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In vivo assessment of anti-inflammatory and antioxidant activities of *Phlomis crinita* polyphenols

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ABSTRACT: The leaves of *Phlomis crinita* are traditionally used in Algerian medicine for the treatment of pain and inflammatory conditions. In order to find a potential application for this native species, the anti-inflammatory and antioxidant effects were investigated on various *in vivo* experimental models, and the total phenolic compounds and flavonoid contents were determined. The carrageenan-induced paw edema method was used to evaluate the anti-inflammatory activity of the extract *in vivo*, while the *in vivo* antioxidant effect was assessed by estimating oxidative stress parameters (MDA, CAT, and SOD). Phytochemical screening revealed the presence of substances with high therapeutic values. *In vivo* anti-inflammatory studies show that plant extract has a significant and dose-dependent impact on the inhibition of edema formation. The maximum percentage inhibition value was 87.79% after 4 h at a concentration of 500 mg/kg. Moreover, the administration of the extract significantly enhanced the activities of antioxidant enzymes in the livers of mice. It significantly ($p < 0.05$) increased CAT and SOD activities and significantly ($p < 0.05$) decreased the MDA level activity, compared to the control inflammatory group. Our findings support that *Phlomis crinita* can be considered as a promising source of therapeutic bioactive compounds.

Keywords: *Phlomis crinita*; Phenolic content; Edema; Stress.

1. INTRODUCTION

The overproduction of reactive oxygen species beyond the antioxidant capacities of biological systems results in oxidative stress that is involved in the occurrence of several diseases, including inflammation. The latter is a defense reaction of the body to the penetration of an infectious agent, antigen or cellular damage. It

is a fundamental biological process and the most common sign of disease [1]. Thus, inflammation and oxidative stress are physiopathological events closely related to a number of chronic diseases, including diabetes, hypertension, cardiovascular diseases, neurodegenerative diseases, cancer, and aging. There is excessive production of free radicals and activation of phagocytes in many inflammatory disorders [2]. Therefore, antioxidant and anti-inflammatory drugs that can reduce inflammation and oxidative stress are required.

Anti-inflammatory therapy is generally conducted by synthetic molecules such as non-steroidal or steroidal anti-inflammatory drugs [3]. However, despite their widespread use, their therapeutic effectiveness appears to be limited because they are frequently linked to severe unfavorable side effects, such as hypertension, gastrointestinal discomfort, ulcers, metabolic disorders, bone marrow depression, etc [4]. As a result, the development of new and safer anti-inflammatory drugs is constantly required. Therefore, to overcome their toxicity, the development of new anti-inflammatory molecules is still needed and natural products such as medicinal plants could potentially serve as a precursor in the production of new drugs to treat inflammation with reduced or no side effects [5, 6]. As a result of their involvement in the physiological processes of living flora, plant components are more compatible with the human body [7].

Lamiaceae is a well-known source of bioactive molecules with strong anti-inflammatory potential. According to The Plant List [8], the genus *Phlomis* L. (*Lamiaceae*) contains more than 100 extant species which are primarily found in the Mediterranean region, Central Asia, and China [9]. *Phlomis* plants have been mentioned as herbal remedies and used in traditional medicine for the treatment of various conditions such as diabetes, gastric ulcer, hemorrhoids, inflammation, and the healing of wounds [10]. These activities are attributed to the fact that aerial parts of *Phlomis* contain a variety of secondary bioactive compounds, including phenylpropanoids, iridoids, diterpenoids, phenylethanoids, alkaloids [11] and phenolic compounds dominated by flavonoids [12], which are considered to be effective as anti-inflammatory and antioxidant agents.

To the best of our knowledge, there is no report studying the *in vivo* activities of *Phlomis crinita* polyphenols. Therefore, the current study was designed to investigate the *in vivo* anti-inflammatory activity of *Phlomis crinita*. Moreover, considering that antioxidants and free radical scavengers can exert an anti-inflammatory effect, the extract was also evaluated for *in vivo* antioxidant activity.

