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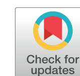
Research article

Bioactive phenolics fraction of *Hedera helix* L. (Common Ivy Leaf) standardized extract ameliorates LPS-induced acute lung injury in the mouse model through the inhibition of proinflammatory cytokines and oxidative stress



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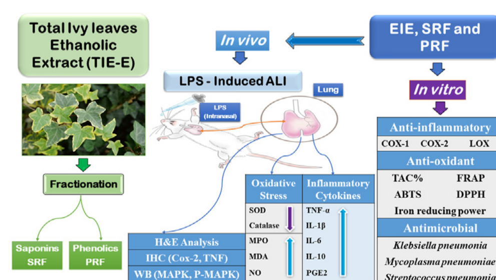
HIGHLIGHTS

- Ivy leaf is a traditional perennial edible herb used as an anti-inflammatory agent for respiratory disorders.
- The plant significantly reduced the serum oxidative stress biomarkers and inflammatory cytokines in the *in-vivo* acute lung inflammation model induced by LPS.
- Also, it had antimicrobial activity.
- Phenolics not saponins are responsible for the activity of the plant.

GRAPHICAL ABSTRACT



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ABSTRACT

Hedera helix L. (family Araliaceae) is classified as a conventional plant used as a medicinal product in the cure and prevention of upper respiratory tract inflammation and infection due to its secretolytic and broncholytic effects. Our research was conducted to authenticate the anti-inflammatory effect of ivy leaves extract in the prevention of acute lung injury (ALI) caused by intranasal administration of lipopolysaccharides (LPS). *In-vitro* antimicrobial, anti-inflammatory, and anti-oxidant were evaluated, in addition to the *in-vivo* acute lung inflammation model induced by LPS in mice. The animals were divided into seven groups randomly (each group containing 10 mice): control negative (saline only), control positive (LPS group), standard (Dexamethasone 2 mg/kg), ethanolic ivy leaves extract (EIE, 100 mg/kg), ethanolic ivy leaves extract (EIE, 200 mg/kg), saponin rich fraction (SRF, 100 mg/kg) and phenolic rich fraction (PRF, 100 mg/kg). Right lungs were homogenized to determine the levels of SOD, MDA, catalase, IL-10, TNF-α, NO, IL-1β, IL-6, PGE2, and MPO. Left lungs were excised for histopathology and histomorphometry. Immunohistochemistry of Cox-2 and TNF-α levels were measured. Additionally, Western blotting was used to determine the levels of phosphorylated MAPK. Also, the ethanolic extract was also standardized through HPLC analysis for its content of rutin.The data showed that the oral supplementation with EIE, 200 mg/kg significantly ($P < 0.05$) decreased the pro-inflammatory mediators, and oxidative stress biomarkers induced by LPS. Interestingly, the phenolics showed promising activity, therefore they are responsible for the

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action. In conclusion, the standardized ivy leaf extract could be advised for acute lung injury for its antimicrobial, anti-oxidant, and anti-inflammatory activities.

1. Introduction

Herbal medicine and products derived from plants which have been used for treating several pathological disorders have been enormous in recent decades, because of their numerous health outcomes and fewer toxic or serious symptoms in comparison to synthetic chemicals (Okba et al., 2021).

The ivy leaves as known in English or *Hedera helix* L. in Latin (Araliaceae), growing in most of the territory of Europe and in the western regions of Ukraine, are used in folklore medicine for numerous conditions, including respiratory disorders with expectorant activity to treat cough, cold and acute or chronic bronchial disorders (bronchitis) (Holzinger and Chenot, 2011). A lot of triterpene saponins were previously isolated, namely α-hederin, hederasaponin-C, hederacoside-E, and hederacoside-F, in addition to phenolic acids (caffeic, chlorogenic, neochlorogenic, dicaffeoyl-quinic, rosmarinic, dihydroxybenzoic protocatechuic, p-coumaric), and flavonoids (quercetin, kaempferol, rutin, isoquercitrin, astragalin, kaempferol rutinoside) (Ramadan et al., 2020). Ivy leaf extracts have demonstrated mucolytic, antispasmodic, bronchodilator, and antibacterial effects (Sieben et al., 2009), and so are administered for productive acute cough, upper respiratory tract disorders, and chronic inflammatory bronchial conditions (Lutsenko et al., 2010). These extracts are able to stimulate the gastric mucosa and, in turn, increase the secretion of bronchial mucus by parasympathicotonic reflexes due to the presence of a low amount of emetine in ivy leaves extracts (Roşca-Casian et al., 2017). Hederasaponins C, E, and F may also reduce bronchial inflammation by disrupting leukotriene signaling, as bradykinin and prostaglandin responses (Gepdiremen et al., 2005). It was recently reported that the antispasmodic and mucolytic actions of ivy leaf dried extracts depend on increased bronchial ß2-adrenergic reactivity due to suppression of G-protein-coupled receptor kinase 2 (GRK2)-mediated β2-adrenergic receptor (ß2AR) phosphorylation and associated desensitization induced by the triterpenoid saponin α-hederin (Schulte-Michels et al., 2016).

Lung inflammation is considered a serious health problem, especially the acute type caused by a lot of pathologies such as sepsis, trauma, pneumonia, or administration of toxic substances by inhalation. Therefore, patients progress to hypoxemic respiratory failure with high mortality rates. Despite the significant advances in the medical strategy over the last few decades, there are still few effective medications to treat ALI (Matthay et al., 2003). ALI is usually associated with cell damage (epithelial cells of alveoli and endothelial cells of capillaries) which is accompanied by different types of edema (pulmonary, interstitial, or alveolar), acute hypoxia, and inflammatory cell infiltration especially leukocytes (Jiang et al., 2016). The infiltration of leukocytes and neutrophils has a significant effect on the initiation of inflammatory responses. Pathologically, ALI is characterized by small lung volume and abnormal lung efficacy with imponderable blood flow ratio and ventilation. In the case of severe conditions, it led to respiratory distress syndrome, the infiltration developed from both sides of the lung, and finally reach to respiratory failure (Cheng et al., 2016).

Lipopolysaccharides (LPS) are the main content in the gram-negative bacteria cell wall. It is released into the extracellular media to perform its specific bioactivity only after bacterial lysis or any external damage. LPS are known as endotoxins due to their certain toxic effects. LPS are commonly used as an efficient external substance that induces inflammatory reactions accompanied by immune diseases in animals such as mice and rats (Tian et al., 2019). LPS is used to cause sepsis in animals associated with characteristic ALI with inflammatory cell sequestration inside the lung, and in turn, increases the permeability of capillaries with significant alveolar edema has occurred. ALI usually occurs with hypoxia, damage to alveoli or capillaries, and severe inflammatory conditions, followed by organ failure in the late stages (Ma et al., 2015).

After systemic administration of LPS and induction of ALI, the inflammatory cytokines are generated and increased via NF-κB cascade signal pathways. The immune response of the body becomes imbalanced, and this also helps in the excessive generation of cytokines (Mokhtari-Zaer et al., 2020). For all these reasons, the intratracheal and intranasal administration of LPS are the common methods used to induce ALI to study their pathogenesis and the ability of novel substances for treatment (Itoh et al., 2007).

To authenticate the conventional use of ivy leaves in the cure of respiratory disorders (Mendel et al., 2011), the authors investigated ethanolic ivy leaves extract and its fractions on the modulation of acute lung inflammation, illustrating the mechanisms of action against LPS-induced organ damage and tracing the metabolites responsible for these actions.

2. Materials and methods

2.1. Preparation of extract from plant

Ethanolic ivy leaves extract was prepared from fresh *Hedera helix* L. leaves which were gathered from a specific orchard located in Egypt. The plant has been documented by Mrs. Teresa Labib, Head of the Taxonomists at Orman Botanic Garden. A voucher specimen (No. 5.3.2020) was deposited in the Herbarium of Pharmacognosy Department, Pharmacy Faculty, Cairo University, Egypt. The 70% ethanol was used for extraction of air-dried powder (2 kg) of ivy leaves using homogenizer Mpressure to dryness (300 g).

