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PHYTOCHEMICAL ANALYSIS, ANTIOXIDANT, ANTI-INFLAMMATORY AND IMMUNOMODULATORY EFFECTS OF METHANOLIC EXTRACT OF *JUNIPERUS PHOENICEA*

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ABSTRACT. This work aims to evaluate the antioxidant, anti-inflammatory and immunomodulatory effects of *Juniperus phoenicea* extract. Polyphenol and flavonoid contents were estimated using colorimetric methods. The antioxidant potential was assessed using 2, 2-diphenyl-l-picrylhy- drazyl (DPPH) radical scavenging, iron chelation, and anti-hemolytic assays. Croton oil-induced ear edema and carrageenan-induced paw edema models were used to evaluate the anti-inflammatory activity. Furthermore, the hemagglutination test was used to assess the immunomodulatory activity. The total polyphenol and flavonoid contents in this extract were 138.15 ± 11.87 µg GAE/mg and 27.5 ± 1.2 µg QE/mg of dry extract, respectively. The methanolic extract exhibited a strong scavenging activity against DPPH radical (IC_{50} = 0.051 ± 0.002 mg/mL), while in iron chelation it presented low activity (EC_{50} = 3.61 ± 0.46 mg/mL) compared to the standard EDTA. The results showed that *J. phoenicea* extract exhibited high protection of erythrocytes in 2, 2′,-azobis (2 amidinopropane) dihydrochloride (AAPH)-induced hemolysis. Treatment with *J. phoenicea* extract (200 and 400 mg/kg) exhibited significant anti-edematogenic in both inflammatory models induced by croton oil and carrageenan. However, these doses did not show any significant increase/decrease on the antibody titer. This study indicates that *J. phoenicea* extract possesses antioxidant and anti-inflammatory effects, confirming the use of this plant in folk medicine.

Keywords: *DPPH, flavonoids, hemagglutination, iron chelation, Juniperus phoenicea, polyphenols.*

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INTRODUCTION

Oxidative stress (OS) arises from an imbalance between the synthesis of reactive oxygen species (ROS) and the capacity of the biological system's ability to neutralize them [1]. ROS are reactive molecules that include both radicals, which have a single unpaired electron, such as the superoxide radical anion ($O_2^{\bullet-}$), and non-radical species such as hydrogen peroxide (H_2O_2) [2]. Excessive production of ROS causes lipid peroxidation as well as DNA and protein damage [3]. Moreover, it has been demonstrated that an excess of ROS is known to promote the production of pro-inflammatory cytokines, transcription factors, and chemokines, ultimately leading to tissue damage [4].

Inflammation is a complex reaction to injury, infection, or tissue damage. It is often associated with pain, fever, swelling, and redness. Moreover, it can cause physiological dysfunction [5]. In this context, acute inflammation is a swift biological response that plays a crucial role in wound healing, however, chronic inflammation is a prolonged, long-lasting condition that arises in diseases such as rheumatoid arthritis, osteoarthritis, and cancer [6].

The immune system plays a crucial role in defending the body against harmful microorganisms. Once the immune system is stimulated, it responds promptly by synthesizing a range of cytokines, chemokines, and inflammatory mediators [7]. Immunomodulators are substances that regulate the immune system, by either enhancing or suppressing its activity [8]. They are essential for managing inflammatory and immune-related diseases, which result from immune system disorders [9].

Although a wide range of anti-inflammatory and immunomodulatory drugs are available, their prolonged use is often restricted by severe adverse effects and high costs, which pose a challenge to effective disease management. Consequently, research is actively underway to find suitable alternatives to these conventional treatments [10].The significance of herbal remedies for human health is attracting more attention, generating increased interest in plant-based traditional medicine. Scientists and researchers are exploring the antioxidant properties of plants as they have the potential to reduce oxidative damage that may trigger multiple diseases [11]. Plants serve as an immense source of therapeutic and pharmaceutical substances due to their abundance of bioactive compounds with medicinal properties [12]. *J. phoenicea* is a native tree of the Cupressaceae family, found in the Mediterranean basin. It predominantly grows in the mountains of Algeria [13]. This plant has traditionally been used to treat rheumatism, diabetes, urinary tract diseases, and bronchopulmonary conditions, as well as to enhance appetite [14].

Most studies on Algerian *J. phoenicea* focus on its antioxidant and anti-inflammatory activities *in vitro*. For instance, research by Ghouti et al. [13] and Zemmouli et al. [15] indicates that both aqueous and hydroethanolic *J. phoenicea* extracts exhibit antioxidant and anti-inflammatory properties. However, no available reports exist on its immunomodulatory effects or *in vivo* anti-inflammatory activity.

