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ARTICLE

In vitro Antioxidant and Anti-Inflammatory Effects of the Hydro-Methanolic Extract of *Phlomis crinita* from North Algeria

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Abstract: *Phlomis crinita* is a medicinal plant commonly used in Algerian folk medicine as a remedy for ulcers, haemorrhoids and healing of wounds. The current study aimed to evaluate the anti-inflammatory and antioxidant effects of this plant and to provide a scientific basis for its use. Protein denaturation and membrane stabilization assays were used to assess the anti-inflammatory action in vitro. DPPH free radical-scavenging and reducing power tests were used to determine the antioxidant activity. The plant methanol extract exhibited potential anti-inflammatory activity, demonstrating its ability to prevent protein denaturation and stabilize the membrane of human red blood cells. It had IC_{50} values of 143.64 ± 2.94 and 119.63 ± 0.80 µg/mL, respectively, compared to diclofenac sodium (IC_{50} value of 60.60 ± 2.55 and 53.47 ± 0.93 µg/mL, respectively). *P. crinita* showed strong antioxidant DPPH scavenging activity (IC_{50} = 82.07±0.37 µg/mL) compared to ascorbic acid (62.48±0.56) and exhibited a strong reducing potential. The results suggested that the plant might be a potential candidate for oxidative stress prevention and inflammatory disease treatment.

Keywords: *Phlomis crinita*, Phenolic compounds, Inflammation, Stress.

Introduction

Inflammation is a defense mechanism that protects tissues from chemical, mechanical, or microbial stimulation. The main cellular constituents involved in the inflammatory response comprise monocytes/macrophages, polymorphonuclear leukocytes and endothelial cells[1]. Upon stimulation of these cells through the respiratory burst, an augmented utilization of oxygen ensues, leading to an increased production of cytokines, reactive oxygen species (ROS), and various other inflammatory mediators. The ROS generated during the inflammatory response facilitate it; however, sustained generation of these factors can induce oxidative stress and contribute to the development of chronic inflammatory disorders.

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Inflammation and oxidative stress are commonly recognized to be linked processes[2,3].

Both steroidal (SAIDs) and non-steroidal anti-inflammatory drugs (NSAIDs) have their place in treating inflammation or irritation. Long-term use of these drugs causes renal toxicity, duodenal ulcers and gastric ulcers[4,5]. As a result, looking for alternatives appears both necessary and beneficial. Medicinal plants that contain a diverse range of compounds could lead to the discovery of novel anti-inflammatory agents. Traditional and folklore remedies play an essential role in global health services[6]. Plants and plant extracts are used in the treatment of around three quarters of the world's population[7].

Among the largest genera in the *Lamiacea* family, the genus *Phlomis L.* encompasses over 100 species, distributed from the Mediterranean region to China and central Asia[8,9]. Many of these species are employed as therapeutic agents for ulcers, inflammation, haemorrhoids and wound healing. Additionally, they are recommended for managing digestive complaints and as prophylactics against cardiovascular diseases, liver complications, kidney disorders and bone diseases[10,11].

Numerous investigations have highlighted *Phlomis* species as valuable natural reservoirs of diverse bioactive constituents, including phenylethanoids, alkaloids, iridoids, phenylpropanoids, diterpenoids[12], as well as phenolic compounds primarily comprised of phenolic acids and flavonoids[8,13]. This chemical combination provides the species with a range of advantageous bioactivities, including intestinal anti-inflammatory properties[14], antimutagenicity[15], antiulcerogenic activity[16], enzyme inhibitory, antioxidant activities[8] and antidiabetic effect[17].

Due to its size and variety of climates, Algeria is renowned for its extensive flora[11]. *P. crinita*, one of the species of the *Phlomis* genus found in Algeria, is extensively employed for treating wounds and burns. This is achieved by preparing a poultice using minced leaves[18].

To the best of our knowledge, only limited research has been realized regarding assessing the in vitro antioxidant activity of polyphenols from *P. crinita* leaves, and no studies have been done on the in vitro anti-inflammatory properties of these plant phenolic compounds. Consequently, this study aims to investigate the in vitro antioxidant and anti-inflammatory effects of polyphenols obtained from the leaves of *P. crinita.*

Materials and Methods

Chemicals used in this work include methanol (99.9%), hexane (99%) and dextrose from Merck, Germany, bovine serum albumin (BSA), 1,2-diphenylpicrylhydrazyl (DPPH), ascorbic acid and ferric chloride from Sigma-Aldrich, USA, NaCl from Biochem, France, sodium citrate from Park Scientific Limited, UK, citric acid and HCl from Qualikems, India, potassium ferricyanide and TCA from LabChem, Indonesia and diclofenac sodium from Generis Farmaceutica, Portugal.