2. MATERIALS AND METHODS

2.1. Polyphenols extraction

The plant leaves were collected during the month of April 2019 in the Ouled Rabeh region (Jijel, Algeria). Fresh *Phlomis crinita* leaves were washed with running water to remove dust and other particles, then dried for a few days in the oven at a relatively stable temperature of (25 °C) until a fixed weight (dry matter) was obtained. The dried plant material was then ground with an electric grinder into a fine powder to obtain a homogeneous granular structure.

50 g of the ground plant material was macerated in 500 ml of methanol/water mixture (80/20: V/V) under magnetic agitation and at room temperature. This maceration is repeated three times successively, with renewal of the solvent every 24 hours. The hydro-alcoholic macerate obtained was subjected to double filtration on Whatman N°1 filter paper. The resulting filtrate was concentrated at the rotary evaporator (Heidolph, LABOROT 4003) at 40°C. After drying in the oven (45°C) for 24 hours, the extract obtained was used for phytochemical and biological tests [13].

2.2. Total phenolic content determination

Total phenol content of the extract was determined using the method of Othman et al. [14]. A volume of

200 µL of the plant extract was added to 1500 µL (1/10 dilution) of the Folin reagent. The mixture was kept at room temperature for 5 min, and then 1500 µL of 7.5% Na_2CO_3 solution was added to promote an alkaline medium to start the redox reaction. The final reaction mixture was then agitated and incubated in the dark for 1 hour at room temperature. The absorbance was determined at 750 nm against a blank containing all the reagents except the extract. A calibration curve is performed in parallel under the same operating conditions using gallic acid. Total phenol contents were expressed as mg Gallic Acid Equivalents (GAE) per gram of crude extract (CE).

2.3. Total flavonoid content determination

The flavonoid content in extracts was determined according to [15]. 1.5 ml of diluted sample or standard were mixed with an equal volume of 2% $AlCl_3$. After incubation at room temperature for 30 min, the yellow color of the mixture was measured at 430 nm. Under the same conditions and in the same way, the absorbances of series of standard solutions of quercetin were measured. The total flavonoid concentration, expressed in mg quercetin equivalent (EQ)/g extract, is calculated from the calibration line.

2.4. Animals

Swiss albino mice from both sex (25 to 30 g), obtained from Pasteur Institute, were used for the present study. The animals were kept at room temperature (25 ± 2 °C) with a 12 hour light/dark cycle and free access to food and water. The animals were acclimatized to the laboratory environment for one week prior to the experiment. The animal experiment was approved by the Institutional Animal Ethical Committee of Bejaia University, Algeria (Approval N°: 03/C.E.D/UB/2023) and was performed in compliance with the Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes.

2.5. Acute toxicity test

To evaluate the acute toxicity of *P. crinita*, five groups of healthy mice (six per group) fasted for 12 h prior to the experiment and were orally administered the extract at a dose of 1–5 g/kg body weight; the control group received 10 mL/kg distilled water. Animals were observed for 1 hour continuously, then hourly for 4 hours and finally after 24 h for any toxic manifestations or mortality. Subsequent observations were made for a further week for any signs of delayed toxicity.

2.6. Anti-inflammatory activity

Carrageenan-induced paw inflammation was generated according to the method described by Winter et al. [16]. Animals were fasted overnight and divided randomly into separate groups of six and treated orally in the following manner:

Group I: administered distilled water (normal control).

Group II: administered distilled water (inflammatory control).

Group III: administered 100 mg/kg bw (body weight) *Phlomis crinita* extract.

Group IV: administered 250 mg/kg bw *Phlomis crinita* extract.

Group V: administered 500 mg/kg bw *Phlomis crinita* extract.

Group VI: administered 50 mg/kg bw diclofenac sodium (standard).

Paw edema was induced by injecting 0.1 mL of 1% carrageenan in saline subcutaneously into the plantar region of the right hind paw after 1 h of administration of the respective drug treatment to each group. The left hind paw volumes were measured with a calibrated digital thickness gauge (Shanghai, China) at hourly intervals up to 4 h.