Therefore, the present study aims to assess the antioxidant, anti-inflammatory, and immunomodulatory effects of the methanolic extract of the aerial part of *J. phoenicea.*

RESULTS

Characterization of polyphenols by HPLC

Figure 1 represents the chromatograms of *J. phoenicea* extract and standards. Multiple compounds were identified in the methanolic extract of *J. phoenicea* which contained high concentrations of Gallic acid (5206.22 µg/g), hesperetin (4972.69 µg/g), catechin (4215.88 µg/g) and chlorogenic acid (3201.39 µg/g). However the kaempferol and ellagic acid were absent in this extract or not detectable. The Phytoconstituents concentrations in *J. phoenicea* extract are presented in the table 1.

Table 1. The Phytoconstituents concentrations of *J. phoenicea* extract.

Polyphenols	**Extract** (Concentration (µg/g))	**Standard** (Concentration (µg/ml))
Gallic acid	5206.22	302.26
Chlorogenic acid	3201.39	435.51
Catechin	4215.88	386.88
Methyl gallate	49.16	337.23
Caffeic acid	194.71	267.82
Syringic acid	353.64	302.37
Rutin	1297.54	367.40
Ellagic acid	ND	1016.95
Coumaric acid	593.03	681.46
Vanillin	162.99	411.03
Ferulic acid	1438.14	417.77
Naringenin	285.85	386.62
Rosmarinic acid	63.82	566.02
Daidzein	124.22	378.43
Querectin	13.42	299.16
Cinnamic acid	15.22	653.28
Kaempferol	ND	339.31
Hesperetin	4972.69	489.55

ND, non detected

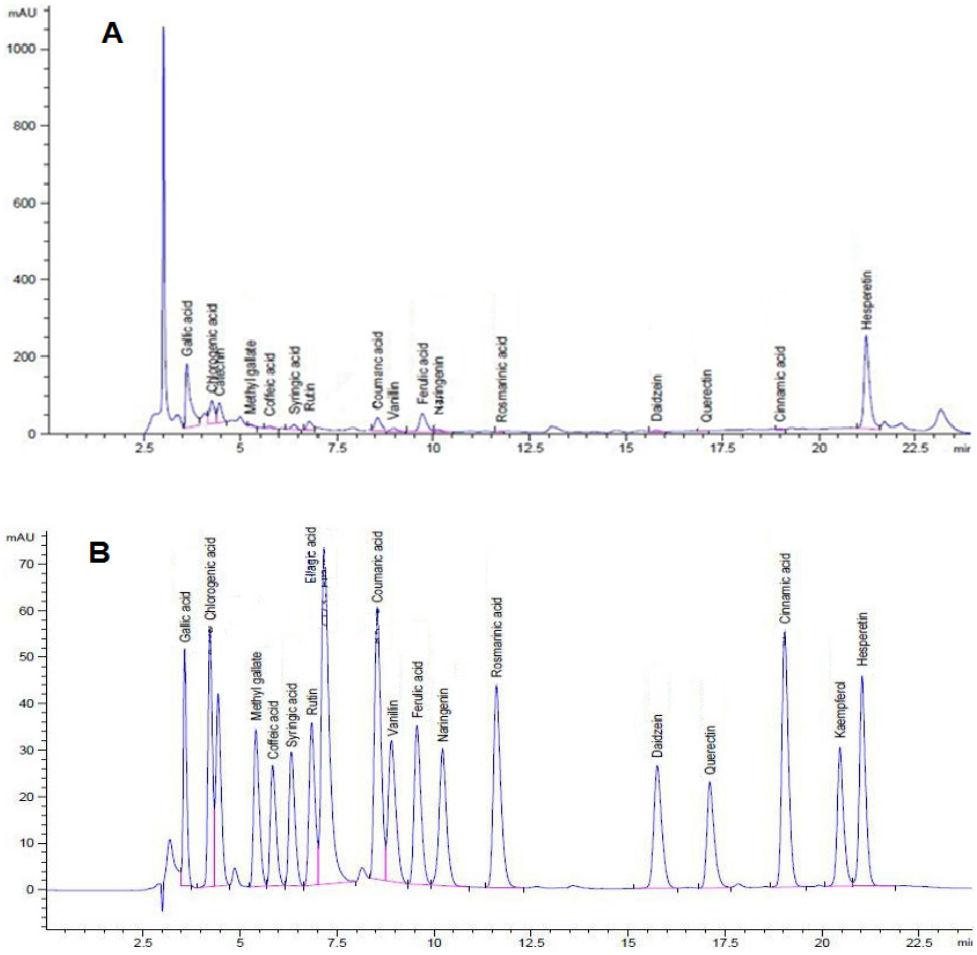

Figure 1. HPLC chromatograms; **A,** *J. phoenicea* extract;
B, Polyphenols standards.

Total polyphenol and flavonoid contents

The methanol extract of *J. phoenicea* produced a yield of 24.73%. The total polyphenol content was 138.15 ± 11.87 µg GAE/mg of dry extract, while the flavonoid content was 27.50 ± 1.20 µg QE/mg of dry extract.

