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HAL Authorization

1 **The proline isomerase Pin1 inhibitors PiB and juglone protect rats against**
2 **lipopolysaccharide-induced respiratory inflammation**

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21

22 **Abstract**

23

24 The peptidyl-prolyl cis/trans-isomerase, NIMA-interacting 1 (Pin1), is involved in several
25 cellular functions by changing the conformation and activity of phosphorylated proteins. Its key
26 role in cellular signalling makes Pin1 an appealing target for the development of
27 pharmacological agents to treat diseases such as inflammation. Pulmonary inflammation is a
28 major disease resulting from the activation of immune cells by microbes or pollutants. In this
29 study, we examined the effects of two Pin1 inhibitors, PiB and juglone, on lipopolysaccharide
30 (LPS)-induced pulmonary inflammation in rats. Our results demonstrate that the intraperitoneal
31 (i.p.) administration of 3 mg/kg of PiB and juglone mitigates LPS-induced pulmonary
32 inflammation by reducing haemorrhage, vascular injury, pulmonary oedema and immune cell
33 accumulation in the airway region. Furthermore, these Pin1 inhibitors were found to protect rats
34 against the overproduction of inflammatory markers by inhibiting myeloperoxidase (MPO) and
35 lactate dehydrogenase (LDH) activities, as well as reducing plasma C-reactive protein (C-RP).
36 Additionally, PiB and juglone protected the rats from nitric oxide synthase (NOS) hyperactivity,
37 preventing oxidative stress by decreasing pro-oxidant markers and restoring the antioxidant
38 enzymes such as catalase, superoxide dismutase (SOD) and glutathione peroxidase (GPx). The
39 use of juglone and PiB could therefore be an effective means of alleviating acute respiratory
40 inflammation by modulating inflammatory and oxidant pathways and preserving lung
41 microstructure.

42

43 **Keywords:** Lung inflammation, Pin1, PiB, juglone, inflammatory markers

44 **Highlights**

45

46 • Juglone and PiB reduce vascular injury during lipopolysaccharide-induced lung
47 inflammation.

48

49 • Juglone and PiB protect rats from LPS-induced oxidative stress in the lungs.

50

51 • Juglone and PiB reduce pro-inflammatory markers during pulmonary inflammation.

52 **Abbreviations**

53

54 ALP, alkaline phosphatase; ANOVA, Analysis of variance; AOPPs, Advanced oxidation
55 protein products; ATSAL, Tunisian Association of Laboratory Animal Science; BAL,
56 Bronchoalveolar lavage; C-RP, C-reactive protein; DMSO, Dimethyl sulfoxide; DTNB, 5,5'-
57 dithio-bis-(2-nitrobenzoic acid); EDTA, Ethylenediaminetetraacetic acid; fMLP, N-formyl-
58 methionyl-leucyl-phenylalanine; GM-CSF, granulocyte-macrophage colony stimulating factor;
59 GPx, Glutathione peroxidase; GSH, Glutathione; i.n., Intranasal; i.p., Intraperitoneal; LDH,
60 Lactate dehydrogenase; LPS, Lipopolysaccharide; MAPK, mitogen-activated protein kinase
61 MAPK; MDA, Malondialdehyde; MPO, Myeloperoxidase; NADPH, Nicotinamide adenine
62 dinucleotide phosphate hydrogen; NF- κ B, phosphorylated nuclear factor kappa B; NO, nitric
63 oxide; NOS, Nitric oxide synthase; Nrf2, nuclear factor erythroid2-related factor 2; OD, Optical
64 density; PBS, phosphate buffered saline; Pin1, peptidyl-prolyl cis-trans isomerase NIMA-
65 interacting 1; SEM, Standard error of the mean; SOD, Superoxide dismutase; TCA,
66 Trichloroacetic acid.

67

68 **1. Introduction**

69

70 Acute pulmonary inflammation is one of the most common lung diseases. It is
71 characterized by lung epithelial barrier dysfunction (Carnesecchi et al., 2012; Liu et al., 2023),
72 oedema due to high vascular permeability caused by the accumulation of inflammatory cells in
73 the lungs (Bayarri et al., 2021; Chen et al., 2010), and oxidative stress (Ward, 2010). Bacterial
74 lipopolysaccharides are a major pro-inflammatory agent that can cause lung inflammation (Ware
75 et al., 2000). The excessive release of reactive oxygen species (ROS) and reactive nitrogen
76 species (RNS) causes lipid and protein damage (Di Meo et al., 2016). During inflammation,
77 various inflammatory cells, including neutrophils, macrophages and eosinophils, invade the
78 affected tissue and alveolar spaces. There, they interact with lung mesenchymal cells to regulate
79 different inflammatory pathways (Bezerra et al., 2023; Jerkic et al., 2023). High infiltration of
80 phagocytic cells into the alveolar spaces (Lee et al., 2021) triggers an oxidative burst through the
81 upregulation of nicotinamide adenine dinucleotide phosphate hydrogen (NADPH) oxidase
82 isoforms, producing superoxide anion ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), hydroxyl radicals
83 (Bezerra et al., 2023; Park et al., 2008) and hypochlorous acid (Aratani, 2018). During
84 inflammatory responses, macrophages and vascular endothelial cells activate nitric oxide
85 synthase to produce nitric oxide (NO), thereby regulating the immune response (Sharma et al.,
86 2007; Syed et al., 2016).

87 Peptidyl-prolyl cis/trans-isomerase, NIMA-interacting 1 (Pin1) is a key enzyme in immune
88 cell function (Shen et al., 2008). Pin1 is involved in the *cis-trans* isomerization of
89 phosphorylated serine/threonine-proline residues in various proteins (Kurakula et al., 2018).
90 Furthermore, Pin1 has been implicated in priming of NADPH oxidase through the
91 phosphorylation of the p47phox component in neutrophils, either by lipopolysaccharide (LPS) or

92 granulocyte-macrophage colony-stimulating factor (GM-CSF) (Boussetta et al., 2010; Boussetta
93 et al., 2024; Liu et al., 2019). Studies have also shown that Pin1 is involved in controlling NO
94 synthases (Ruan et al., 2011) and positively regulates the phosphorylated nuclear factor kappa B
95 (NF- κ B) pathway (Chen et al., 2018; He et al., 2023), as well as the nuclear factor erythroid 2-
96 related factor 2 (Nrf2) pathway (Kim et al., 2010; Stefano et al., 2016). Pin1 activity has been
97 associated with various signalling pathways, protein interactions and diseases (Wu et al., 2024;
98 Zhang et al., 2022).

99 The Pin1 knockout mouse model has been used to study the role of the Pin1 protein in
100 various diseases (Dong et al., 2021; Uchida et al., 2003). The use of unrelated Pin1
101 pharmacological inhibitors, such as PiB and juglone, in an alternative pathway is a promising
102 approach for modulating cancer cell proliferation and preventing neuro-inflammation (Peng et
103 al., 2015; Uchida et al., 2003). Juglone (5-hydroxy-1,4-naphthoquinone) has been extracted from
104 walnut trees (Cosmulescu et al., 2014). This bioactive molecule has been shown to exhibit broad-
105 spectrum anti-cancer activity (Erkoç Kaya and Dursunoğlu, 2023; Zhong et al., 2024). It is also
106 widely recognized as a preventive agent against liver and kidney damage (Şenol et al., 2023a;
107 Şenol et al., 2023b).

