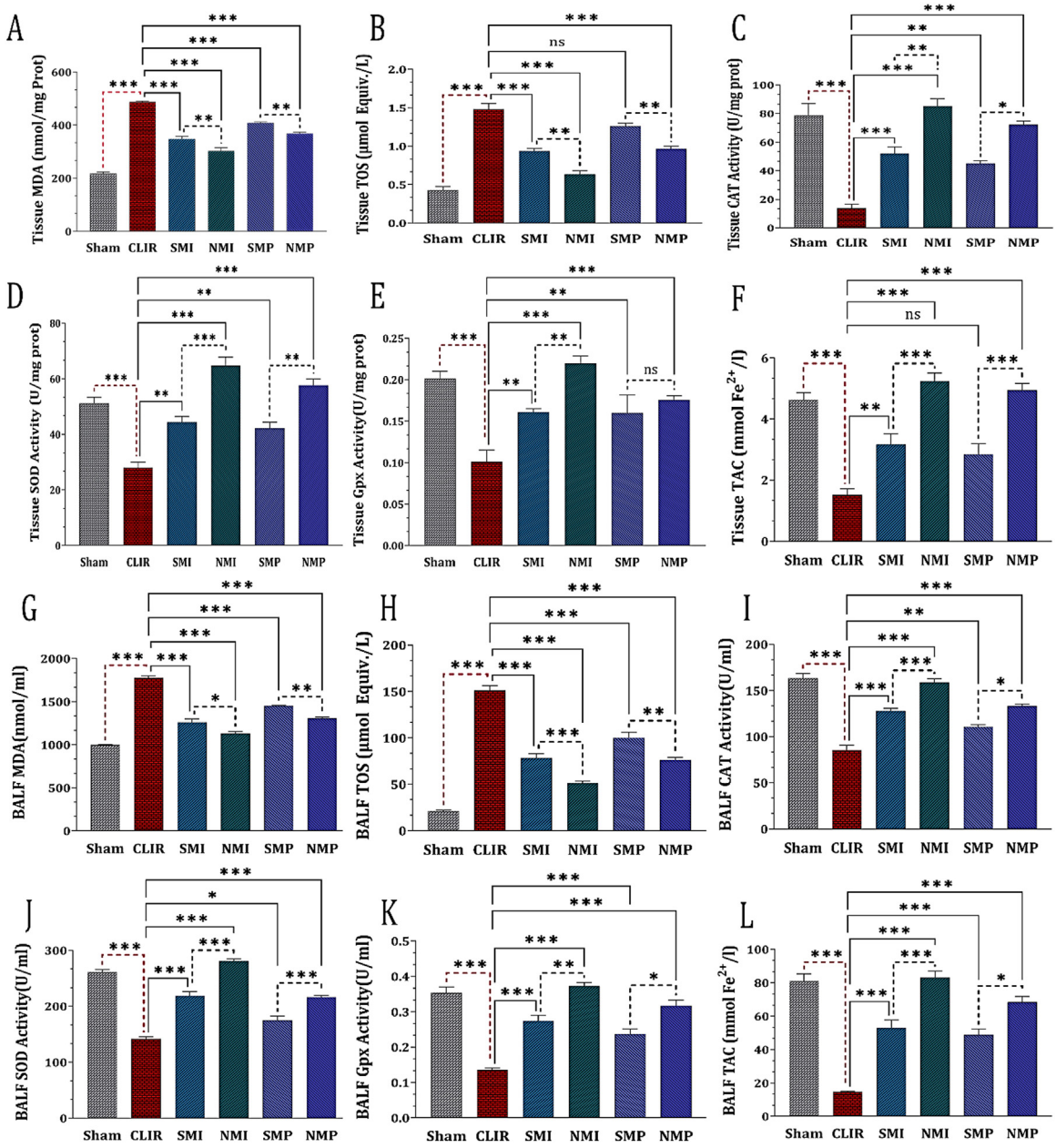


**Figure 3.** The effects of inhalation and intraperitoneal administration of simple and niosomal forms of myrtenol on oxidant and antioxidant factors (A–F) in lung tissue and (G–L) BALF of rats. Data are mean ± SEM. $n = 7$ in each group. Sham: sham group, CLIR: control lung ischemia reperfusion injury, SMI: inhalation of simple form myrtenol, NMI: inhalation of niosomal form of myrtenol, SMP: IP injection of simple form of myrtenol, MNP: IP injection of niosomal form of myrtenol. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

radicals, is one of the most important enzymes of the endogenous antioxidant defenses for the organism. The antioxidant role of myrtenol may be due to the increase in expression of this enzyme and other antioxidant defense enzymes.[29,30]