Antioxidant activity

DPPH radical scavenging activity

DPPH free radical scavenging activity was determined by the IC_{50}values (IC_{50} represents the concentration of the sample required to inhibit 50% of the DPPH free radicals). The methanol extract of *J. phoenicea* demonstrated strong free radical scavenging activity with an IC_{50} value of 0.051 ± 0.002 mg/mL, in comparison to the synthetic antioxidant BHT (IC_{50} = 0.047 ± 0.0022 mg/mL).

Ferrous iron chelating effect

The capacity to chelate ferrous ions was expressed as the mean of the EC_{50} values (EC_{50} represents the effective concentration that chelates 50% of Fe^{2+}). The methanol extract of *J. phoenicea* showed an EC_{50} value of 3.61 ± 0.46 mg/mL. For comparison, EDTA showed an EC_{50} value of 3 ± 0.53 µg/mL.

Anti-hemolytic activity

The methanolic extract of *J. phoenicea* exhibited a strong ability to protect the erythrocyte membrane against hemolysis ($p < 0.001$) with HT_{50} = 74.93 ± 4.78 min compared to the negative control (AAPH) which reduced the hemolysis half-time (HT_{50} = 32.73 ± 1.83 min). Vitamin C served as standard (50 µg/mL) demonstrated an HT_{50} = 70.90 ± 6.43 min (Figure 2).

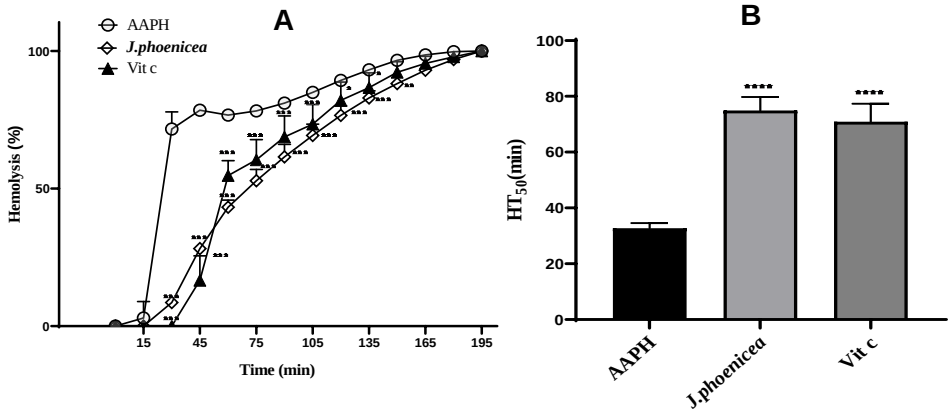

Figure 2. The kinetics of *J. phoenicea* extract and vitamin C action on AAPH-induced hemolysis of red blood cells (RBCs), (**A**). Half-Hemolysis Time (HT_{50}) for the analyzed compounds (**B**). Vit C: vitamin C. Values are presented as the mean ± SD (n=4). *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001 when compared with the control.

Acute toxicity study

The oral administration of the methanolic extract of *J. phoenicea* (2000 and 5000 mg/kg) in mice demonstrated no signs of toxicity or mortality during the 14 days of the experiment. For the evaluation of the biological activities two doses were selected (200 and 400 mg/kg body weight).

Anti-inflammatory activity

Croton oil-induced ear edema in mice

Treating mice with the methanolic extracts of *J. phoenicea* (200 and 400 mg/kg) in croton oil-induced ear edema resulted in a significant reduction of edema ($p < 0.001$) after 6 hours (0.062 ± 0.009 mm, 0.026 ± 0.005 mm, respectively) compared to the control mice group, which showed an increase in the thickness of the right ear (0.138 ± 0.015 mm). Diclofenac at 50 mg/kg produced a significant reduction ($p < 0.001$) of edema (0.022 ± 0.007 mm) in comparison to the control group (Figure 3).

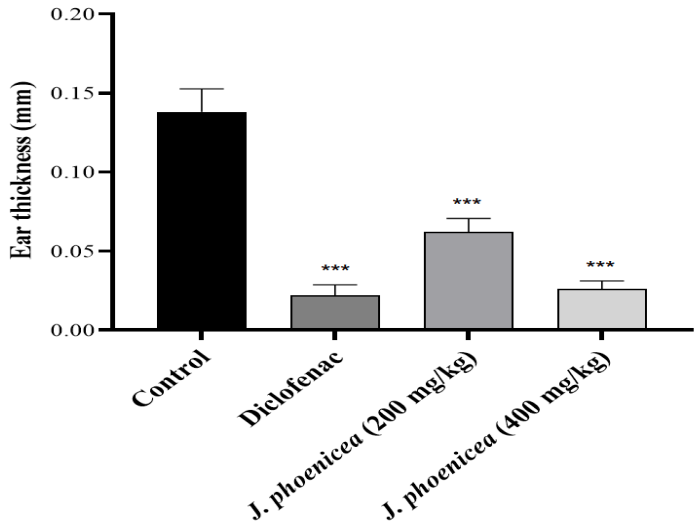

Figure 3. Effect of *J. phoenicea* extract on croton oil-induced ear edema in mice. Values are presented as the mean ± SEM (n=5), ***p < 0.001 when compared with the control group.