2.2. Preparation of saponins and phenolics rich fractions

The previously prepared extract was used for the preparation of 2 fractions (saponins and flavonoids rich fractions) according to the method of Shokry et al. (2022) in our previous research.

2.3. Chemicals and reagents

Lipopolysaccharides (LPSs) of *Escherichia coli* and Dexamethasone sodium phosphate were bought from Sigma-Aldrich (St. Louis, MO, USA). Mouse IL-1β (Catalogue No.: SEA563Ra), IL- 6 (Catalogue No.: SEA079Ra), IL-10 (Catalogue No.: MBS355232), and TNF-α (Catalogue No.: abx050220) ELISA kits were obtained from Sinogeneclon Co. Ltd. (Hangzhou, China). Superoxidase dismutase (SOD) (Catalogue No.: K335-100), myeloperoxidase (MPO) (Catalogue No.: E4581-100), malondialdehyde (MDA) (Catalogue No.: K739-100), Catalase (Catalogue No.: MBS8243260), Nitric Oxide (NO) (Catalogue No.: K252-200), PGE2 (Catalogue No.: ER1800) kits were bought from Bio-diagnostic Company, Dokki, Egypt.

2.4. Determination of total phenolics, flavonoids, and terpenes contents

The total phenolic content was detected by the Folin-Ciocalteu method using standard gallic acid (El-shiekh et al., 2019). The absorbance was measured at 750 nm. The total flavonoid content was detected by aluminum chloride colorimetric assay using standard quercetin (Chatatikun and Chiabchalard, 2013). The vanillin-glacial acetic acid colorimetric assay is the method of detection of total terpenes amount using ursolic acid as a standard (Chang et al., 2012).

2.5. In vitro activities of ethanolic ivy leaves extract (EIE), saponin rich fraction (SRF), and phenolic rich fraction (PRF)

2.5.1. In vitro antimicrobial activity

All strains were prepared from the Regional Center for Mycology and Biotechnology (RCMB), Al-Azhar University, Cairo, Egypt. Antibacterial activity was expressed as the diameter of inhibition zones, which is determined by the agar well diffusion method. Ciprofloxacin (MAST Diagnostics Ltd., Bootle, Merseyside, UK) was used as the standard antibacterial. Each sample was done three times followed by a calculation of the average zone of inhibition. MIC was calculated as the minimum concentration of the sample that inhibits the visible growth of a microorganism after overnight incubation.

2.5.2. In vitro anti-inflammatory activity

In vitro cyclooxygenases (COX) and lipoxygenase (LOX) inhibition assays were determined using CAYMAN CHEMICALS colorimetric inhibitor screening assay kits for EIE, SRF and PRF before the *in vivo* experiment to determine the anti-inflammatory response by inhibiting the COX-1, COX-2 and LOX enzymes as mentioned by (George et al., 2014), respectively. The efficacy of extracts and standard Celecoxib to inhibit both COX-1 and COX-2 isoenzymes and Sodium aurothiomalate to inhibit LOX was determined as the concentration causing 50% enzyme inhibition (IC_{50}). All determinations were carried out three times.

2.5.3. In vitro anti-oxidant activity

The total anti-oxidant capacity activity (TAC) of tested samples was assessed by the phosphomolybdenum assay (Chaouche et al., 2014). The TAC was expressed in Trolox equivalents (μM/g of extract). The total anti-oxidant potential of the samples was determined by ferric reducing anti-oxidant power (FRAP) colorimetric assay according to the method described by (Chaouche et al., 2014). The free radical scavenging activity of the samples was also detected by the ABTS and DPPH radical assays (Hidalgo and Almajano, 2017). The reducing power of samples was determined according to the method of Chung et al. (2002). Trolox was used as standard. The tested sample was performed three times. Our results were expressed as an IC_{50} value (ug/mL).

2.6. In vivo acute lung inflammation

2.6.1. Animals

Male mice (6–8 weeks, weight 20–25 g) were obtained from the National Research Center, Egypt. Mice were supplied with standard feed and water. The mice adapted to the environmental condition in a sterilized animal house with specific temperature and humidity. All the animals need at least 5 days in this environment before beginning the experiment.

2.6.2. The experimental design

The mice were divided into seven groups randomly (each group contained 10 mice, n = 10): control negative, control positive (LPS group), standard (Dexamethasone 2 mg/kg), ethanolic ivy leaves extract (EIE, 100 mg/kg), ethanolic ivy leaves extract (EIE, 200 mg/kg), Saponin rich fraction (SRF, 100 mg/kg) and phenolic rich fraction (PRF, 100 mg/kg) groups (Süleyman et al., 2003). This study was done according to the instructions of the National Institutes of Health Guide for Care and Use of Laboratory Animals office (IACUC No.: Vet CU01102020229). During the first 7 days of the experiment (Figure 1), all mice were administrated orally with normal saline in the first and second group, dexamethasone in the third group, EIE, 100 and 200 mg/kg in the fourth and fifth group, SRF and PRF in the sixth and seventh group. ALI was done by intranasal administration of 10 μL of 4 mg/ml LPSs solution after 30 min of oral administration of drugs on day 7 of the experiment (Tian et al., 2019). The control negative group was instilled in PBS intranasally. After six hours, the mice were euthanized and lungs were collected. The homogenate was prepared from the lungs of mice for the assay of SOD, MPA, MDA, and catalase contents. Also, the levels of TNF-α, IL-1β, IL-6, IL-10, NO, and PGE2 were measured using different rat ELISA kits according to the manufacturer's instructions.

2.6.3. Western blot assays

After collection of the lungs, the homogenate was prepared with 0.05 M phosphate buffer (pH 7) using a polytron homogenizer at 4 °C. Centrifugation of the homogenate occurs at 10,000 rpm for 20 min to remove the cell debris, damaged cells, nucleus, red blood cells, and mitochondria to obtain the final supernatant. Then, the proteins were extracted and measured protein content in the tissue was measured using

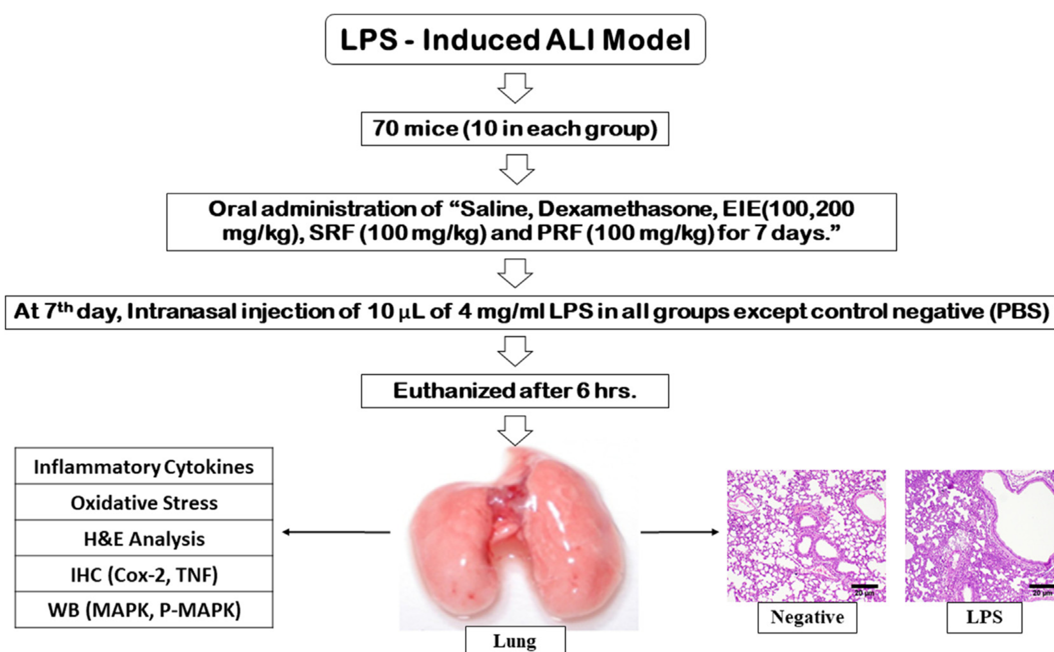

Figure 1. The schema of the in vivo LPS induced ALI in a mouse model. LPS; lipopolysaccharide; EIE; ethanolic ivy leaves extract, SRF; Saponin rich fraction, and PRF; Phenolic rich fraction (n = 10).

the method of (Bradford, 1976) using Lowry Protein, Bio Basic Inc, protein estimation kit. The ReadyPrepTM protein extraction kit (total protein) provided by Bio-Rad Inc (Catalog No: 163–2086) was employed according to the manufacturer's instructions and was added to each sample of the homogenized tissues. The primary antibody of p38-MAPK (Catalog No: CBMAB-1738-YC) and phospho p38-MAPK (Catalog No: ACI00233) was measured according to the manufacturer's instructions.