In the present study, myrtenol significantly prevented the up-regulation of the pro-apoptotic protein Bax and the down-regulation of the anti-apoptotic protein $Bcl_2$. Bax and Bcl-2 are two proteins belonging to the Bcl-2 family that regulate cellular apoptosis appositely.[31] So that Bax promotes and Bcl-2 prevents the apoptosis.[31] Myrtenol completely restored the balance of pro- and anti-apoptotic proteins (Bcl-2/Bax ratio) and prevented lung cell apoptosis.

The comparison of two forms of Myrtenol in both administration methods showed that the niosome form was more effective than the simple form. This effect can be caused by increased absorption and controlled release of Myrtenol. We have developed a controlled release mechanism for myrtenol by slow release of myrtenol from the niosome, which allows continuous exposure of myrtenol to lung cells over a more extended time. In addition, the comparison between the two administration methods showed that the inhalation administration was more effective than the intraperitoneal administration. Aerosol delivery is one of the main methods of delivery of therapeutic agents to respiratory organs in respiratory diseases. This provides an excellent way to bypass drug metabolism and deliver the drug directly to the target organ, thereby increasing drug bioavailability and efficacy.[32] The more effective inhalation method can be caused by the difference in the absorption mechanisms[33] of Myrtenol in the inhalation method compared to intraperitoneal administration. In fact, in the intraperitoneal method, due to primary liver metabolism, a significant part of the bioavailability of Myrtenol is reduced.[32] As a result, a smaller amount of Myrtenol reaches the lungs.

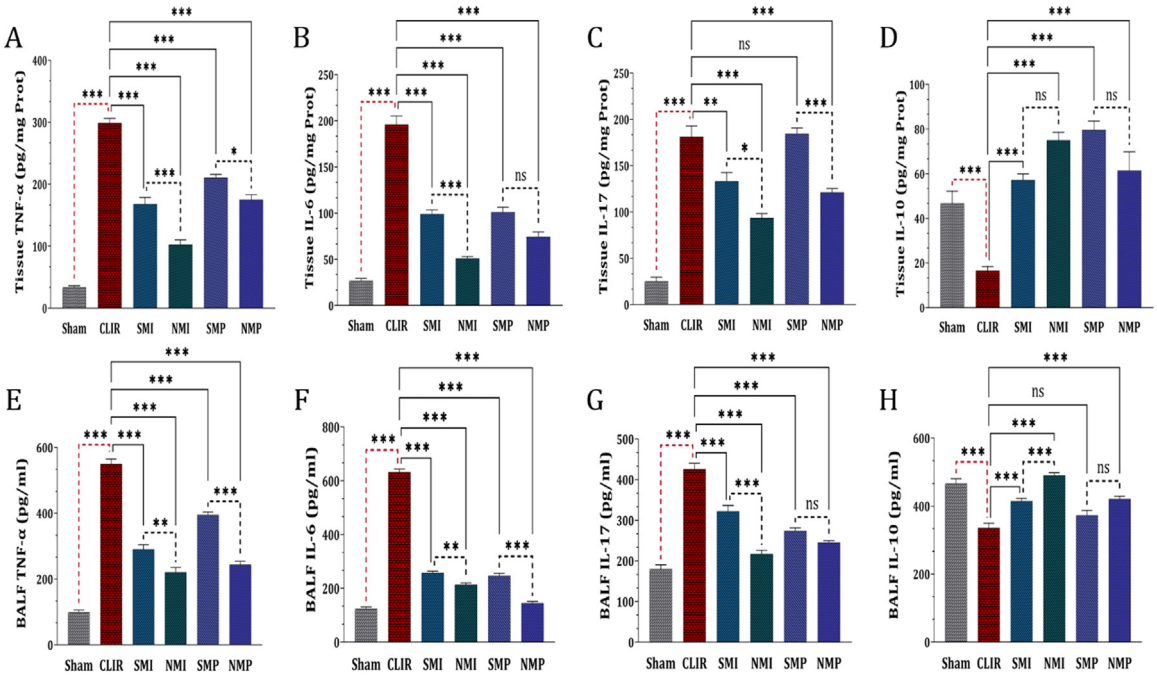


**Figure 4.** The effects of inhalation and intraperitoneal administration simple and niosomal forms of myrtenol on inflammatory and anti-inflammatory cytokines (A-D) in the lung tissue and (E-H) BALF of rats. Data are mean ± SEM. $n = 7$ in each group. Sham: sham group, CLIR: control lung ischemia reperfusion injury, SMI: inhalation of simple form myrtenol, NMI: inhalation of niosomal form of myrtenol, SMP: IP injection of simple form of myrtenol, MNP: IP injection of niosomal form of myrtenol. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