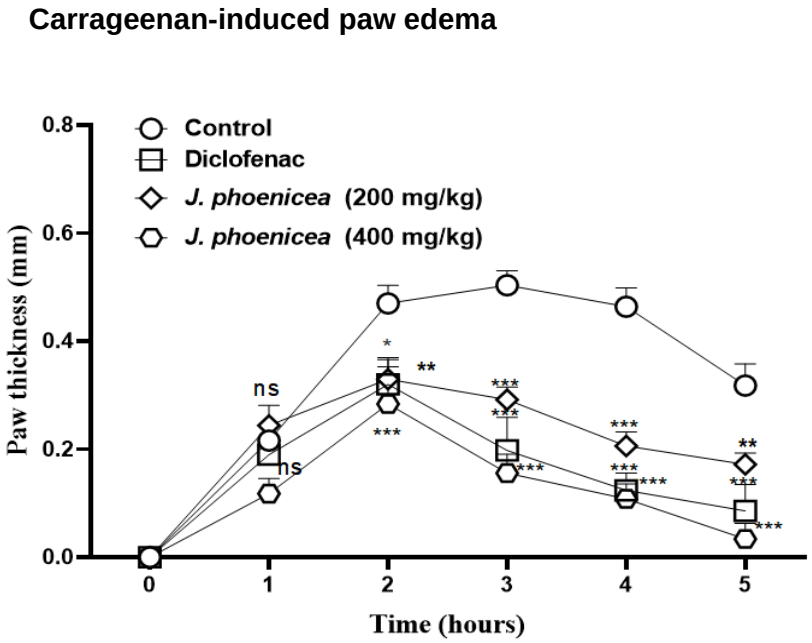

Figure 4. Effect of *J. phoenicea* extract on carrageenan-induced paw edema in mice. Values are presented as the mean ± SEM (n=5). ns: non-significant, *p < 0.05, **p < 0.01, ***p < 0.001 when compared with the control group.

Figure 4 represents the effect of *J. phoenicea* extract on carrageenan-induced paw edema in mice. The mice in the control group that received only the carrageenan solution showed an increase in paw thickness during the 1st, 2nd, and 3rd hour (0.216 ± 0.016 mm, 0.47 ± 0.033 mm, and 0.504 ± 0.027 mm, respectively), after the 3rd hour, which represents the peak of inflammation, a gradual decrease was observed (0.318 ± 0.040 in the 5th h). The group treated with *J. Phoenicea* extract at a dose of 200 mg/kg decreased paw thickness from the 2nd to the 5th h (0.33 ± 0.036 mm and 0.172 ± 0.021 mm, respectively), in comparison to the control group. The results demonstrated that the highest dose of the extract (400 mg/kg) was significantly ($p < 0.001$) more effective in reducing paw volume from the 2nd to the 5th hour (0.284 ± 0.069 mm and 0.034 ± 0.029 mm, respectively), relative to the control group. Diclofenac resulted in a significant reduction in paw edema from the 2nd to the 5th hour (0.32 ± 0.05 mm and 0.086 ± 0.049 mm, respectively), in comparison to the control group.

Humoral antibody response

Figure 5 represents the effect of *J. phoenicea* extract on antibody titer in mice. The results did not show any significant increase in the antibody titer when the methanolic extract of *J. phoenicea* was orally administered at doses of 200 and 400 mg/kg.

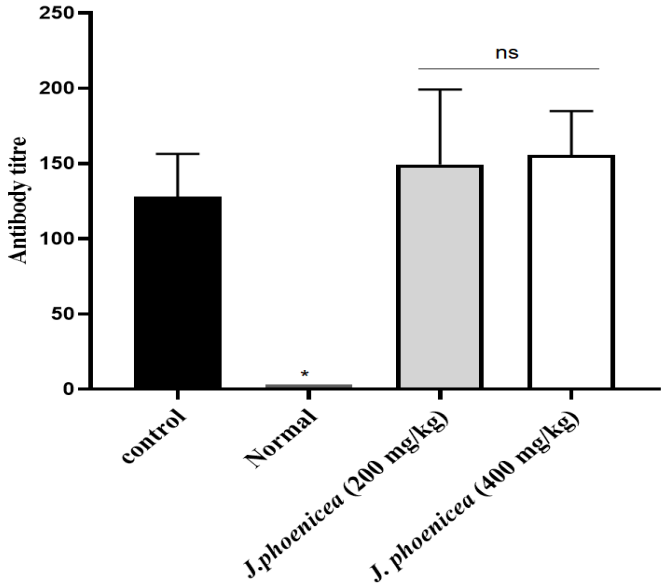

Figure 5. The effect of *J. phoenicea* extract on antibody titer in mice. Values are presented as the mean ± SEM (n = 6). ns: non-significant, *p < 0.05 when compared with the control group.