108 In addition, PiB is a synthetic molecule that inhibits Pin1 activity by masking the active
109 domains involved in substrate binding to the enzyme (Uchida et al., 2003). PiB has been shown
110 to promote inhibitory activity against epithelial-mesenchymal transition in bladder cancer by
111 attenuating signal transducer and activator of transcription 3 (STAT3) and down-regulating the
112 NF- κ B pathway (Moretto-Zita et al., 2024; Nakada et al., 2019). Our laboratory's studies have
113 demonstrated that PiB and juglone can inhibit N-formyl-methionyl-leucyl-phenylalanine
114 (fMLP)-induced human neutrophil activation (Bedouhene et al., 2021) and LPS-induced priming
115 of ROS production (Liu et al., 2019). Furthermore, juglone has been shown to inhibit tumor

116 necrosis factor- α (TNF- α)-induced priming of neutrophils and primed neutrophils isolated from
117 patients with rheumatoid arthritis (Boussetta et al., 2010; Boussetta et al., 2024).

118 The aim of this study was to test the effect of two chemically unrelated Pin1 inhibitors,
119 juglone and PiB, on LPS-induced respiratory inflammation in rats. These inhibitors have been
120 shown to inhibit priming and activation of neutrophil functions (Bedouhene et al., 2021;
121 Boussetta et al., 2010; Liu et al., 2019). The dose used in this study was 3 mg/kg, which has
122 previously been shown to be effective in rats (Ahmad et al., 2022). Juglone is a natural
123 compound purified from walnut leaves, whereas PiB is a chemically synthesised compound. As
124 juglone and PiB have very different molecular structures (Bedouhene et al., 2021), obtaining the
125 same results with both inhibitors could support the role of Pin1 in lung inflammation.

126

127 **2. Materials and methods**

128

129 **2.1. Chemicals and reagents**

130 Lipopolysaccharide from *E. coli* O111:B4, juglone (5-hydroxy-1,4-naphthoquinone) and
131 PiB (diethyl-1,3,6,8-tetrahydro-1,3,6,8-tetraoxobenzo[*lmn*]phenanthroline-2,7-diacetate), bovine
132 serum albumin; hexadecyltrimethylammonium bromide; orthodiansidine dihydrochloride;
133 Na₂ CO₃ ; NHCO₃ ; bovine catalase; epinephrine; potassium iodide; chloramine-T; sulfanilic
134 acid, N-(1-naphthyl)ethylenediamine, vanadium chloride, ethylenediaminetetraacetic acid,
135 sodium nitrite, glutathione and trichloroacetic acid, as well as 5,5'-dithiobis(2-nitrobenzoic acid)
136 were purchased from Sigma-Aldrich (Saint-Quentin-Fallavier, France). The Rodent Standard
137 pellet (AIN-93G) was provided by the Society El Badr in the Utique region of Bizerte, Tunisia.
138 Commercially available kits for measuring iron, creatinine, lactate dehydrogenase, alkaline

139 phosphatase activity and C-reactive protein (C-RP) were provided by Biomaghreb Society,
140 Tunisia.

141

142 **2.2. Animal care and experimental design**

143 Thirty-six male Wistar rats weighing 135 ± 15 g were provided by the Pasteur Institute of
144 Tunis. The rats were kept in controlled conditions for 14 days, including a temperature of 22 ± 2
145 °C, a 12-hour light/dark cycle, and free access to laboratory rodent food and water. After this
146 period, the animals were randomly divided into six equal groups of six rats each. During the in
147 vivo experiments, isoflurane (3%) was used as the anaesthetic agent in accordance with the
148 pharmacological guidelines for rodent anaesthesia (Cicero et al., 2018). Briefly, the control and
149 LPS groups were pre-treated with daily intraperitoneal (i.p.) administration of 1% dimethyl
150 sulfoxide (DMSO) diluted in phosphate-buffered saline (PBS) for two days. On day three, the
151 control group received a single acute intranasal (i.n.) administration of PBS, while the LPS group
152 received an acute i.n. administration of LPS (1.5 mg/mL, dissolved in sterile PBS) (Kouki et al.,
153 2023). The injected volume was calculated based on rat body weight using the previously
154 described formula ($\mu\text{L/g}$) (Khadangi et al., 2021). For the juglone groups, the animals received
155 juglone (3 mg/kg dissolved in 1% DMSO) via the i.p. route for two days (Ahmad et al., 2022),
156 followed on day three by the acute administration of PBS (i.n.) for the juglone group, and the
157 acute administration of LPS (i.n.) for the juglone + LPS group. Rats in the PiB group received
158 PiB (3 mg/kg dissolved in 1% DMSO) via the i.p. route for two days under the same conditions,
159 followed by acute i.n. administration of PBS for the PiB group or LPS for the PiB + LPS group
160 (Fig. 1). The dose of both inhibitors was 3 mg/kg and the choice of administration route was
161 based on preliminary experiments and previous studies (Ahmad et al., 2022) to determine the
162 specificity of each component. Additionally, we calculated the doses of PiB and juglone for each
163 rat using the formula described (Erhirhie et al., 2014; 5 mL/kg). Approval for the release of all

164 experiments was granted by the ethical committee of the Tunisian Association of Laboratory
165 Animal Science (approval number No. 0127/2022 ATSAL), in accordance with the NIH
166 guidelines for the care and use of laboratory animals and the recommendations of the European
167 Convention (2010) for the protection and use of vertebrate animals.

168

169 **2.3. Sample collection**

170 Twenty-four hours after intranasal LPS administration, the rats in all groups were quickly
171 sacrificed (Holson, 1992). Their blood was collected in heparinised tubes and immediately
172 centrifuged using a refrigerated centrifuge (Eppendorf 5415 R). The resulting plasma was
173 transferred to sterile Eppendorf tubes and stored at -80°C for biochemical analysis, determination
174 of oxidative stress parameters and measurement of inflammatory markers. Minor modifications
175 were made to the bronchoalveolar lavage (BAL) process based on the techniques published by
176 Dhlamini et al. (2022) and Khadangi et al. (2021). This process involved carefully removing
177 both lungs, ligating the tracheas to preserve the anatomical structure of the lungs, and washing
178 the tissues with ice-cold saline. In brief, 300 µL of PBS solution was injected into the left lung,
179 collected in a sterile syringe and rinsed twice to obtain a total fluid volume of 535 ± 10 µL. The
180 obtained liquid was then centrifuged at 290 g and 4 °C for 10 minutes. The resulting supernatant
181 was recovered and stored at -25 °C for subsequent analysis. Additionally, each lung tissue
182 sample was divided into three parts: two parts were used for histological analysis and oedema
183 index assessment, and the remaining part was stored at -25 °C for examination of pulmonary
184 oxidative markers.

185

186 **2.4. Assessment of pulmonary oedema**

187 The presence of pulmonary oedema was determined by measuring the relative weight of
188 the lungs using the formula described by El-Alfy et al. (2012). Freshly prepared tissues from all

189 groups were weighed initially, then dried at 80°C until their weight remained unchanged. This
190 enabled us to calculate the wet-to-dry weight ratio, which provides an indication of vascular
191 permeability and water content in pulmonary tissues. The right lung tissue was excised and
192 homogenised in cold PBS (0.01 M, pH 7.4) using an Ultra-Turrax homogeniser (IKA T10 basic)
193 on ice. The pulmonary homogenate samples were then centrifuged at 7,247 g for 20 minutes at 4
194 °C in a refrigerated centrifuge (5415 R). The pellet was then removed and the resulting
195 supernatant was divided into Eppendorf tubes and stored at -25°C for further analysis.

196

197 **2.5. Protein vascular leakage and lung permeability index**

198 To assess vascular leakage in pulmonary tissues, total protein concentrations in BAL and
199 lung homogenate were measured according to the Lowry method (Lowry et al., 1951), and the
200 results were expressed as milligrams per milliliter (mg/mL). Additionally, the permeability index
201 was calculated by determining the ratio of total protein concentrations in BAL and plasma (Xu et
202 al., 2014).