2.6.4. Histopathology and histomorphometry

The left lung samples were fixed in 10% neutral-buffered formalin for 48 h and embedded in paraffin blocks. Then blocks were cut into 4–5μm sections. Lung tissues were stained with hematoxylin and eosin (H&E) for evaluation and grading of lung inflammation; and alcian blue stain to assess goblet cell metaplasia and airway mucus responses, as described by (Bancroft and Gamble, 2008). Tissue sections were visualized using Olympus BX43 light microscope connected to a digital camera (DP27) and CellSens dimensions software.

Lung inflammation was graded by counting the layers of inflammatory cells around the airways and blood vessels as previously illustrated (Huang et al., 2019). Concisely, the slides were scored as follows, 0: normal, 1: a few cells, 2: a ring of cells, one cell layer deep; 3: a ring of cells, two to four cells deep, 4: a ring of cells of, more than four cells deep. Scoring of inflammation was performed on at least five fields for each animal (6mice/group/400x). Data were expressed as median ± SD using Kruskal–Wallis test, followed by Tukey's Multiple Comparison Test.

Quantitation of goblet cell metaplasia in the bronchi and bronchioles was expressed as mean ± SD of the goblet cells numbers per 100μm of the basement membrane in four sections of each animal (6mice/group/400x), applying one-way analysis of variance (ANOVA) followed by Dunnett's comparison test using ImageJ® (Lee et al., 2017 & Jensen, 2013).

2.6.5. Immunohistochemistry of pro-inflammatory specific markers (COX-2 and TNF-α)

Sections of the lung were deparaffined and performed according to the technique provided with avidin-biotin-peroxidase complex kits (Wang et al., 2016). Briefly, the primary antibody against COX-2 and TNF-α (1:1200) were incubated with the slides overnight at 4 °C. Then, the slides were washed with PBS three times and incubated with the secondary antibody. Immunoreaction was visualized with a diaminobenzidine substrate and counterstained with hematoxylin. The analyzed data were assessed as mean ± SD of the percentage area (%) of positively stained tissue in five sections of each sample (6mice/group/400x), via one-way analysis of variance (ANOVA) followed by Dunnett's Comparison Test.

2.6.6. Statistical analysis

Values are presented as mean ± SD. One-way ANOVA followed by Duncan's multiple range tests using SPSS version 17, Chicago, IL, USA was used for the evaluation of data and $P < 0.05$ was accepted as statistically significant.

2.7. HPLC analysis

2.7.1. Instrumentation

Chromatographic analysis was done on Agilent Technologies 1100 series HPLC system, with a quaternary pump, degasser G1322A, autosamplling injector, and a diode-array detector. The separation was operated on an Eclipse XDB-C18 column (150 × 4.6 mm, particle size 5 μm) with a C18 guard column (Phenomenex, Torrance, CA) at room temperature. The data acquisition and processing were achieved using Agilent Chemstation software.

2.7.2. Sample and standard preparation

The lyophilized ethanolic ivy leaves extract (1 mg/1 mL) was prepared in HPLC methanol and used as a working solution. For constructing the calibration curve, 5 different concentrations (62.5, 125, 250, 500, and 1000 μg/mL) of rutin were prepared. Aliquots of 20 μL of each were injected intothe HPLC system in triplicates. The calibration curve was then constructed by plotting mean peak areas versus corresponding concentrations. The sample solutions were filtered through 0.45 μm membrane filter and degassed in an ultrasonic bath before use. The content of rutin in the extract was then determined from the pre-established standard calibration curve.

2.7.3. Chromatographic conditions

Satisfactory separation was performed at room temperature for 25 min of the run, by gradient elution using 0.1% trifluoroacetic acid (A) and acetonitrile (B). A stepwise gradient elution program was established: 80 - 20% A (0–10 min), 70-30% A (10–15 min), 50-50% A (15–25 min) with flow rate of 1 mL/min and λ = 280 nm.

3. Results

3.1. Phytochemical assessment

Phenolics, flavonoids, and terpenoid contents (n = 3) were assessed as gallic acid equivalent (GAE), quercetin equivalent (QE), and ursolic acid equivalent (UE), respectively as shown in Table 1.

3.2. In vitro antimicrobial activity

The antimicrobial activity against *Klebsiella pneumonia*, *Mycoplasma pneumoniae,* and *Streptococcus pneumonia* is shown in Table 2. Where, the MICs of PRF were promising as compared to ciprofloxacin (n = 3).

Table 1. Total phenolics, flavonoids, and terpenes contents of ethanolic ivy leaves extract, saponins, and Phenolic rich fractions (n = 3).

	EIE	PRF	SRF
Phenolics (GAE/g extract)	32.98 ± 0.95	49.32 ± 2.5	23.91 ± 0.5
Flavonoids (QE/g extract)	43.23 ± 2.8	66.22 ± 3.9	16.20 ± 2.4
Terpenes (UAE/g extract)	167.33 ± 5.6	30.73 ± 1.60	229.93 ± 8.04

EIE; ethanolic ivy leaves extract, SRF; Saponin rich fraction, and PRF; Phenolic rich fraction. Values are presented as mean ± SD. GAE: gallic acid equivalent; QE: quercitin equivalent; UA: ursolic acid equivalent.

Table 2. *In-vitro* antimicrobial activity (MIC in μg/mL) of ethanolic ivy leaves extract, saponins, and Phenolic rich fractions (n = 3).

	EIE	PRF	SRF	Ciprofloxacin
Klebsiella pneumonia	5.1	2.1	98.6	1.95
Mycoplasma pneumoniae	14.2	8.63	125	7.81
Streptococcus pneumonia	2.8	0.59	62.5	0.48

EIE; ethanolic ivy leaves extract, SRF; Saponin rich fraction, and PRF; Phenolic rich fraction. The underline indicate the most significant result for the tested phenolic fraction (PRF).

Table 3. *In-vitro* anti-inflammatory activities of ethanolic ivy leaves extract, saponins, and Phenolic rich fraction (n = 3).

Sample/Standard	COX-1	COX-2	LOX
	IC_{50}, μg/mL		
EIE	301.1 ± 2.2^{c}	3.36 ± 0.8^{c}	68.19 ± 3.9^{c}
PRF	273.5 ± 2.9^{b}	1.5 ± 0.09^{b}	32.56 ± 1.2^{b}
SRF	500 ± 3.3^{c}	7.4 ± 0.3^{d}	361.6 ± 4.6^{d}
Celecoxib	248.9 ± 2.4^{a}	0.26±0^{a}	
Sodium aurothiomalate			23.4 ± 1.2^{a}

EIE; ethanolic ivy leaves extract, SRF; Saponin rich fraction, and PRF; Phenolic rich fraction.
Values are presented as mean ± SD. The different superscripts letters in the table for any value are significantly different from each other (Duncan's significant difference multiple range post-hoc test, $P < 0.05$). The underline indicate the most significant result for the tested phenolic fraction (PRF).

Table 4. *In-vitro* anti-oxidant activities of ethanolic ivy leaves extract, saponins, and Phenolic rich fractions (n = 3).