The inhibition percentage of each group was calculated as follows:

$$\% \text{ Inhibition } = \frac{(P_t - P_0)}{P_0}.100 \qquad \text{Eqn. 1}$$

Where:

P_t: paw volume after carrageenan injection.

P_0: paw volume before carrageenan injection.

2.7. Preparation of mice liver cytosolic fraction

Mice liver cytosolic fraction was obtained according to the method described by Iqbal et al. [17]. At the end of the experimental period, all animals were sacrificed for the determination of the antioxidant status. 1 gram of liver was homogenized with 3 volumes of phosphate buffer (0.1 M, pH = 7.4) containing 1.17% KCl using a Dounce Bucky. First centrifugation of the homogenate at 800 rpm for 15 min at 4°C, eliminates nuclear debris and the resultant supernatant in turn is centrifuged for 45 min at 9600 rpm and 4°C. This recovers the final supernatant containing cytosolic enzymes. Using BSA as a reference, the protein content of the supernatants was assessed using Bradford's method [18].

2.8. Lipid peroxidation assay

Malondialdehyde (MDA) levels were estimated by the method of Okhawa et al. [19]. The method's basic idea is to measure the color produced by the reaction of thiobarbituric acid (TBA) and MDA using spectrophotometry. For this purpose, one gram of frozen liver was washed with NaCl (0.9%) then cut and homogenized with three volumes of 1.15% KCl using a DOUNCE crusher. Then 0.5 ml of the homogenate was added 0.5 ml of TCA 20% and 1 ml of TBA (0.67%). The mixture was heated to 100°C for 15 min, cooled with tap water to stop the reaction then 4 ml of n-butanol were added and the mixture was centrifuged for 15 min at 3000 rpm. At 532 nm, the supernatant's optical density was measured. Using the same assay method and a known quantity of 1,1,3,3-tetraethoxypropane, a standard curve was produced. MDA was measured in mol/g of tissue.

2.9. Extract effect on catalase activity (CAT)

The activity of catalase was determined using the Clairborne method [20]. The principle is based on the disappearance of H_2O_2 in the presence of the enzymatic source at 25°C. For the assay, to a quartz cuvette, substrate solution consisting 1 ml phosphate buffer (0.1 M, pH 7.4), and 0.025 ml of the enzyme source was prepared. The reaction was initiated by adding 0.950 ml of hydrogen peroxide (H_2O_2). Catalase activity calculated as the decomposition of hydrogen peroxide was measured at 240 nm every minute for 2 min. The enzyme activity of catalase was expressed as IU/mg protein according to the following formula:

$$\text{Units/mg protein} = (2.3033/T) \log (A_1 / A_2) / mg \text{ protein} \qquad \text{Eqn. 2}$$

A_1: absorbance at time 0 min.

A_2: absorbance at time 1 min.

T: time interval in minute.

2.10. Extract effect on superoxide dismutase activity (SOD)

The enzyme activity of SOD was evaluated using the Beauchamp and Fridovich (1971) method. [21]. For this purpose, 2 ml of a reaction mixture containing: 50 mM phosphate buffer (pH 7.8), 10^{-2} M methionine, 2×10^{-5} M sodium cyanide (NaCN), 2×10^{-6} M riboflavin, 6.6×10^{-3} M EDTA, and 1.76×10^{-4} M nitroblue

tetrazolium (NBT) were added to 5 µL of the cytosolic fraction. The resulting mixture is subsequently illuminated for 10 minutes by fluorescent lamps. The absorbance was measured by a spectrophotometer at 560 nm. A control is prepared under the same conditions, but without the cytosolic fraction. The amount of enzyme which inhibits the reduction of NBT by 50% was taken as the unit of activity of SOD. The SOD activity results were expressed as U/mg protein.

$$\% \text{ Inhibition} = \frac{DO_{control} - DO_{sample}}{DO_{control}} . 100 \qquad \text{Eqn. 3}$$

$$\text{SOD Units/mg protein} = \% \text{ inhibition} \times 6.35 \qquad \text{Eqn 4.}$$

2.11. Statistical analysis

All the experiments were repeated six times (n=6) and presented as mean ± standard deviation (SD). The data were subjected to analysis of variance (ANOVA), followed by the Tukey–Kramer HSD test (JMP version 7.0 Software). *P* value <0.05 was considered statistically significant.