203

204 **2.6. Enzymatic and non-enzymatic pro-oxidant markers**

205

206 **2.6.1. Myeloperoxidase activity**

207 Neutrophil infiltration was measured by determining myeloperoxidase (MPO) activity
208 using the spectrophotometric method (Goldblum et al., 1985). Briefly, BAL samples were mixed
209 with 0.5% hexadecyltrimethylammonium bromide and sonicated. Following centrifugation at
210 12,000 g for 15 minutes at 4 °C, 50 µL of each supernatant was mixed with 300 µL of PBS and
211 0.167 mg/mL of orthodianisidine dichloride. The reaction was initiated by adding H₂ O₂
212 (0.0005%). MPO activity was determined by measuring the optical density (OD) at 460 nm over
213 10 minutes; enzymatic activity was then determined as one unit (U) of MPO consuming 1 nmol

214 of H₂ O₂ per minute. Data analysis was presented as a percentage of the MPO activity of the
215 group treated only with LPS (the LPS group).

216

217 **2.6.2. Nitric oxide content**

218 The nitric oxide content in BAL, diluted plasma and lung homogenate was evaluated using
219 the spectrophotometric method described by Güzel et al. (2007). This method is based on the use
220 of Griess reagent (1% sulfanilic acid prepared in 5% orthophosphoric acid and 0.1% N-(1-
221 naphthyl)ethylenediamine dissolved in water). In summary, 0.1 mL of each sample was mixed
222 with an equivalent volume of 800 mg/mL vanadium chloride solution in 1 N hydrochloric acid
223 and 0.1 mL of Griess reagent. The tubes were then incubated at room temperature until a pink
224 colour appeared. Absorbance was then measured at a wavelength of 540 nm. Sodium nitrite
225 (NaNO₂) was chosen to plot the calibration curve and calculate NO levels in each sample.
226 Results were expressed in mM/mg for lung homogenate and in mM/L for BAL and plasma
227 samples (Güzel et al., 2007).

228

229 **2.6.3. Malondialdehyde content**

230 We measured malondialdehyde (MDA) levels, the main indicator of lipid peroxidation, in
231 plasma, lung tissue and bronchoalveolar fluid using the colourimetric method described by Yagi
232 (1976). In brief, the samples were mixed in a solution containing 1% butylated hydroxytoluene
233 and 20% trichloroacetic acid (TCA), after which they were centrifuged at 1000 x g for 5 minutes
234 at 4°C. The resulting supernatant was mixed with a solution of 120 mM thiobarbituric acid
235 (TBA) and 0.5% hydrochloric acid, and the mixture was boiled in a water bath (Biobase XMTD-
236 704) at 80 °C for ten minutes. The supernatant was collected and the optical density was
237 determined at 532 nm using a spectrophotometer (Spectro UV-Vis Dual Beam UVS-2800,

238 Labomed, Inc., USA). MDA levels in each sample were determined using the molar extinction
239 coefficient (1.56×10^5 M/cm) (Yagi, 1976).

240

241 **2.6.4. Plasma advanced oxidation protein products**

242 Advanced oxidation protein products (AOPPs) levels in plasma were determined using the
243 method described by Witko-Sarsat et al. (1996). In brief, diluted plasma (5-fold dilution) was
244 vigorously mixed with potassium iodide (1.16 M/L) for six minutes. Then, acetic acid was added
245 to the mixture, which was then vortexed for two minutes. The optical density was then measured
246 at 340 and 490 nm using a spectrophotometer (Spectro UV-Vis Dual Beam UVS-2800, Labomed
247 Inc., USA). AOPP concentration was quantified using a standard curve generated under the same
248 conditions with 10 mM/L chloramine-T. The results were then multiplied by the dilution factor
249 and expressed in $\mu\text{M/L}$ of plasma (Witko-Sarsat et al., 1996).

250

251 **2.7. Enzymatic and non-enzymatic antioxidant markers**

252

253 **2.7.1. Superoxide dismutase activity**

254 The superoxide dismutase (SOD) content of lung homogenates was determined using the
255 spectrophotometric procedure described by Misra and Fridovich (1972), which relies on the
256 autoxidation of epinephrine. In brief, 10 μL of lung sample was incubated in 1970 μL of an
257 alkaline solution (Na_2CO_3 / NHCO_3 , pH = 10.2) containing equal volumes of bovine catalase
258 and epinephrine (10 μL). The absorbance was then measured at 480 nm for five minutes using a
259 spectrophotometer (Spectro UV-Vis Dual Beam UVS-2800, Labomed Inc., USA). The data are
260 expressed as U per minute per mg of tissue protein (Misra and Fridovich, 1972).

261

262 **2.7.2. Catalase activity**

263 Catalase activity was examined in lung tissue using the spectrophotometric method
264 described by Aebi (1984). The homogenised samples were mixed with a phosphate buffer
265 solution (50 mM, pH 7) and H₂ O₂ , after which the detoxification reaction was monitored at
266 240 nm using quartz cuvettes by measuring the decrease in absorbance over a period of three
267 minutes. The results were presented as mM of H₂ O₂ per mg of lung protein (Aebi, 1984).

268

269 **2.7.3. Glutathione peroxidase**

270 Glutathione peroxidase (GPx) activity was determined in plasma using the colorimetric
271 method described by Flohé and Günzler (1984). In brief, 0.1 mL of diluted plasma (3-fold
272 dilution) was added to 100 mM phosphate buffer solution and 4 mM glutathione (GSH), mixed
273 with 5 mM hydrogen peroxide, and incubated for one minute at room temperature. The sample
274 was then centrifuged for five minutes at 1500 g and the supernatant collected. Then, a volume of
275 0.1 mL of the supernatant was mixed with 0.25 mL of 5% TCA diluted in distilled water and
276 0.01 M 5,5'-dithio-bis-(2-nitrobenzoic acid) (DTNB) solution prepared in distilled water. The
277 absorbance was recorded at 412 nm every 15 seconds. The data were then multiplied by the
278 dilution factor of the samples and the results were presented as nmol of GSH per minute per mL
279 (Flohé and Günzler, 1984).

280

281 **2.7.4. Reduced glutathione contents**

282 This is a colorimetric method based on the reaction of GSH with DTNB. Briefly, 0.1 mL
283 of the sample was incubated with 0.4 mL of 10% TCA solution, after which the samples were
284 vortexed and centrifuged for five minutes at 805 g. An equal volume of the resulting supernatant
285 and DTNB solution was then added to a Tris buffer solution (400 mM, pH 8.9) and distilled
286 water. The mixture was incubated until a yellow colour appeared and the absorbance was
287 measured at 412 nm (Ellman, 1959; Yiğit et al., 2019).

288

289 **2.7.5. Thiols marker**

290 Plasma levels of thiol (-SH) markers were determined according to Hu's method (1994).
291 Briefly, 0.1 mL of each plasma sample was mixed with 250 mM phosphate buffer solution (pH
292 8.2) and 0.02 M Ethylenediaminetetraacetic acid (EDTA), and the OD₁ was then determined at
293 412 nm. Then, a volume of 100 µL of DTNB (10 mM) solution was added and the mixture
294 incubated for one hour before determining the OD again. The results were calculated from the
295 difference between OD₂ and OD₁ using the molar extinction coefficient ($13.6 \times 10^3 \text{ M}^{-1} \times$
296 cm^{-1}), and the data were presented as mM/mL (Hu, 1994).

297

298 **2. 8. Plasmatic Inflammatory and Biochemical Markers**

299 Kits for measuring C-RP (Reference, 41012), lactate dehydrogenase (Reference, 20012)
300 and alkaline phosphatase activity (Reference, 13026), provided by the Biomaghreb Society, were
301 used according to the manufacturer's instructions to determine plasmatic inflammatory markers.
302 Furthermore, iron and creatinine levels in plasma were measured using Biomaghreb kits
303 (references 20151 and 20061).

304

305 **2.9. Histological examination**

306 The lung tissue was collected and rinsed with an ice-cold solution before being preserved
307 in a 10% formalin preparation. The lung fragments were dehydrated using ethanol solutions of
308 various concentrations and embedded in paraffin to remove the water. We then stained the 5 µm
309 cutting section with haematoxylin and eosin, before observing it under a Carl Zeiss microscope
310 (Jena, Germany) for histopathological examination.