DISCUSSION

The use of natural antioxidants holds potential for the prevention of inflammation, cancer, diabetes and many other diseases [16]. Medicinal plants have demonstrated their importance as a primary source of biologically active compounds, or phytochemicals [17]. The bioactive compounds found in medicinal plants are predominantly polyphenols. The total phenolic and flavonoid contents of *J. phoenicea* were 138.15 ± 11.87 µg GAE/mg and

27.50 ± 1.20 µg QE/mg, respectively. The results obtained exceeded those reported by Bouassida et al. [18], who estimated the total phenolic and flavonoid contents of the hydroethanolic extract of *J. phoenicea* leaves from Boulifa northwestern part of Tunisia as follows 70.30 ± 0.2 mg GAE/g and 11.33 ± 0.05 mg QE/g, respectively. However, Keskes et al. [19] reported that the hexane and methanol extracts of the leaves of *J. phoenicea* in mid-west Tunisia contained higher phenolic and flavonoid contents than the results of the present study (265 ± 5.8 mg GAE/g, 176 ± 0.52 mg QE/g for the methanol extract and 162.3 ± 3.2 mg GAE/g, 96 ± 0.48 mg QE/g for the hexane extract, respectively). Furthermore, The study carried out by Zemmouli et al. [15] indicated that the aqueous extract of *J. phoenicea* leaves, collected from Biskra province in Algeria and obtained via decoction, reported total phenolic and flavonoid contents of 374.36 ± 0.1 mg GAE/g, 174.02 ± 2.79 mg QE/g, respectively. The amount and composition of phenolic compounds can be variable due to several factors such as the nature of the solvent, polarity, concentration of solvent and type of extraction [20], the season of the collection, the choice of parts and geographic origin [21].

The antioxidants that scavenge radicals to inhibit the initiation of the chain reaction and break the propagation phase are effective as primary antioxidants. However, the secondary antioxidants inhibit the formation of radicals [22]. In this context, two methods are used to assess primary and secondary antioxidant activity, the DPPH scavenging and the iron chelation tests, respectively.

The DPPH radical scavenging assay is intended to measure the ability of the extracts to neutralize the free radical 2,2'-diphenyl-1-picryl hydrazyl (DPPH) by donation of an electron or a hydrogen atom [19]. The ability of the plant extracts to scavenge DPPH is measured by the discoloration of DPPH, from purple to yellow color due to the formation of diphenyl picryl hydrazine. In this study, the methanolic extract of *J. phoenicea* exhibited high antioxidant activity (IC_{50}: 0.051 ± 0.0019 mg/mL). The IC_{50} of the methanolic extract was lower than those reported by Ghouti et al. [13] and Bouassida et al. [18], who assessed the ability of the Algerian Sahara *J. phoenicea* leaves hydroethanolic extract to scavenge the stable radical DPPH (IC_{50}: 12 ± 1 µg/mL), as well as Tunisian *J. phoenicea* leaves hydroethanolic extract (IC_{50}: 12.22 ± 0.02 µg/mL). Previous study have demonstrated a strong correlation between the content of polyphenols and the DPPH scavenging effect [23]. The antioxidant properties of polyphenols are attributed to their low redox potential and their ability to donate multiple electrons or hydrogen atoms [24]. A study conducted by Choi et al. [25] indicates that hesperetin, a flavonoid present in *J. phoenicea* extract demonstrated a strong DPPH free radical-scavenging effect.

Iron and other transition metals promote oxidation through their role as catalysts in radical reactions [26]. Chelating agents, known to form σ-bonds with metals, have been reported to work as secondary antioxidants because they stabilize the oxidized state of the metal ion [27]. In this study the results showed that the methanolic extract of *J. phoenicea* exhibited chelating properties and can bind ferrous ions before ferrozine, with an EC_{50} value of 3.614 ± 0.46 mg/mL. This extract showed a more marked chelating capacity than the results obtained by Taviano et al. [22] who evaluated the ability of Turkey *J. communis* and *J. oxycedrus* methanolic extracts to chelate ferrous ions (EC_{50}= 33.10 ± 0.4 mg/mL and EC_{50} = 6.82 ± 1.15 mg/mL, respectively). According to Taviano, Marino (22) and our findings, there is no correlation between the ferrous ion chelating ability of the extract and their polyphenol and flavonoid concentrations. This may suggest the presence of other compounds, including non-phenolic antioxidants responsible for metal chelation [28].