3. RESULTS

3.1. Determination of total phenolic and flavonoid contents

Total phenolics and flavonoid contents of *Phlomis crinita* extract were analyzed and presented in Table 1. In this study, the plant crude extract was characterized by its high content of total phenolics (412.59 ± 1.73) expressed as mg gallic acid equivalent (GAE) per g of crude extract (CE). Total phenolics estimations were performed using standard gallic acid. Similarly, total flavonoid content equivalent to quercetin (QE) for *Phlomis crinita* was calculated. Our results proved that *Phlomis crinita* also presents significant quantities of flavonoids (83.65 ± 1.62 mg QE/g CE).

Table 1. Total phenols and flavonoids contents in *Phlomis crinita* leaves extract.

Total phenols and flavonoid contents	Concentration
Total phenolic content (mg GAE/g CE)	412.59 ± 1.73
Total flavonoid content (mg QE/g CE)	83.65 ± 1.62

Values are means± SD, n = 6.

3.2. Acute toxicity

In the acute toxicity test, the extracts did not produce any mortality and did not alter the general behavior of animals, even at the highest dose of 5 g/kg.

3.3. Effect of *Phlomis crinita* on carrageenan-induced mice paw edema

The *in vivo* anti-inflammatory activity of *Phlomis crinita* leaves extract has been evaluated using carrageenan-induced paw edema in mice and the results are presented in Table 2. After carrageenan induction, there was a significant increase in edema formation in normal control mice. A single oral treatment with *Phlomis crinita* extract at different doses was capable of reducing (p<0.05) the paw edema volume throughout the experiment. This reduction was time and dose-dependent. The peak edema volume reduction of the extract was recorded with a dose of 500 mg/kg (0.41± 0.05) representing 87.79% inhibition of edema formation at 4 h. Likewise, Diclofenac sodium inhibited (p<0.05) the oedematogenic response evoked by carrageenan in mice with the highest inhibition percentage (91.66%) of paw edema at 4 h after carrageenan injection, which was compared with the extract at a dose of 500 mg/kg bw (85.90%).

Table 2. Effect of *Phlomis crinita* extract on carrageenan-induced hind paw edema in mice.

Treatment	Doses (mg/kg bw)	Right hind paw volume (% inhibition)			
		1 h	2 h	3 h	4 h
Control	–	2.86±0.08	3.12± 0.12	3.29 ±0.07	3.35±0.03
Diclofenac sodium	50	1.12±0.03^{g} (60.83%)	0.73±0.03^{h} (76.60%)	0.55±0.4^{i} (83.28%)	0.28±0.06^{k} (91.66%)
Extract	100	1.87 ±0.04^{a} (34.64%)	1.64±0.02^{b} (47.25%)	1.43±0.03^{cd} (56.54%)	1.24±0.02^{ef} (63.03%)
	250	1.49 ±0.04^{c} (47.90%)	1.35±0.02^{d} (56.73%)	1.11±0.04^{g} (66.26%)	0.74±0.06^{h} (77.97%)
	500	1.34 ± 0.03^{de} (53.14%)	1.16±0.05^{fg} (62.82%)	0.73±0.06^{h} (77.81%)	0.41± 0.05^{j} (87.79%)

Values are expressed as mean ± SD (n = 6). According to Tukey HSD Test, values followed by different subscripts are significantly different (P<0.05).