311

312 **2.10. Statistical analysis**

313 All data analyses are expressed as the mean \pm standard error of the mean (SEM), with six rats per
314 group. The results were obtained using GraphPad Prism 8 software (GraphPad Software, San
315 Diego, CA, USA). One-way analysis of variance (ANOVA) was used to calculate the variation
316 between groups, followed by a Tukey post-test for multiple comparisons between groups. P-
317 values of ≤ 0.05 were considered significant.

318

319 **3. Results**

320

321 **3.1. PiB and juglone are tolerated by rats**

322 Before studying the effect of PiB and juglone on lung inflammation, we assessed their
323 toxic effect on rats. Daily intraperitoneal injection of PiB or juglone at 3 mg/kg did not result in
324 death or significant changes in body weight (see Table 1). Furthermore, as shown in Table 1,
325 LPS administered by the i.n. route induced a downward trend in rat body weight, from $129.2 \pm$
326 4.2 g to 127.9 ± 4.1 g. Both PiB + LPS and juglone + LPS had no effect on body weight. These
327 results demonstrate that PiB and juglone are well tolerated in vivo at the tested doses, both in the
328 absence and presence of LPS.

329

330 **3.2. Macroscopic and microscopic evaluation of the effect of PiB and juglone on LPS-** 331 **induced lung damage**

332 As expected, intranasal administration of LPS to rats resulted in marked pulmonary
333 redness, accompanied by an accumulation of blood cells (pink arrow) and a haemorrhagic
334 trachea (blue arrow), compared to control rats (Fig. 2, left panels). This finding is consistent with
335 our previous observations (Kouki et al., 2023). Our results also show that PiB and juglone alone
336 had no visible effect on the lungs. Furthermore, histological examination revealed that control
337 animals had normal lung microstructures and healthy alveolar walls, with no signs of altered

338 pulmonary vascular tissue (X4 and X40 magnifications). Conversely, LPS administration by the
339 i.n. route (LPS group) revealed thickened alveoli (black arrows, X4 magnification) as a major
340 sign of lung oedema, accompanied by an altered lung microstructure. Erythrocyte accumulation
341 (yellow arrow) and intensive neutrophil and lymphocyte infiltration (red arrow) were observed in
342 the alveolar epithelial spaces at X40 and X100 magnifications. Furthermore, the lungs of the
343 LPS group showed erythrocyte aggregation (yellow arrow), neutrophil infiltration (red arrow),
344 inflammatory zones surrounding the alveoli (black circle) and thickened spaces between the
345 alveoli (yellow arrow) (X100 magnification). Interestingly, using PiB (the PiB + LPS group) or
346 juglone (the juglone + LPS group) reduced tracheal haemorrhaging and protected the lungs
347 against the redness caused by LPS (see Fig. 2). Furthermore, our findings revealed normal lung
348 microstructure at X4 magnification and an absence of thickened alveoli and erythrocyte
349 aggregation at X40 and X100 magnifications. These results suggest that PiB (3 mg/kg, i.p.) or
350 juglone (3 mg/kg, i.p.) reduced the infiltration of inflammatory cells into the airways and
351 alveolar epithelium (X40 and X100 magnifications).

352

353 **3.3. Effect of PiB and juglone on LPS-induced alteration of lung weight and functions**

354 LPS induced a significant increase in relative lung weight ($P < 0.05$) when administered
355 via the i.n. route (0.89 ± 0.03) compared with control rats (0.70 ± 0.04) (Fig. 3A). Our results
356 clearly demonstrate that pre-treating rats with PiB or juglone alone did not affect relative lung
357 weight compared to the control group. However, administering PiB (3 mg/kg, i.p.) before LPS
358 (the PiB + LPS group) or juglone (3 mg/kg, i.p.) before LPS (the juglone + LPS group)
359 significantly reduced the relative lung weight ($P < 0.05$) compared with the LPS group (Fig. 3A).
360 Furthermore, evaluation of the wet-to-dry weight ratio, reflecting oedema, showed that LPS
361 significantly increased ($P < 0.001$) lung oedema (5.50 ± 0.31) compared to the control group
362 (2.67 ± 0.37) (Fig. 3B). Therefore, treatment with PiB or juglone alone showed no signs of

363 oedema compared with the control group. However, juglone (i.p.) or PiB (i.p.) pre-treatment
364 before LPS (juglone + LPS group or PiB + LPS group), respectively, revealed a significant
365 decrease in wet weight and dry ratio.

366 Assessment of total proteins in BAL of rats demonstrated that LPS-induced a significant
367 increase ($P < 0.001$) in BAL protein levels (8.199 ± 0.73 mg/mL) compared to the control group
368 (3.317 ± 0.61 mg/mL) (Fig. 3C). Furthermore, the i.p. administration of PiB or juglone
369 significantly ($P < 0.001$) limited excessive protein release in BAL compared to the LPS group.
370 Additionally, our findings revealed that pre-treatment with juglone or PiB (without LPS) did not
371 impact total protein levels in the BAL compared to the control group (Fig. 3C). Intranasal
372 administration of LPS also significantly increased plasma protein levels ($P < 0.01$) compared to
373 the control group that did not receive LPS (Fig. 3D). Moreover, our data analysis showed that
374 pre-treatment with PiB (3 mg/kg, i.p.) or juglone (3 mg/kg, i.p.) alone did not affect plasma
375 protein levels compared with the control group. Furthermore, the administration of PiB (3
376 mg/kg, i.p.) or juglone (3 mg/kg, i.p.) before LPS significantly protected rats ($P < 0.05$) against
377 excessive plasma protein release compared with the LPS group treated with i.n. LPS alone.

378 One consequence of lung inflammation is the increase in lung permeability (Moldoveanu
379 et al., 2008). We thus assessed this parameter and found that, as expected, compared to the
380 control group, LPS-induced lung inflammation caused a significant increase in lung permeability
381 from 4.325 ± 0.796 to 6.721 ± 0.596 ($P < 0.05$) (Fig. 3E). Therefore, no variation was found in
382 the pulmonary permeability index between rats pre-treated with juglone alone or PiB alone and
383 the rats of the control group. Furthermore, data analysis revealed that the use of juglone or PiB
384 before LPS administration (juglone + LPS group and PiB + LPS group) significantly limits the
385 rise in lung permeability compared to the LPS group (Fig. 3E).

386

387 **3.4. PiB and juglone decrease protein and lipid oxidation and NO production during LPS-**
388 **induced lung inflammation**

389 LPS-induced lung inflammation in humans and animals is accompanied by oxidative
390 stress, resulting in protein oxidation and lipid peroxidation in the inflamed lung, which then
391 diffuses into the blood (Di Meo et al., 2016). Determining MDA levels in the lung and
392 bronchoalveolar fluid using the colorimetric method described by Yagi et al. (1976) showed that
393 rats given only LPS had significantly higher MDA levels in pulmonary homogenate ($P < 0.001$)
394 and BAL ($P < 0.01$) than the control group (Fig. 4A and 4B). Pre-treating the rats with PiB
395 significantly reduced MDA content in BAL ($P < 0.05$) and lung homogenate ($P < 0.01$).
396 Additionally, juglone pre-treatment exhibited potent preventive effects against elevated MDA
397 levels in bronchoalveolar fluid ($P < 0.01$) and pulmonary homogenate ($P < 0.001$) relative to
398 LPS-treated rats. Furthermore, our findings showed that pre-treatment with PiB or juglone (3
399 mg/kg, i.p.) did not affect the state of lipid peroxidation in either BAL or lung homogenate
400 compared to the control group.