Sample/ Standard	TAC% (TE)	FRAP	ABTS	DPPH	Iron reducing power
		IC_{50} (μg/mL)			
EIE	87.16 ± 1.3[b]	79.61±1[c]	101.24 ± 4.2[b]	62.62 ± 2.8[b]	266.64 ± 0.9[c]
PRF	120.5 ± 0.6[c]	41.21 ± 0.5[b]	68.49 ± 3.9[a]	31.17 ± 1.9[a]	184.25 ± 4.2[b]
SRF	69.5 ± 0.33[a]	406.10±5[d]	464.064 ± 7.4[c]	395.64 ± 2.5[c]	317.27 ± 8.6[d]
Trolox	-	15.61 ± 1.4[a]	62.5 ± 2.5[a]	27.47±0[a]	30.95 ± 2.1[a]

EIE; ethanolic ivy leaves extract, SRF; Saponin rich fraction, and PRF; Phenolic rich fraction.
Values are presented as mean ± SD. The different superscripts letters in the table for any value are significantly different from each other (Duncan's significant difference multiple range post-hoc test, $P < 0.05$). The underline indicate the most significant result for the tested phenolic fraction (PRF).

3.3. In vitro anti-inflammatory activity

The results are shown in Table 3. Where, PRF showed the highest anti-inflammatory activity.

3.4. In vitro anti-oxidant activity

The antioxidant potential of the ethanolic extract and fractions was evaluated by five different integral methods *in-vitro*. Where, The PRF exhibited the most powerful effect as shown in Table 4. It showed an IC_{50} of 41.21, 68.49, 31.17 and 184.25 μg/mL in the FRAP, ABTS, DPPH, and iron reducing power assays, respectively. Further, the antioxidant capacity exerted by the fraction was calculated as 120.5 μM trolox acid equivalent/g extract, n = 3.

3.5. In vivo acute lung inflammation

3.5.1. Effect on the oxidative stress biomarkers

The effects of EIE, PRF, and SRF on SOD, MDA, and catalase were investigated (n = 10). Where, EIE, 200 mg/kg and PRF, 100 mg/kg successfully reduced the levels of MDA to normal values compared to LPS group; 0.97 ± 0.08 nmol/mg and 0.87 ± 0.09 nmol/mg, respectively *vs.* 2.8 ± 0.17 nmol/mg (Figure 2). Additionally, these groups reveled in an increment in the levels of SOD and catalase enzymes in lung tissues to promising values; 1.9 ± 0.06 U/mg and 2.03 ± 0.12 U/mg, respectively *vs.* 0.50 ± 0.06 U/mg for SOD and 23.23 ± 1.13 U/mg and 26.40 ± 1.13 U/mg, respectively *vs.* 6.87 ± 0.84 U/mg for catalase (Figure 2).

3.5.2. Effects on the levels of cytokines

The animals treated with EIE, 200 mg/kg, and PRF, 100 mg/kg exhibited a significant reduction in the proinflammatory cytokines (n = 10) including TNF-α, IL-1β, and IL-6, and increased the levels of protective IL-10 cytokine. It is worth highlighting that ivy leaves showed potent anti-inflammatory activity that was better than oral administration of dexamethasone (Figure 3).

3.5.3. Effects on other inflammatory mediators

The other four inflammatory mediators (n = 10) were selected for our study to evaluate the anti-inflammatory activity of ivy leaves in the ALI model. Also, the oral administration of EIE, 200 mg/kg, and PRF, 100 mg/kg significantly reduced the high levels of MPO, PGE-2, and NO induced by LPS (Figure 4). MAPK signaling pathway plays an important role in regulating the inflammatory response, our results (Fig. S1)

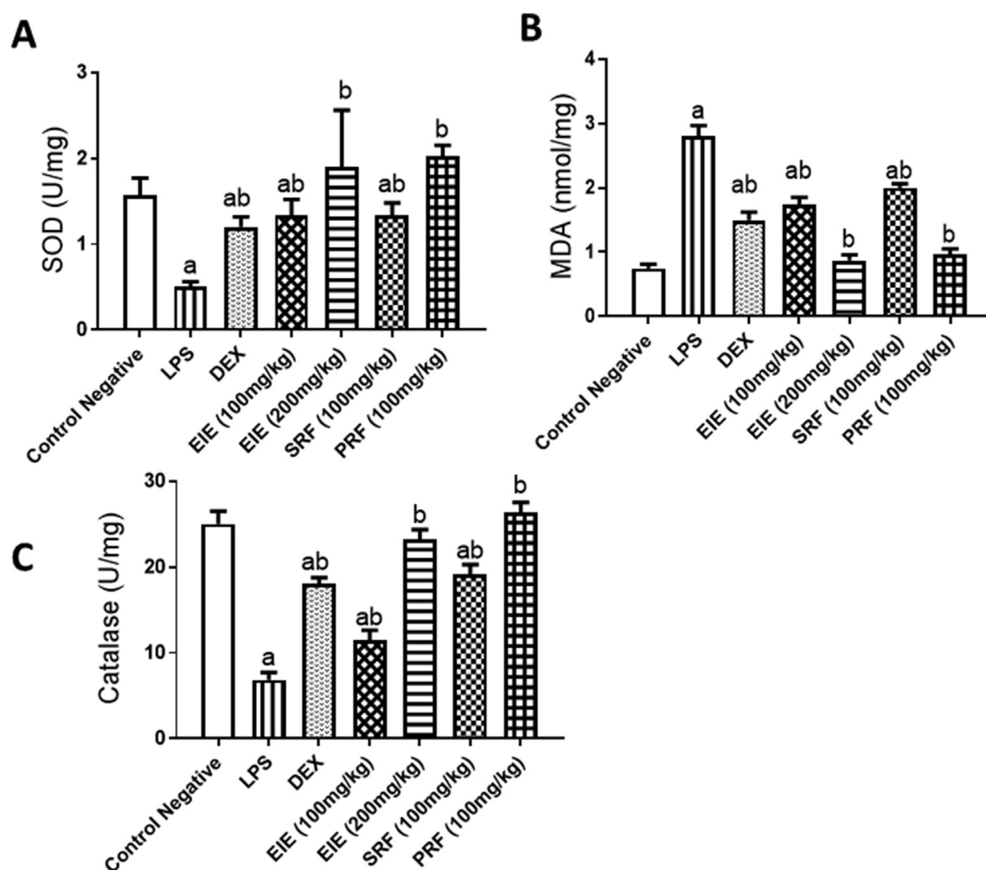

Figure 2. Biomarkers in LPS-stimulated mice. A) SOD (U/mg), B) MDA (nmol/mg), C) Catalase (U/mg). EIE; ethanolic ivy leaves extract, SRF; Saponin rich fraction, and PRF; Phenolic rich fraction. Statistical analysis was carried out by one-way ANOVA followed by Duncan's multiple comparison test. [a]Significant difference from normal control group at P < 0.05. [b]Significant difference from dexamethasone group at P < 0.05. [ab]Significant difference from normal control group and dexamethasone group at P < 0.05, (n = 10).

demonstrated that LPS markedly increased the level of activated p38-MAPK which is activated by a variety of cellular stresses including inflammatory cytokines. Activated p38-MAPK has been shown to be phosphorylated. Additionally, treatment with ivy leaves attenuated the altered expression of p38-MAPK and phospho p38-MAPK after intranasal administration of LPS, and these results are shown in different Western blot replicates in Fig. S2.

3.5.4. Histopathology and histomorphometry

Lung tissues stained with H&E and alcian blue were presented in (Figure 5a). In the control negative group, normal histological structure of the bronchi, bronchiole, and alveoli was observed. In contrast, the LPS group showed a severe infiltration of inflammatory cells associated with goblet cell metaplasia. Both EIE, 200 mg/kg, and PRF, 100 mg/kg significantly suppressed the inflammation of lung; a few inflammatory cells as well as a reduction in proportion of goblet cells metaplasia were observed. The inflammation of lung and goblet cell metaplasia in DEX and EIE, 100 mg/kg also tended to be lower than that in LPS. However, the grade of inflammation in saponins-treated mice was not different from that in the LPS group.