3.4. Effect of extract on MDA level

As shown in Fig. 1, the concentration of MDA in paw tissue was significantly ($p < 0.05$) increased in the carrageenan-induced group (110.25 ± 1.8) when compared with the normal group (40.92 ± 1.94). Treatment with *Phlomis crinita* extract showed a significant ($p < 0.05$) decrease in MDA level when compared to the carrageenan-induced group. 500 mg/Kg tested doses of *P. crinita* extract inhibited lipid peroxidation (50.05 ± 1.31) in a manner that was comparable to that of Diclofenac sodium (48.7 ± 1.55 µmol).

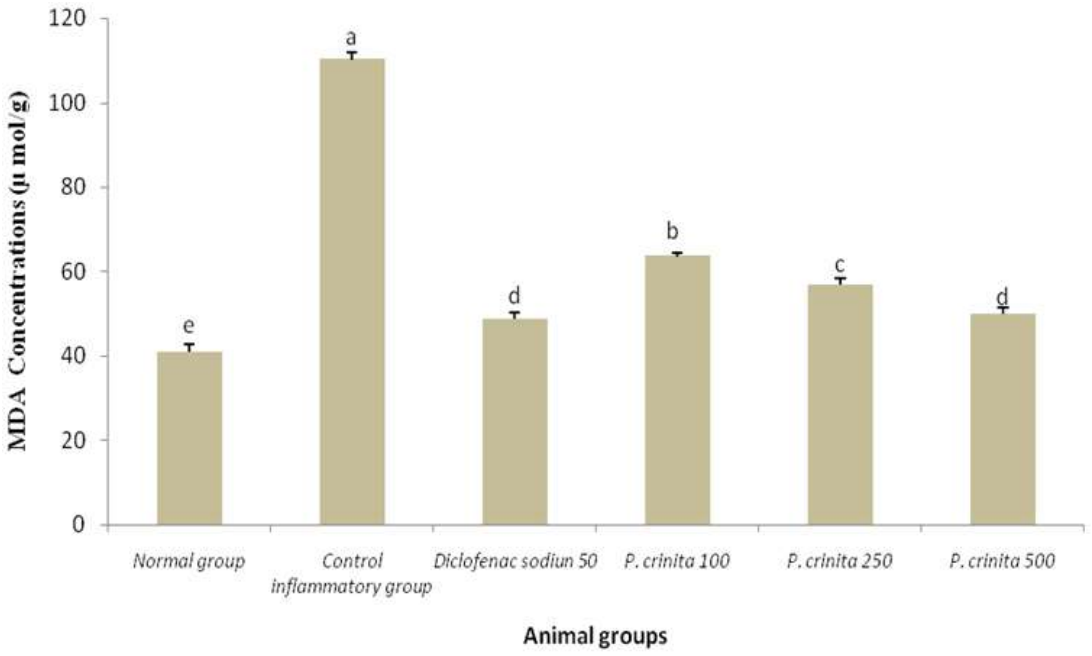

Figure 1. Effect of *Phlomis crinita* extract and controls on liver MDA levels.

Values are means ± SD (n = 6). According to Tukey HSD Test, values followed by different subscripts are significantly different (P<0.05).

3.5. Effect of extract on CAT and SOD activities

The antioxidant status of liver tissue was monitored by evaluating the activities of CAT and SOD in mice administered with test extract and standard. The activities of both antioxidant enzymes declined substantially in the inflammatory group (Fig. 2 and Fig. 3) compared to the normal control group. CAT and SOD levels were increased significantly ($p < 0.05$) in pretreated groups with extract and standard in comparison

to that of the inflammatory control group. *P. crinita* extract manifested a dose-dependent protective effect against carrageenan-induced decreases in antioxidant enzyme activities. Appreciable recovery in the activity of both antioxidant enzymes was observed with the treatment of *Phlomis crinita* extract at a dose of 500 mg/kg. These levels are statistically comparable to the standard.