401 The over-release of NO, a potent inflammatory marker, modulates oxidative stress and
402 inflammatory responses during respiratory disorders. Indeed, measurements of NO levels in lung
403 homogenate clearly demonstrated a significant increase ($P < 0.01$) in the LPS group (2.5 ± 0.22
404 mM/mg) compared to the control group (1.27 ± 0.13 mM/mg) (Fig. 4C). Additionally, our
405 findings showed that pre-treatments with either PiB or juglone alone did not alter NO levels in
406 lung homogenate compared to the control group. Furthermore, our data analysis revealed that
407 juglone provides strong protection against the LPS group. Furthermore, PiB (3 mg/kg, i.p.)
408 triggered a significant decrease ($P < 0.05$) in NO content in the lungs (1.35 ± 0.18 mM/mg)
409 during LPS-induced lung inflammation (Fig. 4C). Conversely, NO levels were dramatically
410 higher in the bronchoalveolar fluids of LPS-treated rats (12.06 ± 1.18 mM/L) than in the control
411 group (5.84 ± 0.89 mM/L) (Fig. 4D). Notably, our findings demonstrated that pre-treatments

412 with PiB (3 mg/kg, i.p.) or juglone (3 mg/kg, i.p.) provided potent protection against NO
413 increases compared to the LPS group.

414 Measurement of MDA content in rat plasma showed that LPS (i.n.) induced a significant
415 increase ($P < 0.001$) compared to the control group (Fig. 5A). Furthermore, pre-treatment with
416 either PiB (3 mg/kg, i.p.) or juglone (3 mg/kg, i.p.) alone did not affect MDA levels in plasma
417 compared to the control group. However, pre-treatment with juglone or PiB prior to LPS
418 administration significantly prevented an increase in plasma MDA levels compared to the LPS
419 group (Fig. 5A).

420 In addition to assessing lipid peroxidation, we also evaluated protein oxidation in the
421 plasma of rats. The results show that LPS induced an increase in plasmatic advanced oxidation
422 protein products (AOPPs) (Fig. 5B). The AOPPs level was $98.4 \pm 3.51 \mu\text{M/L}$ in the LPS group,
423 compared with $65.25 \pm 3.03 \mu\text{M/L}$ in the control group. Our data also showed that AOPPs levels
424 did not change significantly when rats were pre-treated with PiB (3 mg/kg, i.p.) or juglone (3
425 mg/kg, i.p.) alone, compared with the control group. However, juglone pre-treatment
426 significantly prevented the increase in AOPPs induced by intranasal LPS administration ($P <$
427 0.001). Furthermore, pre-treatment with PiB (PiB + LPS group) clearly reduced AOPPs levels (P
428 < 0.01) compared with LPS-treated rats.

429 Compared to the control group (Fig. 5C), All these results show that LPS-induced lung
430 inflammation in rats was accompanied by oxidative stress resulting in lipid and protein
431 oxidation, as well as an increase in NO levels, and that the two Pin1 inhibitors, juglone and PiB,
432 were able to protect rats in this model of lung inflammation.

433 During inflammatory responses, immune cells produce NO by activating nitric oxide
434 synthase (NOS) in the vascular and endothelial systems. Indeed, determining NO levels in
435 plasma shows that LPS resulted in a significant increase ($P < 0.001$) in this inflammatory
436 biomarker ($17.67 \pm 1.53 \mu\text{M/L}$) compared to the control group ($7.24 \pm 0.72 \mu\text{M/L}$). Importantly,

437 our findings showed that pre-treatment with juglone or PiB modulated the NO signalling
438 pathway, resulting in a significant decrease ($P < 0.01$) in plasma NO levels compared to the LPS
439 group. Furthermore, pre-treatment with PiB (3 mg/kg, i.p.) or juglone (3 mg/kg, i.p.) alone did
440 not affect plasmatic NO levels compared to the control group (Fig. 5C). These results show that
441 LPS-induced lung inflammation in rats is accompanied by oxidative stress, resulting in lipid and
442 protein oxidation, as well as increased NO levels. They also demonstrate that the two Pin1
443 inhibitors, juglone and PiB, can protect rats in this lung inflammation model.

444

445 **3.5. Pre-treatments with PiB and juglone protect rats against disruption to the** 446 **antioxidant status during acute lung inflammation.**

447 Superoxide dismutase catalyses the conversion of $O_2^{\bullet -}$ to H_2O_2 and oxygen (O_2).
448 Indeed, measuring the activity of this antioxidant enzyme revealed that LPS significantly
449 decreased SOD activity in lung tissue ($P < 0.001$) compared to the control group (Fig. 6A).
450 Furthermore, PiB (3 mg/kg, i.p.) or juglone (3 mg/kg, i.p.) did not affect this enzymatic activity
451 compared to the control group. The results clearly showed that pre-treatment with PiB ($P < 0.05$)
452 or juglone ($P < 0.01$) provided significant protection against decreased SOD activity in lung
453 homogenate compared to rats treated with LPS alone.

454 Determination of catalase activity in pulmonary homogenates (Fig. 6B) showed that the
455 LPS rat group exhibited a significant reduction ($P < 0.001$) in antioxidant enzymatic activity
456 compared to the control group. Furthermore, catalase activity was unaffected by PiB or juglone
457 alone. Our findings clearly demonstrated that PiB pre-treatment significantly restored catalase
458 activity compared to the LPS rat group ($P < 0.05$). Additionally, juglone significantly ($P < 0.001$)
459 protected rats against reduced catalase activity compared to the LPS group.

460 The results presented in Fig. 6C revealed that LPS (i.n.) significantly decreased reduced
461 GSH levels in lung homogenate ($P < 0.001$) and plasma ($P < 0.001$) (Fig. 7A) compared to the

462 control group. Furthermore, the presence of PiB or juglone did not affect GSH content in plasma
463 or lung homogenate compared to the control rat group. Interestingly, pre-treatment with PiB in
464 the PiB + LPS group provided significant protection ($P < 0.001$) against a decrease in GSH
465 levels in plasma ($P < 0.001$) and tissue homogenate ($P < 0.05$) compared with the LPS group.
466 Furthermore, juglone (3 mg/kg, i.p.) in the juglone + LPS group protected rats against variations
467 in GSH levels, increasing them in plasma ($P < 0.01$) and pulmonary homogenate ($P < 0.001$)
468 compared to the LPS group.

469 LPS-induced oxidative stress and inflammation may result from an increase in ROS
470 production, a decrease in antioxidant enzymes and agents, or both. Here, we demonstrate that
471 LPS treatment decreased the activity of GPx, a major antioxidant enzyme, from 0.12 ± 0.01
472 nmol/mL in the control group to 0.04 ± 0.01 nmol/mL in the LPS-treated group (Fig. 7B).
473 Furthermore, PiB or juglone administered alone did not affect this enzymatic activity. However,
474 pre-treatment with PiB or juglone significantly reversed the down-regulation of GPx activity (P
475 < 0.05 and $P < 0.01$, respectively) compared to the LPS group (Fig. 7B).

476 Total plasma thiols revealed a significant decrease ($P < 0.001$) in these compounds in the
477 LPS rat group compared to the control group. Furthermore, PiB (3 mg/kg, i.p.) or juglone (3
478 mg/kg, i.p.) did not affect this antioxidant marker compared to the control group. Our findings
479 showed that juglone pre-treatment significantly ($P < 0.001$) protected the (juglone + LPS) group
480 against the LPS-induced reduction in plasma thiol levels. Furthermore, pre-treatment with PiB
481 for the (PiB + LPS) group significantly prevented the reduction in total thiols caused by LPS
482 treatment (Fig. 7C).