The histopathological results were confirmed by investigation of the inflammatory score and number of goblet cells per 100 μm of the basement membrane of bronchi and bronchioles as shown in (Fig. 5b&c). Scoring investigation of lung inflammation revealed more significantly in the LPS-treated mice compared to control negative, EIE, 200 mg/kg and PRF, 100 mg/kg groups ($P < 0.001$). Moreover, the inflammatory grade was slightly suppressed in DEX and EIE, 100 mg/kg mice compared to LPS mice ($P < 0.05$). No significant difference was recorded between the LPS and Saponins groups (Figure 5b).

The number of goblet cells which positively stained with alcian blue was significantly increased in the LPS and saponin groups compared to the control negative group ($P < 0.001$). Administration of EIE, 100 mg/kg or DEX, suppressed goblet cell hyperplasia ($P < 0.01$). Besides, the goblet cell number was not significantly different in EIE, 200 mg/kg, and PRF, 100 mg/kg groups compared to the control negative group (Figure 5c).

3.5.5. Immunohistochemistry

The immunohistochemistry expression of TNF-α and COX-2 were shown in (Figure 6). The strongest expression of both pro-inflammatory markers was noted in the LPS and saponin groups in the form of brown granules. In the EIE, 200 mg/kg, and PRF, 100 mg/kg groups, significant suppression of COX-2 and TNF-α expression was recorded and its appearance was like that in the control negative group. Treatment with EIE, 100 mg/kg showed a weak immunoreaction. Besides, the expression levels of both COX-2 and TNFα were slightly downgraded in the DEX treated group compared with the LPS group.

3.5.6. Standardization of the ethanolic ivy leaves extract (EIE)

The calibration curve was plotted and the contents of rutin in the EIE, evaluated by the proposed method, was 127.5 mg/g extract. Where phenolics were found the metabolites responsible for their activity were also found (Figures S3-S5).

4. Discussion

ALI is frequently triggered by capillary endothelial cell and alveolar epithelial damage induced by various types of direct or indirect injurious

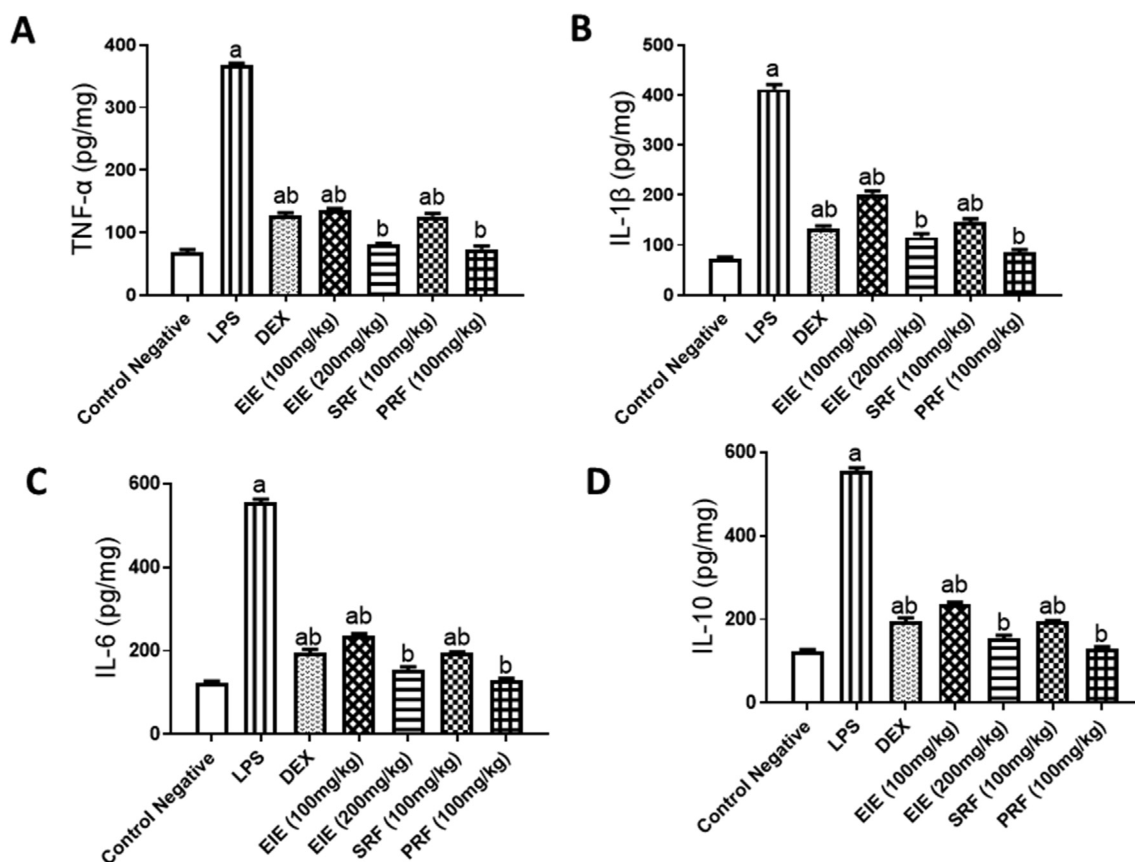

Figure 3. Effect of ethanolic ivy leaves extract, saponins and Phenolic rich fractions on the levels of cytokines in LPS-stimulated mice. A) TNF-α (pg/mg), B) IL-1β (pg/mg), C) IL-6 (pg/mg), D) IL-10 (pg/mg). EIE; ethanolic ivy leaves extract, SRF; Saponin rich fraction, and PRF; Phenolic rich fraction. Statistical analysis was carried out by one-way ANOVA followed by Duncan's multiple comparison test. [a]Significant difference from normal control group at P < 0.05. [b]Significant difference from dexamethasone group at P < 0.05. [ab]Significant difference from normal control group and dexamethasone group at P < 0.05, (n = 10).