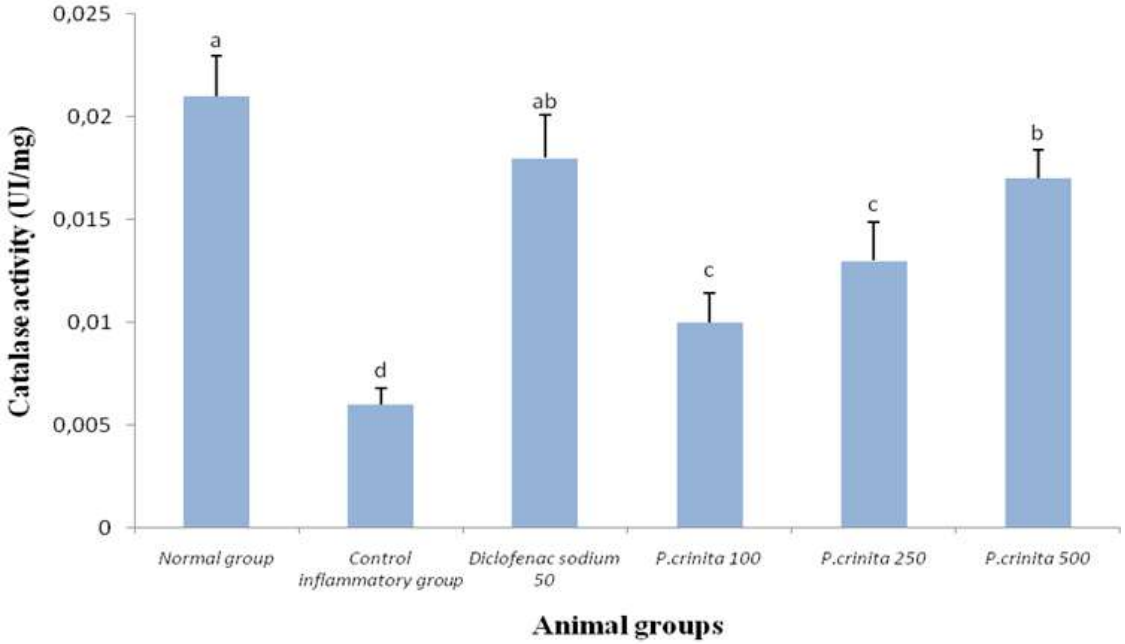

Figure 2. Effect of *Phlomis crinita* extract and controls on liver CAT activity.

Values are means ± SD (n = 6). According to Tukey HSD Test, values followed by different subscripts are significantly different (P<0.05).