Past studies have also shown that drug-loaded niosomes increase drug stability and enable controlled release, improving drug delivery.[34] This study used non-ionic surfactants to create biocompatible and non-toxic nanocarriers. Additionally, we used cholesterol to stabilize the niosomes. The composition of the nanocarriers affects their stability, solubility, and delivery efficiency. In the present study, loading myrtenol in nanocarriers increased its therapeutic effects in the LIRI rat model.

In summary, currently, there is no clinical therapeutic agent for preventing lung ischemia-reperfusion injury, and treatment options are limited to supportive care. Previous studies on drug delivery have mainly focused on developing various types of nanocarriers, such as liposomes, to enhance the solubility and stability of other drugs. Other studies have reported improvements in the effects of other drugs under laboratory conditions. However, there is a need for a delivery system that provides both increased absorption and controlled release of the drug inside the body. However, no study has examined the preventive effects of a simple form or niosomal myrtenol on preventing lung ischemia-reperfusion injury.

Our study differs from previous studies in several key ways. First, we used nanoniosomes, a different type of nanocarrier, to encapsulate myrtenol. Unlike liposomes and polymeric nanoparticles,

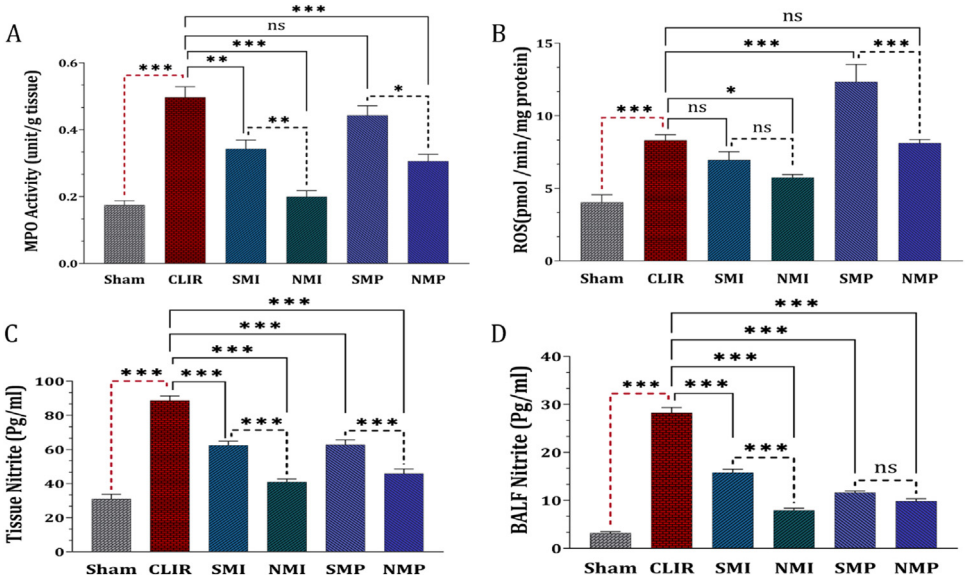


**Figure 5.** The effects of inhalation and intraperitoneal administration simple and niosomal forms of myrtenol on (A) MPO, (B) ROS and (C and D) nitrite content in the lung tissue and BALF. Data are mean ± SEM. $n = 7$ in each group. Sham: sham group, CLIR: control lung ischemia reperfusion injury, SMI: inhalation of simple form myrtenol, NMI: inhalation of niosomal form of myrtenol, SMP: IP injection of simple form of myrtenol, MNP: IP injection of niosomal form of myrtenol. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