483
484 **3.6. PiB and juglone modulate pro-inflammatory markers during LPS-induced acute lung**
485 **inflammation**

486 As shown in Fig. 8A, MPO enzymatic activity in LPS-treated rats increased significantly
487 ($P < 0.001$) by 68% compared to the control rat group. Furthermore, pre-treatment with PiB (PiB
488 group) or juglone (juglone group) did not affect MPO activity compared to the control group.
489 However, data analysis revealed that PiB pre-treatment prevented neutrophil infiltration of
490 bronchoalveolar fluid by significantly decreasing MPO activity ($P < 0.001$) compared to rats
491 treated with LPS alone. Moreover, our results clearly showed that juglone pre-treatment
492 significantly protected rats against LPS by reducing MPO activity by 65.53% compared to the
493 LPS-treated rat group (Fig. 8A).

494 Measurements of C-RP, a key biomarker of acute inflammation, showed that LPS (via the
495 i.n route) caused a significant increase ($P < 0.001$) in plasma C-RP levels, reaching 9.94 ± 0.90
496 mg/dL compared to 3.53 ± 0.29 mg/dL in the control group. However, rats treated with PiB or
497 juglone showed no statistically significant change in plasma C-RP levels compared to the control
498 group. Furthermore, our findings revealed that pre-treatment with juglone reduced plasmatic
499 CRP levels by 50% ($P < 0.001$) compared with LPS-treated rats. Animals in the PiB + LPS
500 group exhibited a significant reduction ($P < 0.01$) in plasma C-RP during LPS administration
501 (Fig. 8B).

502 Lactate dehydrogenase (LDH) activity is involved in converting pyruvate to lactate in rat
503 plasma. As shown in Fig. 8C, measurements of this activity revealed that LPS triggered a
504 significant increase in LDH activity ($P < 0.001$), raising it to 54.42 ± 5.805 U/L compared to the
505 control rat group (19.79 ± 1.799 U/L). Furthermore, administration of PiB (PiB group) and
506 juglone (juglone group) demonstrated non-significant variation in this enzymatic activity
507 compared to the healthy group. Furthermore, our results showed that juglone (3 mg/kg, i.p.) and
508 PiB (3 mg/kg, i.p.) prevented a significant ($P < 0.01$) increase in LDH activity during pulmonary
509 inflammation (Fig. 8C). Additionally, measurement of alkaline phosphatase (ALP) activity
510 revealed that LPS significantly increased this enzyme activity by 9.7 ± 0.56 U/L compared to the

511 control group. PiB (3 mg/kg) induced a significant decrease ($P < 0.05$) in ALP activity, reducing
512 it to 6.30 ± 0.74 U/L compared to rats treated only with LPS. Furthermore, pre-treatment with
513 juglone was found to significantly decrease ALP activity ($P < 0.001$) by 5.17 ± 0.82 U/L, as
514 compared to rats in the LPS group (Fig. 8D).

515 As shown in Fig. 8E, plasmatic iron measurements revealed a significant ($P < 0.001$)
516 increase in plasma iron content in LPS-treated rats (61.26 ± 8.41 $\mu\text{M/L}$) compared to the control
517 group (16.50 ± 6.57 $\mu\text{M/L}$). Furthermore, our data analysis showed that pre-treatment with PiB
518 or juglone did not affect plasma iron status compared to the control group. However, the
519 presence of PiB or juglone was found to significantly reduce plasma iron levels by 42.8% and
520 37.6%, respectively, compared to the LPS group.

521 Plasma creatinine is a useful biomarker for examining renal dysfunction during an
522 inflammatory response. Determining creatinine levels in rat plasma (Fig. 8F) showed that
523 treatment with LPS (i.n.) led to a significant increase in these biomarkers ($P < 0.001$) compared
524 to the control group. Furthermore, the use of PiB or juglone had no adverse effects on plasma
525 creatinine levels compared with the control group ($P < 0.05$). Additionally, our findings revealed
526 that PiB and juglone significantly inhibited the increase in plasma creatinine levels compared to
527 the LPS rat group ($P < 0.05$).

528

529 **4. Discussion**

530 Pulmonary inflammation is one of the most common lung diseases, causing significant
531 health issues worldwide. It is characterised by dysfunction of the lung epithelial barrier
532 (Carnesecchi et al., 2012; Liu et al., 2023), which is caused by the accumulation and activation
533 of inflammatory cells, such as neutrophils, macrophages, and eosinophils, in the alveolar spaces.
534 Pin1 is a key enzyme that controls the function of these immune cells (Shen et al., 2008). In this
535 study, we found that two Pin1 inhibitors protected rats from LPS-induced lung inflammation.

536 Lipopolysaccharide, a glycolipid found in Gram-negative bacteria, has been shown to
537 activate various oxidative and pro-inflammatory pathways (Gorąca and Józefowicz-Okonkwo,
538 2007; Rahman et al., 2006). During lung inflammation, LPS has been found to upregulate the
539 NADPH oxidase system via the i.n. route (Kouki et al., 2023). In vitro, LPS has been shown to
540 activate Pin1 and upregulate the NADPH oxidase system by phosphorylating Ser345 of the
541 p47phox subunit (Liu et al., 2019). Consequently, the excessive release of hydrogen peroxide
542 and MPO could contribute to lung injury (Hampton et al., 1998; Liu et al., 2019; Nauseef, 2007).
543 Furthermore, this free radical reacts with NO to induce lipid peroxidation and protein oxidation
544 (Chabot et al., 1998). Therefore, limiting the release of ROS and RNS is a promising approach
545 for modulating lung injury.

546 Pin1 knockout models can prevent endotoxin shock by modulating various inflammatory
547 targets and macrophage activity (Akiyama et al., 2011). However, genetic mutations can
548 exacerbate the inflammatory response (Alm et al., 2010). In this case, we investigated the
549 pharmacological application of PiB and juglone to control the oxidative and inflammatory
550 responses enhanced by LPS. In this study, we clearly demonstrated that the effects of LPS
551 resulted in haemorrhaging, reddening, pulmonary oedema and increased lung permeability.
552 These outcomes are attributed to substantial neutrophil infiltration into the epithelial layer and
553 increased vascular permeability (Moldoveanu et al., 2008). Interestingly, the use of juglone and
554 PiB was found to protect rats from pulmonary haemorrhage, reduce alveolar space thickening
555 and decrease pulmonary oedema and permeability index. These results are consistent with
556 previous studies which showed that juglone can significantly prevent pulmonary oedema (Hu et
557 al., 2020). Furthermore, PiB's protective effects could be attributed to its inhibitory effect on
558 neutrophil chemotaxis during inflammatory responses (Bedouhene et al., 2021).
559 Myeloperoxidase activity is a key indicator of immune cell infiltration into inflamed tissue
560 (Meyer et al., 2017). Here, we demonstrate that LPS induces a significant increase in this

561 enzymatic activity, which is consistent with our previous study (Kouki et al., 2023). However,
562 PiB and juglone exhibited a substantial protective effect against the increase in MPO activity,
563 confirming the effect of juglone in equine laminitis (Mouithys-Mickalad et al., 2021) and colitis
564 (Alizadeh Nobakht et al., 2023).

565 Alveolar cells and various inflammatory cells, such as neutrophils and macrophages, are
566 known to express NOS (Moncada et al., 1991), which can be stimulated by LPS to produce NO
567 (Li et al., 2014; Nathan et al., 1994). In the present study, we observed that LPS induced
568 substantial NO production in plasma, lung tissue and bronchoalveolar lavage, whereas rats
569 challenged with juglone and PiB exhibited a notable decrease in NO production. LPS can induce
570 over-expression of Pin1 in various diseases, thereby enhancing NO production (Cho et al., 2015;
571 Fagiani et al., 2021; Ruan et al., 2011). Additionally, pharmacological Pin1 inhibitors, such as
572 juglone, can limit NO production (Pektas et al., 2018). Conversely, decreased NO levels may be
573 associated with inhibition of the protein kinase B pathway (Ahmad et al., 2019; Van der Vliet et
574 al., 2000). Furthermore, increased NO disrupts the balance of oxidative stress (Wu et al., 2019).
575 In the present study, we demonstrated that LPS significantly increased plasmatic levels of
576 protein oxidation and lipid peroxidation in plasma, lung homogenate and bronchoalveolar lavage
577 fluid.