An oven (Memmert, Germany) was used for plant drying and anti-inflammatory test incubations. A rotatory evaporator (Heidolph, Germany) was used to prepare the plant extracts. A UV-visible spectrophotometer (Shimadzu, Japan) was used to measure biochemical parameters.

Plant Material Collection and Extraction

The leaves of *P. crinita* (Lamiaceae) were gathered in Jijel, Ouled Rabeh, in April 2019 (Algeria). The taxonomic identification of this species was confirmed by Dr. Gaëlle Saladin (researcher in plant biology at the University of Limoges, France). A specimen of *P. crinita* has been deposited at the Herbarium of the Faculty of Sciences and Techniques at the University of Limoges with the Voucher reference Pc1.Lam.2023. After being subjected to a thorough rinsing with running water, fresh leaves of *P. crinita* were air-dried before undergoing baking at a temperature of 40 °C until reaching a predetermined weight. The resulting dried plant material was finely powdered using an electric grinder and subsequently stored in opaque glass bottles.

Polyphenols were extracted through a maceration process using a mixture of methanol and water in an 80:20 ratio (v/v). The solid-liquid ratio was set at 1:10 (w/v), and the maceration was conducted for 72 h at room temperature (24 ± 2 °C). Subsequently, the solvent was filtered, and the extracted material was subjected to hexane defatting, following the prescribed procedure by Yu et al.[19]. The recovery of the methanol phase was performed three times during the process, and it was then condensed at 40 °C under reduced pressure in a

rotary evaporator. The final dried extract was kept at 4 °C in the dark until used.

Inhibition of Protein Denaturation

The inhibition of albumin denaturation technique, developed by Mizushima and Kobayashi[20] and modified slightly, was performed to investigate the *P. crinita*'s anti-inflammatory activity. For this, 500 µL of a solution of 1% bovine serum albumin (BSA) was added to 100 µL of *P. crinita* extract (50–500 µg/mL) reconstituted in methanol. The pH of the reaction mixture was adjusted using 1 N HCl. The mixture was incubated at 37 ºC for 20 min and then heated to 51 ºC for 20 min. The turbidity was measured at 660 nm. Diclofenac sodium was used as a standard. All experiments were performed in six replicates. The inhibition of protein denaturation was calculated as follows:

$$\%\text{inhibition} = \frac{(A_{control} - A_{sample})}{A_{control}} \times 100\%$$

where $A_{control}$ is the absorbance without sample, and A_{sample} is the absorbance of sample/standard. From the linear regression curves of %inhibition versus concentration, the concentration of the extract or drug that results in 50% inhibition of BSA denaturation was calculated (IC_{50}).

Membrane Stabilization Test

The membrane stabilization test was done by applying the method of Shinde et al.[21] The blood was collected from a healthy human volunteer who had abstained from NSAIDs for two weeks before the experiment. The obtained blood was mixed with the sterilized Alsever's solution (2% dextrose, 0.42% sodium chloride, 0.05% citric acid and 0.8% sodium citrate in distilled water). The blood was centrifuged at 3000 rpm for 10 min before performing a set of three washes with isosaline (0.85%, at pH 7.2). After calculating the blood volume, 10% v/v isosaline suspension of the blood was prepared. The experimental setup involved the individual mixing of phosphate buffer (pH 7.4, 0.15 M), hyposaline solution (0.42 %), and a 10% blood suspension (0.5 mL) with different concentrations of the extract (ranging from 50 µg/mL to 500 µg/mL). Diclofenac sodium as a reference and a control group, where distilled water was used instead of hyposaline to achieve complete hemolysis (100%), were also prepared. All the centrifuge tubes containing the reaction mixture underwent a 30-min incubation period at 37 °C and a 20-min centrifugation at 3000 rpm. The absorbance of the supernatant was measured at 560 nm.