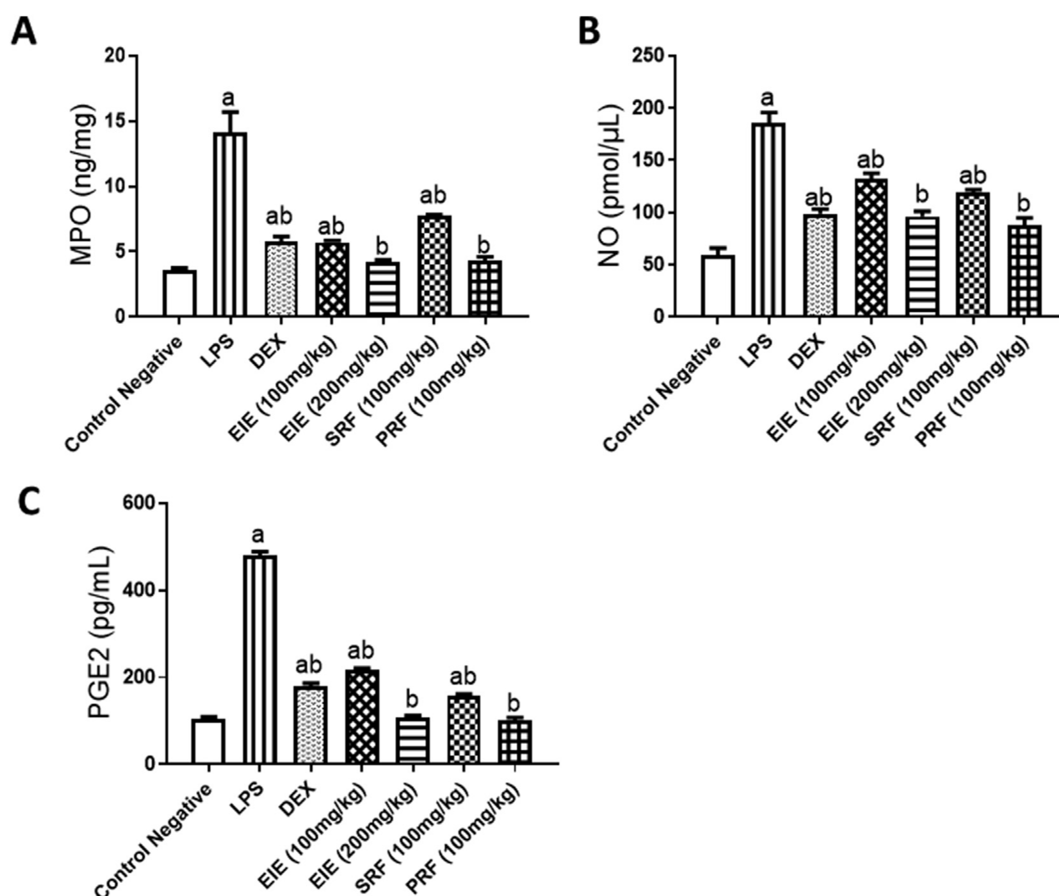

Figure 4. Effect of ethanolic ivy leaves extract, saponins and Phenolic rich fractions on MPO, NO and PGE2 in LPS-stimulated mice. A) MPO (ng/mg), B) NO (pmol/µL), C) PGE2 (pg/mL). EIE; ethanolic ivy leaves extract, SRF; Saponin rich fraction, and PRF; Phenolic rich fraction. Statistical analysis was carried out by one-way ANOVA followed by Duncan's multiple comparison test. [a]Significant difference from normal control group at $P < 0.05$. [b]Significant difference from dexamethasone group at $P < 0.05$. [ab]Significant difference from normal control group and dexamethasone group at $P < 0.05$, ($n = 10$).

effects, and this is accompanied by acute hypoxic respiratory insufficiency, interstitial, or alveolar oedema, and diffuse pulmonary. Lipopolysaccharides (LPSs) are the main constituent of gram-negative bacteria cell wall, and they are released outside the cell to do specific biological function after the bacteria has been exposed to any external factors lead to death or damage or in case of reproduction. They are also known as endotoxins due to their high toxic effect inside the hosts. LPSs are used to induce ALI, where they are the best choice to trigger the inflammatory process and disorders of immune systems in rat, mice, or most of laboratory animals (Tian et al., 2019).

In our study, we evidenced for the first time that ivy leaves can significantly protect the mice from LPS-induced lung injury, where the Phenolic rich fraction is responsible for the activity not the saponins. ALI is a common disease affecting the health of people worldwide, while many medications are used in cases of lung injury and inflammation for prevention or control. There is no effective treatment, so new therapeutic candidates for this disease must be identified.

Ivy leaves are used as a traditional plant for different diseases since a long time of years. Their extracts are commonly used in folklore for the control of respiratory diseases as they can relax bronchial smooth muscle (bronocospasmolytic and bronchodilator), increase mucus production (mucolytic), inhibit inflammation, reduce fever and excessive sweating, and treat bacterial, protozoal, and fungal infections (Shokry et al., 2021). All these uses are related to high amount of different types of biological substances that may be the main cause of these pharmacological effects, including triterpene saponins, flavonoids, coumarins, polyacetylenes, anthocyanins, sterols, alkaloids, amino acids, vitamins, carbohydrates, and volatile oils (Yu et al., 2016).

Antimicrobial activity of the EIE, PRF, and SRF against pneumonia strains; *Klebsiella pneumonia*, *Mycoplasma pneumoniae*, and *Streptococcus pneumonia* were studied *in-vitro*. And they showed good activity, where the PRF was the most active as compared to ciprofloxacin. Additionally, they were tested for their *in-vitro* anti-inflammatory activity by COX-1, COX-2, and LOX. Furthermore, for their anti-oxidant activity using five complementary methods; TAC, FRAP, ABTS, DPPH and iron reducing power. Our results investigated that the tested PRF possessed the highest inhibitory activity as a COX inhibitor in comparison with the standard celecoxib and as a LOX inhibitor comparable to the standard sodium aurothiomalate. Alongside, it showed a strong anti-oxidant activity as compared to Trolox. These promising effects investigated that Ivy leaves can act as a potent anti-inflammatory and anti-oxidant activities *in-vivo*. To test this hypothesis, the *in-vivo* anti-inflammatory effects were evaluated using the LPS induced acute lung inflammation (ALI) model.

Induction of inflammation in lungs by LPSs in mice is a very fast easy experiment to study the pathway of ALI (Jansson et al., 2005). LPS intranasally induces inflammation of lung due to leukocyte infiltration (Puljic et al., 2007). The characteristic features of ALI are injury of alveolar epithelial and endothelial cells and inflammatory cells sequestration in the alveoli. This is usually associated with lung oedema and increased inflammatory cytokines. Without effective medicinal products in our lives, ALI caused high morbidity and mortality rates (Wu et al., 2016) (Shen et al., 2017). observed in their studies these previous significant pathological changes which occur in LPS- induced ALI in mice associated with hemorrhagic changes, formation of hyaline membrane, complete damage in alveoli, and alveolar congestion. All these specific harmful effects were detected in the lung tissues of animals. The animals

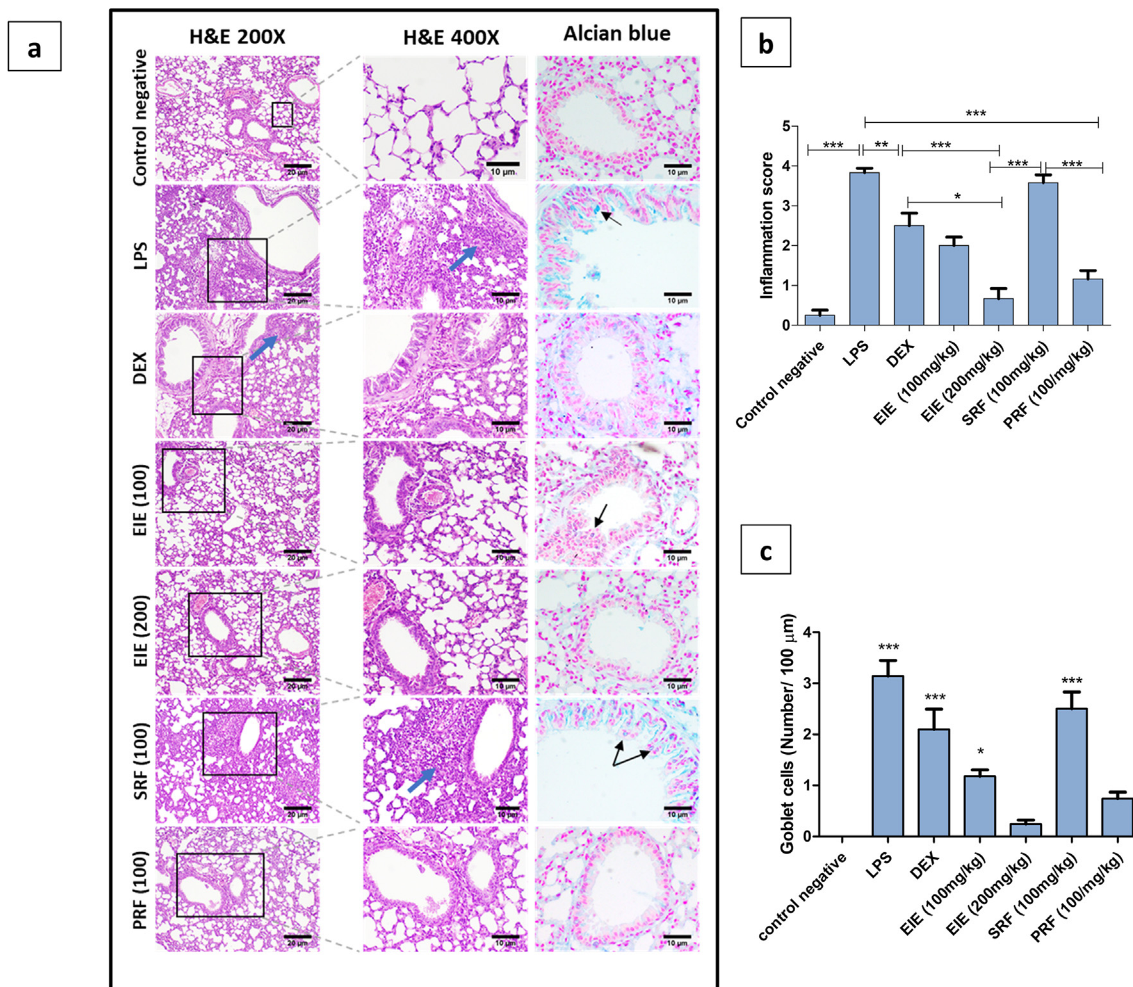

Figure 5. (a) Photomicrographs of lung sections stained with (H&E and Alcian blue); (b) Inflammation in lung; (c) Goblet cells number per 100μm of the basement membrane. Notable black arrows on the figure point to goblet cell metaplasia and blue arrows point to focal aggregation of neutrophils. Data are expressed as mean ± SD (*P < 0.05, **P < 0.01, ***P < 0.001). EIE; ethanolic ivy leaves extract, SRF; Saponin rich fraction, and PRF; Phenolic rich fraction, (n = 10).

in this group were administered LPS only in this experiment. Thus, the mice model for induction of ALI is a successful easy model that occurred using LPS.