578 The increase in MDA content during respiratory inflammation (Chen et al., 2020; Dikmen
579 et al., 2021; Zhao and Du, 2020) is related to the overproduction of ROS, which alters membrane
580 function and reduces fluidity (Bedouhene et al., 2021; Dhlamini et al., 2022). Pre-treatment with
581 PiB and juglone was found to protect rats against increased AOPPs and significantly limit
582 plasmatic lipid peroxidation. This is consistent with Zhou et al.'s (2015) report of the protective
583 effects of both molecules on liver fibrosis through reducing lipid peroxidation.

584 Glutathione is an endogenous antioxidant which reduces ROS and RNS (Pastore and
585 Piemonte, 2012). The antioxidant profile in plasma and lung homogenate revealed that LPS

586 significantly reduced GSH content in both, as reported by Abdelmageed et al. (2016).
587 Furthermore, during ROS overproduction, the antioxidant enzyme GPx utilises GSH as a co-
588 substrate to reduce H_2O_2 (Zhao et al., 2019). Our results showed that LPS-challenged rats
589 presented a significant decrease in plasmatic GPx activity and total plasma thiols, which are
590 early clinical markers used to assess oxidative stress during lung disease (Zinellu et al., 2016).
591 Interestingly, we demonstrated that Pin1 inhibitors (PiB and juglone) have a potent preventive
592 effect against the reduction of these antioxidant markers. During redox homeostasis, the Nrf2
593 complex associates with Keap1 proteins to remain in an inactive state (Li et al., 2018). In our
594 study, we demonstrated that the administration of LPS dramatically inhibits SOD and catalase
595 activities, in line with our previous research (Kouki et al., 2023). Furthermore, Zhao and Du.
596 (2020) clearly demonstrated that LPS significantly decreases GSH content and the activities of
597 SOD and catalase. Indeed, LPS inhibits the Nrf2 signalling pathway (Shivling et al., 2023) by
598 activating the Pin1 target, thereby increasing p38 mitogen-activated protein kinase (MAPK)
599 expression and the ubiquitination of the Nrf2 target (Kim et al., 2010; Ozleyen et al., 2025; Zhao
600 et al., 2021). Conversely, juglone use upregulates the Nrf2 complex by activating different
601 antioxidant genes involved in controlling GPx, catalase and SOD activities, thereby restoring
602 redox balance (Chen et al., 2021). Moreover, in breast cancer, Pin1 activity inhibits the Nrf2
603 pathway by interacting with Nrf2 and Keap1 (Saeidi et al., 2022). Conversely, the use of PiB as a
604 selective Pin1 inhibitor (Yu et al., 2020) may indirectly contribute to Nrf2 activation by
605 inhibiting Pin1 isomerase activity and reducing the ubiquitination of Nrf2 targets. Interestingly,
606 the pharmacological use of Pin1 inhibitors in cases of pulmonary injury, and their potential role
607 in restoring SOD and catalase activities, can be explained by inhibiting the Pin1 target, thereby
608 reducing the ubiquitination of genes that encode antioxidant enzymes (Kim et al., 2010; Ozleyen
609 et al., 2025). This inhibition also down-regulates the MAPK signalling target and reduces ROS

610 production via the inhibition of NADPH oxidase in phagocyte cells. This, in turn, restores the
611 activity of antioxidant markers (Boussetta et al., 2024; Liu et al., 2019; Zhao et al., 2021).

612 Additionally, LDH enzyme activity is a marker of inflammation closely linked to acute
613 lung injury (McFadden et al., 1991). Our study indeed revealed a significant increase in this
614 activity in the LPS group. Furthermore, PiB and juglone were found to significantly prevent the
615 increase in LDH activity during acute lung inflammation, in agreement with the report of Song et
616 al. (2017). Furthermore, the putative protective effect of PiB was found to be associated with the
617 inhibition of Pin1 activity (Chen et al., 2018).

618 Administering LPS via the nasal route results in a significant increase in C-RP (Ekman et
619 al., 2009), which is positively correlated with LDH activity and aids the diagnosis of acute lung
620 disease (Nakatsu et al., 2016; Poggiali et al., 2020). Furthermore, pre-treatments with PiB or
621 juglone significantly lowered CRP levels, which can be explained by the inhibition of Pin1
622 activity. This is closely correlated with the excessive release of CRP during pneumonia
623 (Nechama et al., 2018; Xue et al., 2020). Furthermore, Pin1 knockout mice are protected from
624 non-alcoholic steatohepatitis and liver disease due to reduced release of NF- κ B-related pro-
625 inflammatory markers (Nakatsu et al., 2016).

626 Our results showed that LPS increased plasma iron levels (Xiong et al., 2004). PiB and
627 juglone exert significant protection against iron overload by down-regulating the NF- κ B pathway
628 (Atkinson et al., 2009). Our results also showed that LPS could alter creatine metabolism, a
629 finding supported by Gu et al. (2019). However, the presence of PiB and juglone limits creatine
630 metabolic disorders. Our data analysis was supported by literature reporting the protective effects
631 of Pin1 inhibitors against Nrf2/HO-1-dependent increases in creatinine levels (Yu et al., 2022).
632 Administering only Pin1 inhibitors (juglone and PiB) at a dose of 3 mg/kg to rats helped protect
633 them from harmful nitrogen and oxygen-derived free radicals, reduced MPO activity and
634 normalised various biochemical and inflammatory markers. These results suggest that

635 researchers could use these molecules to screen the effects on the control of inflammatory
636 cytokines and chemokines during lung inflammation.

637 Despite the biological characteristics identified in the present study, the anti-inflammatory
638 properties of PiB and juglone remain largely unexplored. This is due to the insufficient volume
639 of samples from the LPS rodent model (Kohman et al., 2010). Furthermore, genetic variability
640 between humans and rodents in pulmonary disease (Alm et al., 2010) and the presence of
641 signalling targets dependent on the timing of sample collection (Domscheit et al., 2020)
642 complicate matters. These factors include the infiltration of neutrophil cells into the
643 inflammatory area (Bozinovski et al., 2004). This encourages researchers to use sheep or pig
644 models, which are known for their high sensitivity to LPS and great genetic similarity to humans
645 (Kohman et al., 2010; Wyns et al., 2015), to clarify the pharmacodynamic properties of these
646 molecules, localise Pin1 in lung tissue and investigate their roles in respiratory bursts using
647 isolated inflammatory cells from rats and patients with respiratory diseases.

648

649 **5. Conclusion**

650 The present study clearly demonstrated that pre-treatment with juglone and PiB protected
651 rats against deterioration of the lung microstructure during LPS-induced acute lung
652 inflammation. Our findings revealed that juglone and PiB protect against oxidative stress and
653 increased inflammatory markers by modulating the target of NOS, decreasing inflammatory cell
654 infiltration and restoring antioxidant enzyme activity. Using juglone and PiB could therefore be
655 an effective way to stabilise oxidative targets and modulate inflammatory responses during acute
656 lung inflammation.

657 **CRedit authorship contribution statement**

658 **Ahmed kouki**: Conceptualization, Methodology, Data curation and Writing – original draft,
659 **Abdelaziz Souli**: Investigation, Validation, Resources, Writing – review and editing, **Salwa**
660 **Bouabdallah**: Conceptualization, Data curation, Resources and Validation, **Wafa Ferjani**: Data
661 curation, formal analysis and Validation, **Pham My-Chan Dang**: Investigation, Resources,
662 Writing – review and editing, **Mossadok Ben-Attia**: Project administration, Supervision,
663 Writing – original draft, Writing – review and editing, **Jamel El-Benna**: Project administration,
664 Supervision, Funding acquisition, Writing – original draft, Writing – review and editing. All
665 authors revised and approved the submitted paper.