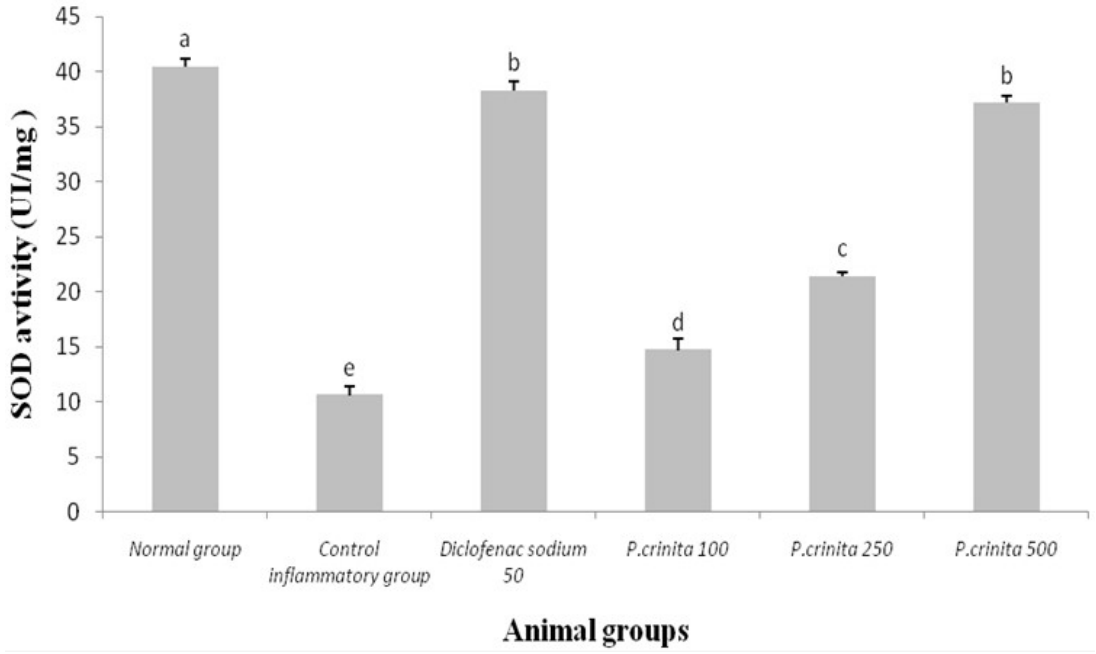

Figure 3. Effect of *Phlomis crinita* extract and controls on liver SOD activity.

Values are means ± SD (n = 6). According to Tukey HSD Test, values followed by different subscripts are significantly different (P<0.05).

4. DISCUSSION

In recent years, the search for phytochemicals possessing antioxidant and anti-inflammatory properties has increased due to their potential use in the therapy of various chronic and infectious diseases. The present study was designed to establish the scientific evidence for the usage of *Phlomis crinita* plant in inflammatory diseases. This work represents the first attempt to provide pharmacological evidence of *in vivo* anti-inflammatory and antioxidant effects of *P. crinita* polyphenols.

Phenolic compounds, in fact, are a large class of plant secondary metabolites that make up a major group of phytochemicals in plants, having significant pharmacological effects [22]. So, it is interesting to sort out the total content of these compounds in the used part of the plant. Results of the phytochemical analysis indicated that the leave extract contained a notable amount of phenolic compounds and presented a high level of flavonoids. Our results are higher than those obtained by Merouane et al. [23] who reported that the amount polyphenols and flavonoids were 117.96 ± 1.70 µg GAE/mg and 42.72± 0.53 respectively when studying three populations of *P. crinita*. The species *P. crinita* seems to have the highest amounts of phenolic compounds when compared to other members belonging *Phlomis* genus such as *P. armeniaca* showing 55.22 ± 1.95 and 54.39 ± 2.77 µg GAE/mg in methanolic and aqueous extracts, respectively [24]. These differences can be attributed to the botanical variety, plant maturity, extraction and analytical methods, and geographic origin of the plants.

Inflammation is a common symptom of many chronic diseases. It is a normal defensive reaction in response to infection and tissue injury [25]. Anti-inflammatory drugs have shown dose-dependent activity to prevent inflammation, but these are related to severe adverse effects like gastrointestinal complications: gastric irritation, ulcer, etc [26] so, herbal medicines have made a comeback to improve our basic health needs.

As stated elsewhere, edema is a fundamental and essential parameter for evaluating the anti-inflammatory potential of extracts/drugs [27]. In this study, the anti-inflammatory effect of *Phlomis crinita* leaves extract was conducted in the carrageenan-induced paw edema model [16], an established and well-known experimental model for the study of anti-inflammatory activity of compounds which assesses the degree of inflammation and efficacy of test drugs, especially at the acute stage [28].

Carrageenan is a non-antigenic phlogistic substance with no apparent systemic activity [27, 29]. It causes an inflammatory response by activating the release of inflammatory mediators, such as histamine, bradykinin, and serotonin. These mediators increase vasodilatation and promote the vasculature's permeability leading to the accumulation of fluids at the site of the inflammation, manifesting as edema. Its effects appear rapidly after injection and reach their maximum value 4 to 6 h later.