LPS significantly increased the reactive oxygen species (ROS) inside the cells associated with inflammatory responses (Lin et al., 2016). Increased levels of ROS led to a reduction in the SOD effect and an increase in the MDA effect with neutrophil infiltration in lung (Li et al., 2015. Our results showed that ethanolic ivy leaves extract administration not only significantly inhibit lung inflammation but also increases SOD and decreases MDA effects. Our data suggest that the protective effect of EIE, 200 mg/kg against LPS-induced acute lung injury may be linked to its effective antioxidant activity. The authors also investigated the fractions of ivy leaves to relate the activity with the metabolites. Where, PRF showed the activity of ivy leaves as a potent anti-oxidant.

Exposure to excessive amounts of oxygen for long time will decrease the ability to control the excessive ROS. At the same time, ROS caused various pulmonary dysfunction, tissue injury, intracellular edema and lipid peroxidation (Yu et al., 2015). ALI is followed by oxidative stress due to exhaustion of high oxygen levels that results in the production of superoxide anions during neutrophil over-stimulation (Tsai et al., 2017). Previous studies showed that MDA and SOD were the most affected parameters in the pathogenesis of ALI caused by LPS (Jing et al., 2015). MDA is one of the most important indexes of lipid peroxidation and tissue damage (Kumari et al., 2015). Natural compounds have protective effects that can modulate abnormal levels of MDA caused by oxidant and inflammatory stimuli such as LPS (Locatelli et al., 2018). SOD is one of the most important initiators of transcription factors, so it plays a significant effect in the generations of inflammatory cytokines (Shokry et al., 2022).

Catalase is one of the important antioxidant biomarkers. There was a significant reduction in the amount of enzyme activity to LPS group. The supplementation of EIE, 200 mg/kg and PRF, 100 mg/kg successfully promoted the activity of catalase.

Vital staffing of neutrophils is one of the most important constituents of initial innate immune response in the lung tissue in front of any pathological agent. the cells of neutrophils are the first immune cells which migrated and accumulated at the place of infection and express many of cytotoxic markers (Nanashima et al., 2008). ALI which is induced by endotoxemia is characterized by accumulation of neutrophils in the lungs, express pro-inflammatory markers, such as IL-1β and TNF-α, and at the end, reaches the pulmonary injury (Parsey et al., 1998). The inflammatory process accompanied by macrophages is associated with the excessive production of inflammatory mediators, such as NO, PGE2, and COX-2, and pro-inflammatory cytokines, including TNF-α, IL-1β, and IL-6 (Kim et al., 2018).

LPS is a specific substance that induces inflammation associated with stimulation of cytokines attracted with inflammation. TNF-α, IL-1β, and IL-6 are the specific markers accumulated in the inflammatory pathway of lung inflammation (Cribbs et al., 2010). These cytokines, as well as other pro-inflammatory mediators, stimulate and exaggerate the

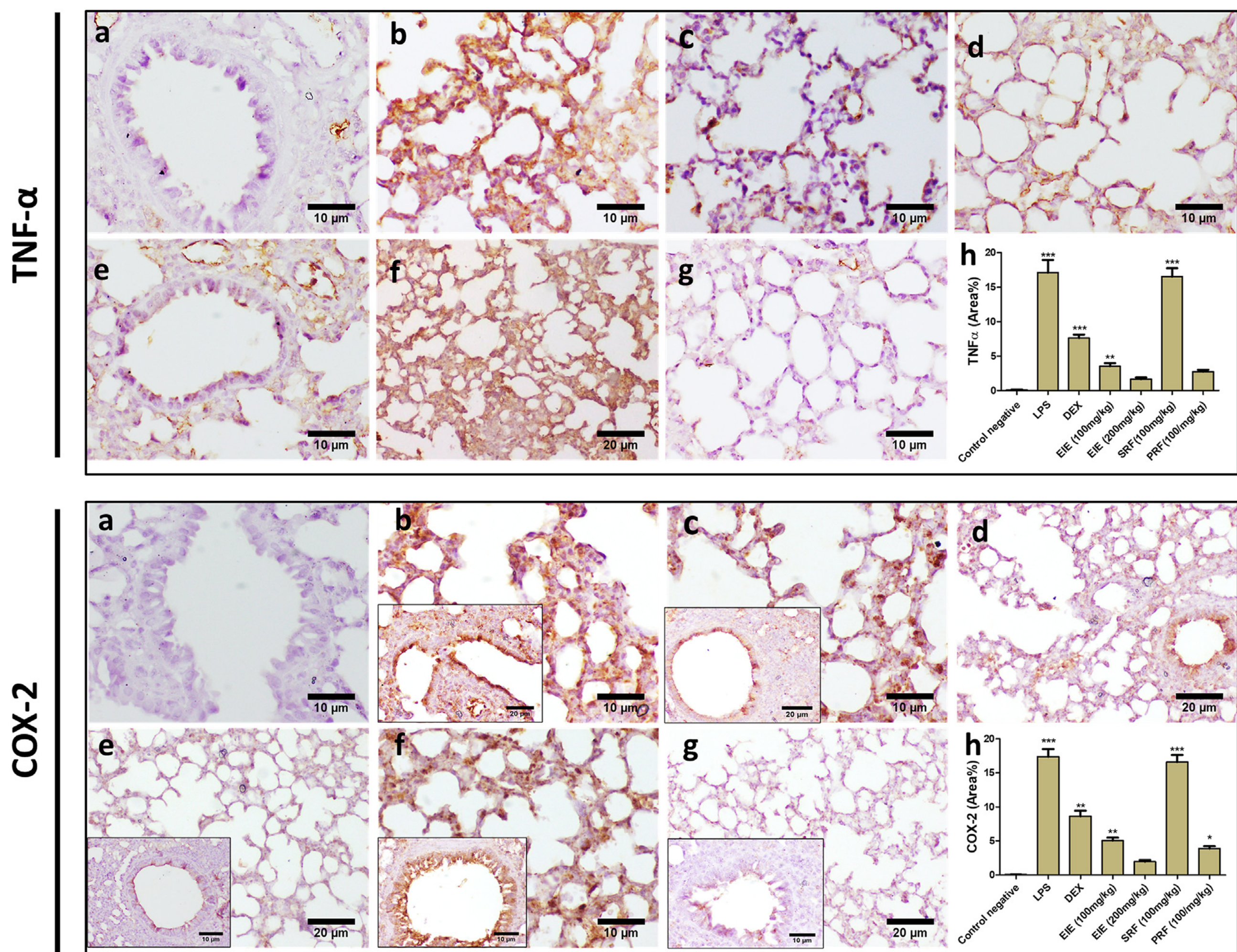

Figure 6. Photomicrographs of lung stained by TNF-α and COX-2 immunohistochemical staining. (a) Control negative group; (b) LPS group; (c) DEX group; (d) EIE (100 mg/kg) group; (e) EIE (200 mg/kg) group; (f) SRF (100 mg/kg); (g) PRF (100 mg/kg); (h) Immunohistochemical analysis for area% of insulin expression. Data expressed as mean ± SD (*P < 0.05, **P < 0.01, ***P < 0.001). EIE; ethanolic ivy leaves extract, SRF; Saponin rich fraction, and PRF; Phenolic rich fraction, (n = 10).

inflammatory response in ALI (Bhatia and Moochhala, 2004). Monocytes and macrophages infiltrated into the site of inflammation produce TNF-α acting as the first and most important endogenous initiator in the process of inflammation. It can start the process of inflammation, cause damage in vascular endothelial cells, and induce the production of other markers from the cells, such as IL-6, from alveolar epithelial cells (Goodman et al., 2003). In this study, we found that TNF-α, IL-1β, and IL-6 upgraded to 5 times in tissues after LPS treatment with great evidence. So, EIE and PRF pretreatments significantly decreased their levels.