Erythrocytes hemolysis is commonly utilized as a model for studying oxidative damage to cell membranes induced by free radicals [29]. Peroxyl radicals are produced through the thermal degradation of AAPH, a water-soluble azo-based compound, under oxygen-rich condition [30]. These radicals target the membranes of erythrocytes to trigger lipid peroxidation and lead to hemolysis [29]. The methanolic extract of *J. phoenicea* exhibited strong anti-hemolytic activity by prolonging the duration required for 50% hemolysis of RBCs. These results are in agreement with those of Zemmouli et al. [15] which showed that the aqueous extract of Algerian *J. phoenicea* leaves collected from Biskra exhibited high protection of erythrocytes. Anti-hemolytic effect of *J. phoenicea* extract can be due to the different phenolic compounds which neutralize free radicals by their antioxidant properties and enhance erythrocytes' resistance to oxidative stress [31]. Furthermore, polyphenols bind to membrane phospholipids and shield them against lipid degradation [32]. Catechin, a type of flavonoid found in *J. phoenicea* extract has previously exhibited strong protection against AAPH-induced damage, according to Grzesik et al. [33].

Acute or chronic inflammation stimulates the production of inflammatory mediators such as tumor necrosis factor-α (TNF-α), prostaglandin E2 (PGE2), and nitric oxide (NO). Excessive inflammation can contribute to the development of diseases, including atherosclerosis, cardiovascular disease, and immune dysfunction [34].

In this study, croton oil and carrageenan-induced inflammatory models are used to evaluate the anti-inflammatory activity of our extract. The topical application of croton oil induces an inflammatory response. Tetradecanoyl-phorbol acetate (TPA) represents the primary irritant in croton oil which activates protein kinase C (PKC), phospholipase A2, lipoxygenase enzyme and induces the expression of COX-2, and pro-inflammatory cytokines [35]. The oral

administration of *J. phoenicea* at doses of 200 and 400 mg/kg exhibited a strong reduction of ear edema. This reduction may be due to chlorogenic acid and hesperetin identified in the *J. phoenicea* extract. Chlorogenic acid participates in modulating enzyme activity such as lipoxygenase and cycloxygenase [35]. As for hesperetin that inhibits the activity of the COX-1 and COX-2 reactions [36].

The carrageenan-induced paw edema serves as a model for acute inflammation biphasic [37]. The initial phase is mediated by serotonins, kinins, and histamines and the later phase is characterized by overproduction of prostaglandin [38]. Our results show that the methanolic extract of *J. phoenicea* at the doses of 200 and 400 mg/kg exhibited a strong reduction of paw edema in the later phase. These results are in agreement with those of Lafraxo et al. [39] which showed that the hydroethanolic extract of *J. phoenicea* leaves from Morocco reduced carrageenan-induced paw edema in rats. The anti-inflammatory effect can be due to Gallic acid and hesperetin which represent the major components of *J. phoenicea* extract [39 ,25].In this context, previous studies showed that hesperetin reduced the levels of NO and PGE2 [25] and gallic acid has been demonstrated to exhibit potent anti-inflammatory effects by targeting the MAPK (mitogen-activated protein kinase) and NF-κB (nuclear factor kappa B) signaling pathways [34].

Immunomodulators are a class of drugs that modify the activity of the immune system, either increasing or reducing its normal functions [40].The immune system can be regulated by various plant extracts. In this regard, the effect of *J. phoenicea* extract on humoral response was tested by hemagglutination test. This response is mediated by antibody molecules secreted by plasma cells [41]. The oral administration of both doses of *J. phoenicea* did not show any significant increase or decrease in the antibody titer. However, a previous study by Ali et al. [42], which determined the effect of the methanolic extract of *J. squamata* (100 and 200 mg/kg) on cell-mediated immunity (Delayed type hypersensitivity (DHT)), showed a significant change in DTH response.

CONCLUSIONS

The findings of the current study demonstrated that the methanolic extract of *J. phoenicea* exhibited strong *in vitro* antioxidant and *in vivo* anti-inflammatory effects. Nevertheless, no significant increase in the antibody titer was observed with this extract. These results suggest that the plant extract analyzed could serve as a potential natural alternative for treating inflammatory diseases.

EXPERIMENTAL SECTION

Chemicals, reagents and equipment

Folin-Ciocalteu reagent, sodium carbonate, gallic acid, trichloride aluminum, quercetin, 2,2'-diphenyl-1-picryl-hydrazyl (DPPH), butylated hydroxytoluene (BHT), methanol, ferrozine, $FeCl_2$, ethylene diamine tetraacetic (EDTA), 2, 2′,-azobis (2 amidinopropane) dihydrochloride (AAPH), phosphate-buffered saline (PBS), acetone , croton oil, carrageenan, NaCl and Alsever's solution were used in this study. All chemicals and reagents were of analytical grade.The extract was concentrated using a rotary evaporator (BÜCHI, Germany). Spectrophotometric measurements were performed using a DRAWELL (DV-8000) spectrophotometer. The AAPH-induced oxidative hemolysis was monitored at 630 nm using a 96-well microplate reader (ELX 800, BioTek Instruments, Winooski, VT, USA).