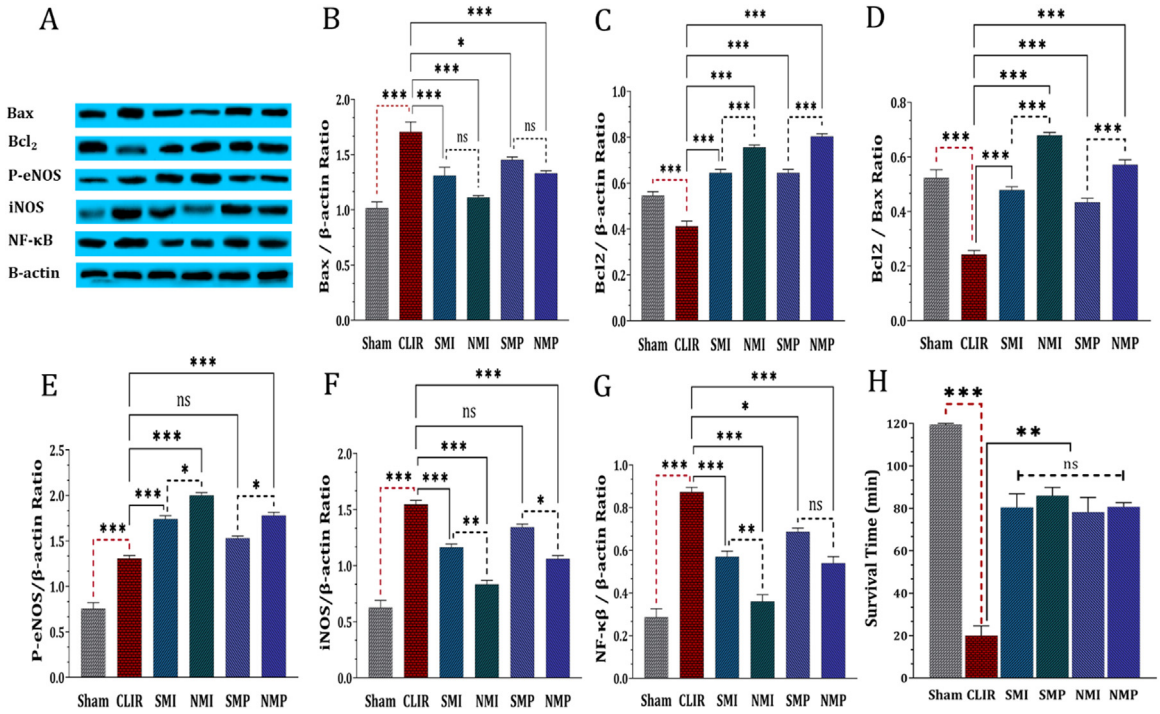


**Figure 6.** The effects of inhalation and intraperitoneal administration of simple and niosomal forms of myrtenol on (B) Bax, (C) Bcl2, (D) Bcl2/Bax ratio, (E) phosphorylated-eNOS, (F) iNOS, (G) NF-kB in lung tissue and (H) survival time of rats. Data are mean ± SEM. $n = 7$ in each group. Sham: sham group, CLIR: control lung ischemia reperfusion injury, SMI: inhalation of simple form myrtenol, NMI: inhalation of niosomal form of myrtenol, SMP: IP injection of simple form of myrtenol, MNP: IP injection of niosomal form of myrtenol. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

nanoniosomes are composed of a mixture of phospholipids, cholesterol, and surfactants, giving them a unique combination of properties suitable for drug delivery. Second, we developed a controlled release mechanism for myrtenol that allows for sustained exposure of the drug to lung cells over a more extended time. Also, due to the more effective effects of niosome form, our study shows the improvement of myrtenol absorption by lung cells, which is an essential factor in determining the drug's effectiveness.

We must "honestly" acknowledge that, in this study, we did not venture beyond the dosimetry methods detailed in the manuscript, and this study could be considered as a pilot study for the evaluation of the effects of myrtenol inhalation form on lung ischemia-reperfusion injury. Also, the estimate of human doses for efficacy in humans from animal models is generally not very reliable. The actual doses in humans need to be determined in humans following appropriate preclinical toxicology studies.

## Conclusion

Overall, for the first time, the results showed the protective role of myrtenol, especially the inhalation administration of its niosomal form, against lung ischemia reperfusion injury. Myrtenol prevents damage caused by ischemia reperfusion by creating a balance between inflammatory and anti-inflammatory, oxidant and antioxidant, and, apoptotic and anti-apoptotic factors. These findings may help us to propose the niosomes form of myrtenol as a potential target for additional studies towards developing a new drug for the prevention and treatment of LIRI injury.

## Declaration of Competing Interest

The authors declare there is no conflict of interest.

## Acknowledgment

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