666

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670

671 **Data availability**

672 The data analysis produced during this research has been included in the submitted draft.
673 For additional information, please contact the corresponding author.

674

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679

680 **Ethics statement**

681 All experiments were conducted in accordance with the recommendations of the NIH
682 Guide for the Care and Use of Laboratory Animals and were approved by the Ethics Committee
683 of the Tunisian Association for Laboratory Animal Science (approval number 0127/2022
684 ATSAL).

685

686 **Conflict of Interest**

687 All authors declared the absence of any conflicts of interest.

688

689 **Consent to Participate**

690 Not applicable

691

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1046 **Figure legends**

1047

1048 **Fig. 1.** Schematic diagram of the protocol used to investigate the impact of pre-treatments with
1049 PiB and juglone on acute pulmonary inflammation induced by the intranasal administration of
1050 lipopolysaccharide (1.5 mg/mL).

1051

1052 **Fig. 2.** The effects of pre-treatment with PiB and juglone on macroscopic and histological lung
1053 changes in a rat model of acute pulmonary inflammation were investigated. The rats received
1054 PiB (3 mg/kg, intraperitoneally) and juglone (3 mg/kg, intraperitoneally) for two days, followed
1055 by intranasal administration of LPS (1.5 mg/mL) for 24 hours. Histological slides are presented
1056 at different magnifications: X4 (scale bar = 100 μ m), X10 (scale bar = 50 μ m), X40 (scale bar =
1057 10 μ m) and X100 (scale bar = 5 μ m). The blue arrow indicates haemorrhagic trachea, the pink
1058 arrow indicates severe inflammation with accumulation of blood cells, the green arrow indicates
1059 healthy trachea, the black arrow indicates lung oedema, the yellow arrow indicates erythrocyte
1060 aggregation, the red arrow indicates the presence of neutrophils and other inflammatory cells, the
1061 black circle indicates an inflammatory zone and the yellow star indicates thickened spaces
1062 between alveoli.

1063

1064 **Fig. 3.** Effects of pre-treatment with PiB and Juglone on pulmonary oedema, vascular damage,
1065 and lung permeability. Rats were treated with PiB (3 mg/kg, i.p.) and juglone (3 mg/kg, i.p.) for
1066 two days before being treated with LPS (1.5 mg/mL, i.n.) for 24 hours. Relative lung weights
1067 (A), pulmonary oedema (B), bronchoalveolar lavage (C), plasma protein contents (D), and lung
1068 permeability index (E) were assessed. Results are expressed as mean \pm SEM of six rats in each

1069 group with * P < 0.05, ** P < 0.01, *** P < 0.001 compared to the control group; # P < 0.05, ##
1070 P < 0.01, and ### P < 0.001 compared to the LPS group.

1071
1072 **Fig. 4.** The effects of pre-treatment with PiB and juglone on pulmonary oedema, vascular
1073 damage and lung permeability were investigated. Rats were treated with PiB (3 mg/kg,
1074 intraperitoneally) and juglone (3 mg/kg, intraperitoneally) for two days, after which they were
1075 treated with LPS (1.5 mg/mL, intranasally) for 24 hours. Relative lung weights (A), pulmonary
1076 oedema (B), bronchoalveolar lavage (C), plasma protein content (D) and lung permeability index
1077 (E) were assessed. The results are expressed as the mean \pm SEM for six rats in each group, with
1078 * indicating P < 0.05, ** indicating P < 0.01 and *** indicating P < 0.001 compared to the
1079 control group, and # indicating P < 0.05, ## indicating P < 0.01 and ### indicating P < 0.001
1080 compared to the LPS group.

1081
1082 **Fig. 5.** The effects of PiB and juglone on plasma pro-oxidant biomarkers during acute lung
1083 inflammation were investigated. Rats were treated with PiB (3 mg/kg, intraperitoneally) and
1084 juglone (3 mg/kg, intraperitoneally) for two days, after which they were treated with LPS (1.5
1085 mg/mL, intranasally) for 24 hours. Plasma levels of MDA (A), AOPPs (B) and NO (C) were
1086 determined. The results are presented as the mean \pm SEM for six rats in each group, with ***
1087 indicating P < 0.001 compared to the control group, and ## indicating P < 0.01 and ###
1088 indicating P < 0.001 compared to the LPS group.

1089
1090 **Fig. 6.** The effects of pre-treatments with PiB and juglone on antioxidant biomarkers in lung
1091 homogenate. Rats were treated with PiB (3 mg/kg, intraperitoneally) and juglone (3 mg/kg,
1092 intraperitoneally) for two days, followed by intranasal administration of LPS (1.5 mg/mL).

1093 Superoxide dismutase (A), catalase (B) activity and glutathione (GSH) content (C) were then
1094 measured in the lung homogenate. The results are presented as the mean \pm SEM for six rats in
1095 each group. *** indicates $P < 0.001$ compared to the control group, # indicates $P < 0.05$, and ##
1096 indicates $P < 0.01$ and ### indicates $P < 0.001$ compared to the LPS group.

1097

1098 **Fig. 7.** The effects of pre-treatments with PiB and juglone on plasma antioxidant biomarkers
1099 during acute lung inflammation induced by intranasal LPS administration were investigated.
1100 Rats were treated with PiB (3 mg/kg, intraperitoneally) and juglone (3 mg/kg, intraperitoneally)
1101 for two days, after which they received an intranasal administration of LPS (1.5 mg/mL).
1102 Glutathione (GSH) content (A), glutathione peroxidase (B) and total thiols (C) in rat plasma
1103 were then measured. The results are presented as the mean \pm SEM for six rats in each group. ***
1104 indicates $P < 0.001$ compared to the control group, # indicates $P < 0.05$, and ## indicates $P <$
1105 0.01 and ### indicates $P < 0.001$ compared to the LPS group.

1106

1107 **Fig. 8.** The effects of pre-treatment with PiB and juglone on inflammatory biomarkers during
1108 acute lung inflammation induced by intranasal LPS administration were investigated. Rats
1109 received PiB (3 mg/kg, intraperitoneally) and juglone (3 mg/kg, intraperitoneally) for two days
1110 before intranasal administration of LPS (1.5 mg/mL). Then, C-reactive protein levels (A),
1111 myeloperoxidase activity (B), lactate dehydrogenase (C), alkaline phosphatase activity (D), iron
1112 content (E) and creatinine levels (F) were determined in rat plasma. The results are expressed as
1113 the mean \pm SEM for six rats in each group. *** indicates $P < 0.001$ compared to the control
1114 group, # indicates $P < 0.05$, ## indicates $P < 0.01$ and ### indicates $P < 0.001$ compared to the
1115 LPS group.

Table 1. Assessment of rat daily body weight (g) during pre-treatment with PiB (3 mg/kg, i.p.) or Juglone (3 mg/kg, i.p.), and the acute pulmonary inflammation induced by intranasal administration of lipopolysaccharide (1.5 mg/mL, i.n.)

Groups		Control	LPS	juglone	juglone + LPS	PiB	PiB + LPS
Number of rats/group (n)		6	6	6	6	6	6
	Day 1	115.5 ± 1.49	125.9 ± 4.09	124.6 ± 2.56	123.8 ± 1.32	127.0 ± 1.08	120.2 ± 1.33
Pre-treatment phase	Day 2	120.2 ± 1.68	129.2 ± 4.21	124.7 ± 2.27	124.1 ± 3.02	126.2 ± 1.20	123.6 ± 2.07
Induced phase	Day 3	125.9 ± 1.70	127.9 ± 4.12	124.2 ± 0.70	127.8 ± 1.18	125.5 ± 1.27	126.3 ± 1.57

Results are expressed as mean ± SEM of 6 rats per group.