In this investigation, the subcutaneous injection of carrageenan in the mice hind paw caused a significant and time-dependent edema formation that peaked at 4 h (the late phase). However, by treating the animals with the *Phlomis crinita* extract, a significant reduction in paw edema evoked by carrageenan was seen. Our results are consistent with those of Shang et al. [30] who found that *Phlomis umbrosa* Turcz extract had e good dose-dependent anti-inflammatory effect. Also, with those of Li et al. [31] who reported that phlomisoside F (PMF) isolated from *Phlomis younghusbandii* and administered orally could not only significantly decrease rat paw edema in rats and ear edema in mice, but also reduce the vascular permeability in mice. Same findings were also reported by Taşkın et al. [32] who found that *Phlomis pungens* methanolic extract demonstrated a prominent and intensive anti-inflammatory effect with 24.7% inhibitive capacity in the altered edema size after the first hour of carrageenan injections. *Phlomis pungens* methanolic extract inhibitory effect increased during three hours and reached a maximum of 41.9%.

Based on the mechanism of carrageenan-induced inflammation, we suggested that the plant extract contains anti-inflammatory-associated compounds that alter the inflammatory cascade associated with edema. Most current anti-inflammatory drugs function by inhibiting phospholipase A2, which inhibits prostaglandin synthesis [33]. As a result, it is suggestive that the studied plant extract contains bioactive substances that provide anti-inflammatory effects by blocking the action of phospholipase A2.

The anti-inflammatory activity of *P. crinita* extract recorded in this study could be due to the presence of phenolic compounds since the phytochemical screening detected large amounts of polyphenols and flavonoids in our plant extract. It has been demonstrated in previous studies that plants rich in polyphenols present a good anti-inflammatory effect [34, 35]. Lipoxygenase inhibitors also play a crucial role in carrageenan-induced paw edema. In this experiment, the ability of *P. crinita* methanol extract to reduce paw edema volume may also be attributed to its inhibitory activity on the lipoxygenase enzyme.

ROS are associated with the inflammatory response and frequently contribute to the tissue-damaging effects of inflammatory reactions [36-38]. Free radicals cause lipid peroxidation, which alters the structural integrity and functions of cell membranes while increasing MDA content [39]. Hence, MDA content in liver tissues was evaluated as an indicator of oxidative stress. In the present study, pre-treatment with the plant extract reduced the MDA level in a dose-dependent manner when compared to the inflammatory control group. The improvement in liver oxidative stress may be attributed to the removal or prevention of radical oxygen scavenging (ROS) accumulation caused by carrageenan administration [39].

The body has an effective mechanism to prevent and neutralize free radical-induced damage. This is accomplished by a set of endogenous antioxidant enzymes, such as catalase and SOD [40]. In the mice's liver extract, CAT and SOD activities were decreased in the carrageenan-induced group. This decrease in enzymes activity may be explained by the excessive use of the produced enzymes that act as scavengers of free radicals generated during the inflammatory process. In contrast, *Phlomis crinita* extract was able to restore the antioxidant enzymes to near-normal levels. The increased levels of catalase and SOD found in this study suggest that the extract has *in vivo* antioxidant activity and it can reduce the effects of ROS in the biological systems. This beneficial effect may be due to the presence of phytochemical compounds with antioxidant activities, which could contribute to the anti-inflammatory process. In our study, we revealed that the extract has the highest amount of phenolic compounds, particularly flavonoids. Numerous previous studies confirm that flavonoids possess potent antioxidant activities capable of scavenging hydroxyl radicals, superoxide anions, and lipid peroxy radicals [41], implying that they have therapeutic potential with an anti-inflammatory effect [42-44].

5. CONCLUSION

The methanolic extract of *Phlomis crinita* leave extract was found to contain a large amount of flavonoids and polyphenols. The extract also displayed remarkable *in vivo* antioxidant and anti-inflammatory properties. Hence, further investigations are required to isolate phytochemicals that possess potent biological activities. Therefore, *Phlomis crinita* leave extract can be further studied for its pharmacological application in the prevention and treatment of inflammation and oxidative stress-related diseases.

Author's contribution: HB, LB: Design the research, laboratory experiments, and paper writing, NB: Literature search, LBM, KM: Data analysis and interpretation, KA: manuscript correction. All authors read and approved the final version of the manuscript.

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