The experiment was run in six replicates, and the percentage of membrane stabilization or haemolysis inhibition was calculated as follows:

$$\%\text{inhibition} = \frac{(A_{control} - A_{sample})}{A_{control}} \times 100\%$$

The concentration of the extract or drug that inhibits erythrocyte haemolysis by 50% (IC_{50}) was determined graphically.

DPPH Free Radical Scavenging Assay

The extract's capacity to scavenge free radicals was assessed using the DPPH assay described by Brand-Williams et al.[22]. Aliquots (100 µL) of the extract in methanol at various concentrations (50–500 µg/mL) were added to 2.9 mL of a 0.025 g/L methanol solution of DPPH. The mixtures were shaken vigorously and left to stand at room temperature (24 ± 2 °C) in the dark for 30 min. The bleaching of the DPPH color decreased the purple coloring of the reaction mixtures, which was detected at 517 nm using a UV spectrophotometer. The following formula was used to determine the DPPH$^{\bullet}$ radical scavenging capacity:

$$\text{DPPH scavenging activity } \% = \frac{(A_0 - A_1)}{A_0} \times 100\%$$

where A_0 represents the absorbance of the control at 30 min, which contains the entire reagents except the sample, and A_1 is the sample's 30-min absorbance. Six replicates of each test were carried out, then the average was calculated. The positive control was ascorbic acid. EC_{50}, i.e., the concentration of the extract generating 50% of the maximum dose response calculated with linear regression, was also used to report the antiradical activity.

Ferric Reducing Power Assay

The ferric-reducing power was determined using the Oyaizu method[23]. One milliliter of the extract at different concentrations was mixed with 1% potassium ferricyanide [$K_3Fe(CN)_6$] and phosphate buffer (0.2 M, pH 6.6). After being incubated at 50 °C for 20 min, the reaction mixture was acidified with 1 mL of 10% trichloroacetic acid (TCA). The resultant mixture was centrifuged for ten minutes at 3000 rpm. The upper layer of the solution was then

thoroughly mixed with distilled water (1.5 mL) and ferric chloride ($FeCl_3$) solution (150 µL, 0.1%). The findings of the absorbance measurement at 700 nm were then compared to that of the standard (ascorbic acid). Increased absorbance was a sign of greater reducing power. Six replicates of the assay were run.

Statistical Analysis

Each experiment was run six times (n = 6), and the results are shown as mean ± standard deviation (SD). The ANOVA test was used to analyze the data, and then the Tukey-Kramer HSD test was performed (JMP version 7.0 Software). Statistics were considered significant if $p < 0.05$.

Results

Inhibition of Protein Denaturation

The inhibition of heat-induced albumin denaturation was used to test the anti-inflammatory activity of the extract. As presented in Table 1, both extract and standard have the ability to inhibit heat-induced BSA denaturation in a dose-dependent manner. Maximum extract inhibitory effect (72.22 ± 0.71%) was noted at 500 µg/mL, and the IC_{50} value was found to be 143.64± 2.94 µg/mL. Diclofenac sodium showed comparatively higher anti-inflammatory activity (83.02 ± 0.48%) with a lower IC_{50} value (60.60 ± 2.55µg/mL) at the same concentration.

Table 1. *P. crinita* extract and standard impact on protein denaturation and membrane stabilization activities.

Concentrations (µg/mL)	Protein denaturation (%inhibition)		Membrane stabilization (%protection)	
	Extract	Diclofenac sodium	Extract	Diclofenac sodium
50	32.12±0.71^{g}	47.49±0.41^{f}	35.55±0.77^{g}	45.55±0.43^{f}
100	46.28±0.70^{f}	58.00±0.48^{d}	44.89±0.50^{f}	64.09±0.15^{d}
250	55.05±0.73^{e}	68.57±0.76^{c}	62.08±0.70^{e}	73.49±1.54^{c}
500	72.22±0.71^{b}	83.02±0.48^{a}	78.24±0.56^{b}	81.76±1.55^{a}
IC_{50} (µg/mL)	143.64±2.94	60.60±2.55	119.63±0.80	53.47±0.93

Results are presented as mean ± standard deviation (n = 6). Significant differences, as determined by the Tukey HSD Test ($p < 0.05$), are denoted by distinct subscripts accompanying the values. The IC_{50} value was determined using linear regression analysis.