The inflammatory cytokines that errand the development of inflammatory cascades are emphasized. However, other cytokines are classified as anti-inflammatory cytokines, such as IL-10, which stop some of the deleterious actions and effects in endotoxemia-induced ALI. IL-10 is considered an important mediator which protects against LPS effects, and it's lack leads to faster death (Abarca-Vargas and Petricevich, 2018). In our study, we also found considerable increases in IL-10 levels in the groups treated with the ivy leaves and dared with LPS.

MPO is one of the major constituents of neutrophil cytoplasmic granules. Determination of its level is a fast determination of neutrophil accumulation (Shenkar and Abraham, 1999). According to our results, MPO levels have been increased gradually in lung tissues as a response to LPS administration to the lung by the intranasal route. According to our expectation, EIE previous treatment significantly is able to decrease MPO levels in lung tissues. Besides our chemical results, the histopathological study also investigated that the previous treatment with the extract is able to inhibit the neutrophil sequestration in the lungs.

Excessive nitric oxide (NO) levels are produced by the stimulated inducible nitric oxide synthases (iNOS), which is one of the most important constituents in inflammatory process in the air way and lung parenchymal inflammation (Bae et al., 2012). NO levels are upgraded inside the lungs in case of inflammatory diseases followed by stimulation release of pro-inflammatory cytokines like IL-6 and TNF-α (Matata and Galiñanes, 2002). LPS-induced lung damage is mediated by the accumulation of inflammatory cytokines such as TNF-α, IL-1β, IL-6, NO, and PGE2 in the BALF. iNOS and COX-2 enzymes are able to produce NO and PGE2 in inflammatory conditions. However, prostaglandin levels rise immediately in acute inflammation; hence, cells stimulated with LPS only showed an elevation of PGE2. PGE2 is a biological lipid marker which is associated with the process of inflammation and cancer. It is synthesized by phospholipases by free fatty acids liberation from cell membranes, including arachidonic acid (Ricciotti and FitzGerald, 2011). Previous investigators reported that increased levels (Askari et al., 2018).

LPS administration is responsible for the production of high markers of pro-inflammatory cytokines and inflammatory mediators. On another hand, the levels of anti-inflammatory cytokines were decreased. However, the previous treatment with EIE at a dose of 200 mg/kg and PRF in a dose of 100 mg/kg strongly prevented the secretion of TNF-α, IL-1β, IL-6, NO, and PGE2 induced by LPS. Regardless, of being one of the pro-

inflammatory cytokines, IL-6 is characterized by specific anti-inflammatory activity (Menghini et al., 2014).

The mitogen-activated protein kinases (MAPKs) are a family of serine/threonine protein kinases that act a serious effect in controlling the cell response to cytokines, the regulation and differentiation of cell survival/apoptosis and they are critical to producing inflammatory mediators. p38- MAPK is one of the most important proteins in this family (Bayazid et al., 2020). It is stimulated by different types of extracellular stimuli such as pro-inflammatory cytokines; this activation leads to histone changes in promoter regions of specific genes and then a potent increase in transcription factors activity such as nuclear factor κB and enhances the inflammatory process (Gaffey et al., 2013). Accordingly, treatments inhibiting MAPKs have a potential therapeutic role in curing ALI. So, the plant should be recommended for inflammatory disorders and lung inflammation by infectious agents by inhibiting the process of inflammation, decreasing the damage occurred by oxidative stress markers, and apoptosis through inactivation of p38-MAPK.

Immunohistochemical analysis of different lung sections revealed an intensive expression of COX-2 and TNF-α in both LPS and saponins groups, nonetheless, the weakest expression was observed in EIE, 200 mg/kg, and PRF, 100 mg/kg groups. LPS enhanced the release of numerous inflammatory cytokines, including TNF-α (Cribbs et al., 2010). TNF-α, generated by monocytes/macrophages, evoked the production of COX-2 and NF-κB in a time-dependent manner (Maturu et al., 2017). Cyclooxygenase-2 (COX-2) is also involved in the production of prostaglandins that induce multiple cellular deleterious events such as acute lung inflammation, congestion of blood vessels, aggregation of airways platelets, and edema (Vila-del Sol and Fresno, 2005). EIE, 200 mg/kg, and PRF, 100 mg/kg significantly downregulated the expression of TNF-α and COX-2 provoked by LPS. Therefore, the inhibition of these pro-inflammatory chemokines may diminish the inflammatory reaction that is verified by histopathological examination. Besides, the maintenance of the cellular structure could also be attributed to its antioxidant activity, which that confirmed in our study.

Ivy leaf extract contains high amounts of phenolics such as chlorogenic acid, which could inhibit the increased level of cyclooxygenase 2 expression in RAW 264.7 cells stimulated by LPS injection. This occurs due to inhibition of NF-KB-dependent gene activation, so the PGE2 synthesis is reduced (Shan et al., 2009). Chlorogenic acid can decrease iron-induced lipid peroxidation by inhibiting the production of hydroxyl radicals through the formation of chlorogenic acideiron complexes (Kono et al., 1998). 3,5- and 4,5-dicaffeoylquinic acid is another component in ivy leaf extract which inhibit NO-Synthesis in RAW 264.7 cells and the expression of the NO-synthase (iNOS) and the cyclooxygenase-2 (COX-2) (Park et al., 2009). The anti-inflammatory effects of ivy leaf extract are confirmed by leucocyte count reduction in the blood samples for cases suffering from severe chronic bronchitis, and this is allocated to the high content of flavonoids and phenolic acids (Greunke et al., 2015).

Phenolics are confirmed to possess a high anti-inflammatory effect either *in vitro* or *in vivo*. The mechanism for this effect is the ability to inhibit eicosanoid generating enzymes, including phospholipase A2, cyclooxygenases, and lipoxygenases. The treatment decreases the levels of prostanoids from arachidonic acid such as prostaglandins E2 and F2 and thromboxane A2. On the other hand, phenolics can inhibit histamine release, phosphodiesterase, protein kinases, and activation of transcriptase (Rathee et al., 2009).

Extracts of ivy leaves have been to treat various respiratory disorders and they are the most important one which is sold as a natural drug used for cough in many areas all over the world. It is commonly known that ivy leaves have expectorant and bronchospasmolytic effects, and many types of researches were applied to describe their mechanism of action, safety, and pharmacological activities (Yu et al., 2015). Finally, from our study, we could authenticate its efficacy against respiratory disorders due to its antimicrobial, anti-oxidant, and anti-inflammatory potential.

5. Conclusion

Ivy leaves significantly inhibited LPS-induced inflammatory cytokine production and MAPK signalling pathways in addition to the reduction of oxidative stress biomarkers. So, we recommended the standardized extract for ALI for its antimicrobial, anti-inflammatory and anti-oxidant activities. Where, the phenolics are the metabolites responsible for that activity.

Declarations

Author contribution statement

Aya A. Shokry; Riham A. El-Shiekh: Performed the experiments; Analyzed and interpreted the data; contributed reagents, materials, analysis tools or data; Wrote the paper.

Alaa F. Bakr: Analyzed and interpreted the data.

Amer Ramadan; Gehan Kamel: Conceived and designed the experiments.

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Data availability statement

Data will be made available on request.

Declaration of interests statement

The authors declare no conflict of interest.

Additional information

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