Artículo original

Variation of Phenolic Content and Antioxidant Activity in Organs and Populations of *Phlomis crinita* L.

Variación del contenido fenólico y de la actividad antioxidante en órganos y poblaciones de *Phlomis crinita* L.

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ABSTRACT

Introduction: The medicinal plant *Phlomis crinita* L. is widely used in Mediterranean folk medicine for its various pharmacological effects, especially to heal wounds and relieve abdominal pain.

Objective: Determine the phenolic and flavonoid content of hydromethanolic extracts obtained from *Phlomis crinita* L. leaves and flowers, as well as their antioxidant properties.

Methods: Total phenolic and flavonoid content was determined by spectrophotometry with the Folin-Ciocalteu and aluminum chloride methods. Antioxidant activity was estimated by the ß-carotene-linoleic acid system and the 2,2-diphenyl-1-picrylhydrazyl free radical assay.

Results: Phytochemical examination showed that the content of bioactive compounds was clearly greater in leaf extracts than in flower extracts. The content of phenolic compounds varied between the three populations ($p < 0.05$), with the Ouled Benabdelkader population exhibiting the largest content (117.96 ± 1.70 µg GAE/mg). The greatest flavonoid content was found in leaves of Medjadja, followed by Ouled Benabdelkader and el-Nakhla ($p < 0.05$). The leaf extracts with the strongest antioxidant activity were the ones from the Ouled Benabdelkader population, of great importance in terms of DPPH free radical scavenging capacity.

Maximum effect was achieved with a CI50= 73.80 ± 1.58 µg/ml. This potency is highly correlated with flavonoid content.
Conclusions: The study revealed the richness of *P. crinita* in bioactive compounds characterized by great antioxidant properties which make them a potentially valuable natural remedy.
Keywords: *Phlomis crinite*; anti-radical activity; natural antioxidants; medicinal herbs; content of phenolic; flavonoid compounds.

RESUMEN

Introducción: La planta medicinal *Phlomis crinita* L. es muy utilizada en la medicina tradicional mediterránea debido a sus diversos efectos farmacológicos. Es especialmente empleada en la curación de heridas y el tratamiento de dolores abdominales.
Objetivo: Determinar el contenido fenólico y flavonoide, y las propiedades antioxidantes de extractos hidrometanólicos de *Phlomis crinita* L.
Métodos: El contenido total de compuestos fenólicos y flavonoides se determinó espectrofotométricamente mediante los métodos Folin-Ciocalteu y cloruro de aluminio. Se estimó la actividad antioxidante utilizando el sistema de ácido ß-caroteno-linoleico y el ensayo del radical libre 2,2-difenil-1-picrilhidrazilo.
Resultados: El examen fitoquímico mostró una clara superioridad en los extractos de hojas respecto a los de las flores en cuanto al contenido de compuestos bioactivos. El contenido de compuestos fenólicos varió entre las tres poblaciones ($p<0,05$). La muestra de la población de Ouled benabdelkader fue la de mayor contenido (117,96 ± 1,70 µg GAE/mg). El mayor contenido de flavonoides lo mostraron las hojas de Medjadja, seguido por las provenientes de Ouled benabdelkader y el-Nakhla ($p<0,05$). Los extractos de hojas que tuvieron la actividad antioxidante más fuerte fueron los de la población Ouled benabdelkader, con gran importancia en la capacidad secuestradora del radical libre de DPPH. Alcanzó el máximo efecto con una CI_{50}= 73,80 ± 1,58 µg/mL. Esta potencia está altamente correlacionada con el contenido de flavonoides.
Conclusiones: Esta investigación reveló la riqueza de *P. crinita* en compuestos bioactivos, las cuales poseen grandes propiedades antioxidantes que pudieran constituir un valioso remedio natural.
Palabras clave: *Phlomis crinita*; actividad antirradical; antioxidantes naturales; hierbas medicinales; contenido de compuestos fenólicos; flavonoides.