Plant material

J. phoenicea was collected from the region of Ouled Tebben (Setif), northeast of Algeria (35° 48′ 46″ north, 5° 06′ 05″ east), in July 2023 and identified by Pr. Hocine Laouer (Laboratory of Valorization of Natural and Biological Resources, Setif 1 Ferhat Abbas University, Algeria). A voucher specimen was deposited in the Department of Vegetal Biology and Ecology (DVBE0054:2023). The aerial part of *J. phoenicea* was washed and air-dried at room temperature in the dark. The dried material was then ground to powder using an electric grinder.

Preparation of extract

The methanolic extract of *J. Phoenicea* was prepared using the method described by Arrar et al. [43]. The powder of the aerial part (600g) was extracted with 6 L of absolute methanol in a sealed vessel at room temperature for 7 then 5 days. The crude extract was obtained by filtering the extract through Whatman filter paper then evaporated using a rotary evaporator at 45°C. It was desiccated until dryness in an oven. Finally, the extract was stored at 4°C until use.

Animals

Adult female NMRI mice weighing 25–30 g obtained from Pasteur Institute of Algeria (Algiers) were utilized for the study. Before the experiments, the animals were kept in clean plastic cages for 7 days in normal laboratory conditions (temperature, 20 – 22°C; relative humidity, 50 – 70 %, and 12/12 h light/ dark cycle). The animal experiments adhered to the guidelines and procedural details outlined in the Guide for the Care and use of Laboratory Animals (NIH Publication No. 86-23, 1985). Permission for experimental use was obtained from the Laboratory of Applied Biochemistry, Setif 1 Ferhat Abbas University.

Characterization of polyphenols by HPLC

Chemical analysis of the aerial part extract from *J. phoenicea* was performed by high-performance liquid chromatography (HPLC), which was conducted using an Agilent 1260 series. A Zorbax Eclipse Plus C8 column (4.6 mm x 250 mm i.d., 5 µm) was employed for the separation of constituents. A mixture of water (A) and 0.05% trifluoroacetic acid in acetonitrile (B) with a flow rate of 0.9 mL/min served as the mobile phase, which was applied in a linear gradient progression, as follows: 0 min (82% A); 0–1 min (82% A); 1-11 min (75% A); 11-18 min (60% A); 18-22 min (82% A); 22-24 min (82% A). The eluted samples and standards were monitored using a multi-wavelength detector at 280 nm. The injection volume was 5 µL and the column temperature was kept at 40°C.

Determination of total polyphenol and flavonoid contents

The total phenolic content of the methanolic extract was estimated using the Folin-Ciocalteu method according to Amraoui et al. [44]. Briefly, 0.1 mL of methanolic extract of *J. phoenicea* or standard was added to 0.5 mL of Folin-Ciocalteu reagent diluted to 1/10 (v/v) and incubated for 4 minutes at room temperature, then 0.4 mL of sodium carbonate (7.5%) solution was added and the mixture was incubated again at room temperature for 90 min in the dark. Then the absorbance was measured at 765 nm. A calibration curve was created using Gallic acid and the total polyphenol concentration was determined and expressed as micrograms of Gallic acid equivalent per milligram dry weight of extract (µg GAE/mg DW).

The concentration of flavonoid in the methanolic extract of *J. phoenicea* was quantified using aluminum chloride reagent, using the method described by Amraoui et al. [44]. A volume of 0.5 mL of the sample or standard was mixed with 0.5 mL of trichloride aluminum solution (2% in methanol). The mixture was incubated at room temperature in the dark for 10 min then the absorbance was read at 430 nm. The flavonoid content was expressed as microgram of quercetin equivalents per milligram dry weight of extract (µg QE/mg).

Antioxidant activity

DPPH radical scavenging activity

The DPPH (2,2'-diphenyl-1-picryl-hydrazyl) radical scavenging assay was evaluated according to Amraoui et al. [45], by mixing 50 µL of the extract at various concentrations with 1250 µL of a 0.004% DPPH solution (in methanol). The mixture was incubated at room temperature in the dark for 30 min then the absorbance was measured at 517 nm. Butylated hydroxytoluene (BHT) served as the standard. The DPPH radical scavenging activity was evaluated using the following equation:

$$I\ \% = 100 \times (Abs\ control - Abs\ sample)/Abs\ control \quad (1)$$

where: Abs control refers to the absorbance of the DPPH solution, Abs sample represents the absorbance in the presence of the extract or standard. IC_{50} is the concentration of an antioxidant-containing substance required to scavenge 50% of the initial DPPH radicals. The lower the IC_{50} value, the more potent is the substance at scavenging DPPH and this implies a higher antioxidant activity.