Membrane Stabilization Test

Table 1 presents the protective effects of *P. crinita* methanol extract and diclofenac sodium against hyposaline-induced damage on the human red blood cell (HRBC) membrane. Additionally, the plant extract and the standard exhibited significant inhibition of heat-induced hemolysis. This data showed that the concentration of *P. crinita* leaf extract increased the protection of the HRBC membrane. With an IC_{50} value of 119.63 ± 0.80 µg/mL, *P. crinita* demonstrated strong membrane stabilizing activity. This value is about two times higher than the positive control, diclofenac sodium (53.47 ± 0.93 µg/mL).

Antioxidant Assays

Table 2 displays the results of the extract and the standard's ability to scavenge DPPH free radicals. Apparently, the extract has the ability to lower the concentration of DPPH radicals. At 500 µg/mL, the extract reached its peak activity of 84.96 ± 0.89%, while the positive control showed 86.49 ± 0.84% inhibition. The results showed that the *P. crinita* extract has a good DPPH radical scavenging effect with an IC_{50} value of 82.07 ± 0.37 µg/mL. The observed result is comparable to the positive control, ascorbic acid (62.48 ± 0.56 µg/mL).

Table 2. DPPH free radical scavenging activity of *P.crinita* extract and standard.

Concentrations (µg/mL)	DPPH (%inhibition)	
	Extract	Ascorbic acid
50	35.64±0.45^{g}	40.48±0.44^{f}
100	59.69±0.38^{e}	65.34±0.78^{d}
250	73.50±0.61^{c}	79.87±0.76^{b}
500	84.96±0.89^{a}	86.49±0.84^{a}
IC_{50} (µg/mL)	82.07±0.37	62.48±0.56

The data is displayed as mean ± SD (n = 6). Values with different subscripts indicate statistical significance, as determined by the Tukey HSD Test ($p < 0.05$). The IC_{50} value was determined using linear regression analysis.

Ferric Reducing Power Activity

Table 3 compares the reductive potential of *P. crinita* leaf extract to those of the standard ascorbic acid. The reducing power of the extract increased with concentration, as did its antiradical activity. The ferric-reducing ability of the extract is, within the concentration range of 50–250 µg/mL, statistically similar to that of ascorbic acid. However, at a concentration of 500 µg/mL, the extract demonstrates significantly superior reducing power ($p < 0.05$) compared to the reference drug.

Table 3. Ferric-reducing potency of *P. crinita* extract and ascorbic acid.

Concentrations (µg/mL)	Absorbance(700 nm)	
	Extract	Ascorbic acid
50	0.253±0.02^{e}	0.282±0.02^{e}
100	0.382±0.01^{d}	0.416±0.01^{d}
250	0.494±0.01^{c}	0.481±0.02^{c}
500	0.611±0.02^{a}	0.562±0.01^{b}

The data is displayed as mean ± SD (n = 6). Statistical analysis using the Tukey HSD Test ($p < 0.05$) reveals significant differences between values, as denoted by distinct subscripts.

Discussion

Plants are a valuable source of a wide range of secondary metabolites, with broad spectra of pharmacological activities against many diseases, and have been utilized as leads for drug discovery and development[24]. The current study aimed to evaluate the in vitro anti-inflammatory activity and antioxidant potential of the hydro-methanol extract derived from *P. crinita*. The anti-inflammatory potential of *P. crinita* leaf extract was carried out using two in vitro tests: protein denaturation inhibition and erythrocyte membrane stabilization assays. As expected, the different concentrations of the extract and diclofenac sodium, the positive control, demonstrated a dose-dependent effect on the inhibition of protein denaturation and the stabilization of the human red blood cell membrane. IC_{50} values for the extract were respectively 143.64 ± 2.94 µg/mL and 119.63 ± 0.80 µg/mL, which were about two times higher than those for the positive control, diclofenac sodium (60.60 ±2.55 µg/mL and 53.47 ±0.93 µg/mL).