Ferrous iron chelation assay

The ferrous iron chelation assay of *J. phoenicea* extract was evaluated by the ferrozine method described by Guemmaz et al. [46]. A volume of 250 µL of different concentrations of extract was mixed with 50µL of $FeCl_2$ (0.6 Mm in H_2O) and 450 µL of methanol. After incubation at room temperature for 5 min, a volume of 50 µL ferrozine (5 Mm) was added to the mixture, which was then incubated again for 10 min at room temperature. The absorbance of the solution was read at 562 nm and EDTA (Ethylene diamine tetraacetic) served as the standard. The chelating effect of samples was calculated using the following equation:

$$Chelating\ power\ (\%) = 100\ x\ (Ac- As)/ Ac \quad (2)$$

where: Ac: absorbance of the control (absence of chelator), As: absorbance in the presence of extract or EDTA.

Anti-hemolytic activity

The anti-hemolytic activity of *J. phoenicea* extract was evaluated using the method described by Guemmaz et al. [47]. In this test 2, 2′,-azobis (2 amidinopropane) dihydrochloride (AAPH) was used as a free radical generator, which damages the membranes of red blood cells (RBCs) and induces hemolysis. Mice blood was collected in tubes EDTA centrifuged and washed with phosphate-buffered saline (PBS; 10 mM, pH 7.4) three times. Blood cells were adjusted to a concentration of 2%. 80 µL of 2% erythrocyte suspension (in PBS) was added to 20 µL of extract or standard in a microplate then a volume of 136 µL of AAPH dissolved in PBS (300 mM) was added to the mixture. After incubation at 37°C, the absorbance was read at 630 nm, using a 96-well microplate reader. Erythrocytes resistance to radical attack was indicated by the time needed for the lysis of 50% of the erythrocytes (half Hemolysis Time in min: HT_{50}). Vitamin C was used as a standard. The negative control consists of RBCs and AAPH.

Acute toxicity evaluation

The acute oral toxicity of the methanolic extract of *J. phoenicea* was assessed according to the Organization for Economic Corporation and Development (OECD 425) guidelines [48], with slight modifications. The mice were divided into three groups of three each. Group 1 represents the negative control (received only distilled water) and two groups received *J. phoenicea* extract (2000 and 5000 mg/kg body weight), through oral administration. The mice were observed for 14 days for Indicators of toxicity such as changes in general behavior, body weight changes and mortality.

Anti-inflammatory activity

Croton oil-induced ear edema

Croton oil-induced mice ear edema was carried out using the method described by Amraoui et al. [49]. The mice were grouped into four sets of five each; group 1 was used as a negative control which was treated with an aqueous solution, group 2 represented the positive control (treated with Diclofenac (50 mg/ kg)) and two groups were treated with the methanolic extract of *J. phoenicea* (200 and 400 mg/kg). Ear edema was induced by applying 20 µL of a solution containing 80 µg of croton oil prepared in acetone-water solution (1:1 V/V) to the inner surface of each mouse's right ear one hour after oral administration of the extract or Diclofenac. The thickness of the right ear was measured using a digital caliper before and six hours after the croton oil application.

Carrageenan-induced paw edema

Carrageenan-induced paw edema assay was used to evaluate the anti-inflammatory activity of *J. phoenicea* extract according to the method of Amraoui et al. [45]. The mice were divided into four groups of five each. Group 1 represents the negative control which was treated with an aqueous solution, group 2 was used as a positive control (treated with Diclofenac 50 mg/ kg), and two groups were treated with the *J. phoenicea* extract (200 and 400 mg/kg). Mice were injected with 0.02 mL of carrageenan suspension (1% in NaCl 0.9%) at the subplantar region in the right paw of each mouse one hour after oral administration of the extract or Diclofenac. The thickness of the paws was measured using a digital caliper before and after carrageenan application at 1, 2, 3, 4, and 5 h.

Humoral antibody response

The humoral response to sheep red blood cells (SRBC) was assessed using the hemagglutination test according to the method described by Aichour et al. [50], with slight modifications. Sheep red blood cells (SRBC)

were mixed with Alsever's solution, washed two times with 0.9% NaCl and centrifuged each time. The red blood cells were adjusted to a concentration of 10^9 cells/mL. The mice were divided into four groups of six each. Group1 represents the healthy control (non-immune), group 2 was used as a negative control, both were treated with an aqueous solution, and two groups were treated with the *J. phoenicea* extract (200 and 400 mg/kg) on days -3, -2, -1, 0, 1, 2 and 3. Groups 2, 3, and 4 were immunized intraperitoneally (i.p) on day 0 with 10^9 cells/mL SRBC. Blood samples were collected from each mouse on day 8. A volume of 50 µL of serum samples was diluted two fold in microtitration plates and mixed with 50 µL of SRBC suspension (1%), then incubated for one hour. The antibody titer represents the final dilution of serum samples that caused hemagglutination.

Statistical analysis

The data are presented as the mean of triplicates ± SD for *in vitro* experiments, whereas *in vivo* results are expressed as the mean ± SEM. Statistical differences were analyzed using ANOVA, followed by Dunnett's test for multiple comparisons, utilizing GraphPad Prism software (version 8.0). A significance threshold of $p \leq 0.05$ was applied.

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Conflict of Interest
The authors declare no competing interests.

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