One of the well-established causes of inflammation and arthritic diseases is the denaturation of protein molecules. According to Mizushima[25], one of the primary mechanisms of action of NSAIDs is the protection against protein denaturation. Therefore, developing anti-inflammatory drugs that can prevent protein

denaturation would be worthy. The modification of electrostatic properties, hydrophobic, hydrogen and disulfide bonds, which results in the disappearance of their secondary and tertiary structures and causes an inflammatory response, is one potential mechanism for this denaturation[20,26]. By triggering the production of self-antigens in vivo, this denaturation induces an inflammatory response[26,27]. Several anti-inflammatory medications, including diclofenac, have demonstrated dose-dependent inhibition of heat-induced protein denaturation[28]. Plant extracts or drugs are thought to inhibit BSA denaturation through their capacity to bind protein. In fact, regions of threonine and lysine residues rich in tyrosine and aliphatics were found to be two binding sites on BSA[29].

Additionally, membrane stabilization is a commonly used method for assessing the in vitro anti-inflammatory capability of human red blood cells[30]. The stabilization of the erythrocyte membrane suggests the ability of the tested extract to stabilize the lysosomal membrane because it is similar to the lysosomal membrane and is induced to lyse in a hypotonic medium[31,32]. The exact mechanism causing this membrane stabilization is still unknown; however, it is conceivable that the extract produces this effect through the augmentation of the surface area to volume ratio of the cells, achieved by either expanding the membrane or reducing the cell size while interacting with membrane proteins[21]. The observed anti-inflammatory activity of the *P. crinita* extract recorded in this study may be attributed to its phytochemical compounds, particularly polyphenols. According to Boutennoun et al.[33], the plant extract contained a high amount of total phenolics and flavonoids (412.59 ± 1.73 mg GAE/g CE and 83.65 ± 1.62 mg QE/g CE, respectively). Previous studies have established a positive correlation between high levels of polyphenols in plants and their anti-inflammatory properties[33-35].

The inflammatory response is associated with reactive oxygen species (ROS), which often play a role in the detrimental effects of inflammatory reactions on tissues[36,37]. Lipid peroxidation, which is brought on by free radicals, alters the structural integrity and operations of cell membranes while increasing MDA levels[38].

As a result, the in vitro antioxidant capacity of the *P. crinita* extract was evaluated using the DPPH and reducing power assays. DPPH, a stable free radical, has been extensively employed to assess the free radical scavenging potential of plant extracts and antioxidants[39]. Additionally, the ability of antioxidants to scavenge the DPPH free radical is widely attributed to their hydrogen-donating capacity[40].

In the current investigation, there was an increase in DPPH radical scavenging activity with elevated plant extract concentration, indicating an improved ability to donate hydrogen ions that is proportional to the number of gained electrons[41,42], which is an indication that this extract was capable of strong radical scavenging. According to these findings, the plant extract obviously contains significant amounts of secondary metabolites with potent antioxidant properties. By transforming the stable DPPH radical into a yellowish diphenylpicrylhydrazine derivative, polyphenols in the *P. crinita* extract may be involved in their free radical reactions. This conclusion finds support in existing literature, which consistently demonstrates a positive correlation between phenolic compounds and DPPH radical scavenging activity[43,44].

Compounds' reducing power reveals their ability to donate electrons, which is connected to their antioxidant activity. An important mechanism of phenolic antioxidant action is its electron-donating activity, and Fe^{3+}reduction is frequently used as an indicator of this activity. In the reducing power assay, antioxidants within the sample extract donate electrons, leading to the conversion of Fe^{3+} to Fe^{2+}. The formation of Perl's Prussian blue ($Fe_4[Fe(CN)_6]_3$) at 700 nm can then be used to monitor the amount of Fe^{2+} complex that has formed. An increase in the reaction mixture's absorbance indicates an improvement in its ability to reduce Fe^{3+}[45,46]. Our findings indicated that the extract had a significant reducing activity related to extract concentration.

The extract displayed the highest reducing capacity at 500 µg/mL, exceeding the standard. The *P. crinita* leaf extract contains a significant amount of phenolics, as previously discovered[9], and it can, therefore be assumed that these phenolics are what give the extract its strong reducing power. This is in line with numerous studies that have demonstrated a strong correlation between a plant's reducing power and its total phenolic content[44].

Conclusion

The findings of the present study demonstrate that the methanol extract of *P. crinita* leaves has significant in vitro antioxidant and anti-inflammatory activities. Our results support the plant's potential as a therapy for inflammation and related diseases. This plant is a low-cost origin of vital bioactive components with potential for herbal medicine development. Further studies are necessary to purify the bioactive compounds that are responsible for biological activities.

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