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Introduction

Phlomis L. including about 100 species, is one of the fascinate genus of *Lamiaceae* family due to its large traditional uses in its repartition area starting from the Mediterranean region to the central Asia and China.[1] The phytotherapeutic uses of *Phlomis* species have been mentioned since ancient times as traditional remedy[2] for ulcer, inflammation, hemorrhoids and healing of wounds.[3,4] They are also recommended in the treatment of gastrointestinal complains,[5] and as prophylactics against liver, kidney, bone and cardiovascular diseases.[6]

Phytochemical investigation of the extracts from *Phlomis* genus showed that it is rich in various bioactive molecules including phenylpropanoids, irridoids, diterpenoids, phenylethanoids, alkaloids[7] and phenolic compounds represented mainly by phenolic acids and flavonoids.[1,8,9] This worthy chemical mixture granted the beneficial biological activities of these species.[1,4,9,10,11,12]

Flavonoids and other phenolic compounds are the common antioxidants in medicinal herbs, they may intervene against reactive oxygen species (ROS) and act as antibacterial, anti-cancer, anti-inflammatory, immune system promoting and cardioprotective agents in human health, curing and preventing many diseases.[13]

During last decades search for phenolic compounds from medicinal plants have gained increased interest. Members of *Phlomis* genus have received notable interest; unfortunately rare are the reports on phenolic compounds and antioxidant potencies of *P. crinita*. In the other hand, the available data focused on phytotherapeutic aspect of this species[4,11] have ignored some examinations, regarded valuable for phytotherapy, such as determination of phenolic and flavonoid amounts, combination of different assays in the evaluation of antioxidant activity. Thus, this investigation evaluates the phenolic and flavonoid contents and the antioxidant properties of *P. crinita* locally named *Khayat El-djerah* (wound healing) and widely used in local traditional medicine as remedy of wounds and abdominal troubles.

Therefore, the aim of this paper was to investigate about mentioned *Phlomis* species in comparison approach between leaves and flowers potentialities obtained from three populations, with respect to their phenolicand flavonoid contents, and their antioxidant activity evaluated by two *in vitro* complementary assays.

Methods

Plant material

Aerial parts (leaves and flowers) of *P. crinita* were harvested during flowering season in May 2017 from three populations located in the localities called

Medjadja, El-Nakhla and Ouledbenabdelkader within Chlef province, North-West of Algeria. Leaves and flowers were separately dried under shade at room temperature and finely powdered then stored in hermetic bottles until extraction.

Preparation of extracts

The extraction was performed according to the previous research by *Merouane, et al.*[14] 500 mg of fine powder of *P. crinita* was macerated in 80% methanol at room temperature under continuous shaking (WIS-10, Daihan Scientific co. Ltd., Korea) for 24 h. The extracts were filtered with Whatman filter paper and the residues were rinsed twice with 10 ml of 80% methanol. The total extracts were concentrated by rotaryvapor (Büchi, Flawil, Switzerland) followed with lyophilisation (Christ Alpha 1-4 LD plus, Germany) to give a crude extract which was kept at 4 ºC until analysis.

Assay for total phenolic content

The total phenolic content was determined by the spectrophotometric method given by *Thummajitsakul, et al.*[15] involving Folin-Ciocalteu reagent and gallic acid as standard with slight modifications. The reaction mixture was prepared by mixing 0.1 mL of extract prepared at 2 mg/ml and 2.5 ml of 10% Folin-Ciocalteu's reagent in water. After 10 min, 2.5 ml of 7.5% $NaHCO_3$ was added. The mixture was incubated in dark at room temperature for 45 min and the absorbance was read at 765 nm using UV-Vis spectrophotometer (Optizen 2120, Mecasys Co. Ltd., Korea) against blank contemporaneously prepared by replacing extract with methanol. All tests were carried out in triplicate. The phenolic content was expressed as µg Gallic acid equivalents/mg of extract (µg GAE/mg) based on calibration line constructed from standard solution of Gallic acid using concentrations ranging from 10 to 80 µg/ml.

Assay for total flavonoid content

The determination of total flavonoids content was based on the method described by *Souza et al.*[16] with slight modifications. In brief, 0.5 ml of extract prepared at 2 mg/ml was mixed with 0.1 ml of 2% aluminum chloride ($AlCl_3$) dissolved in methanol, and 0.5 mL of methanol. The mixture was allowed to stand for one hour in dark at room temperature and measured spectrophotometrically (Optizen 2120, Mecasys Co. Ltd., Korea) at 420 nm against blank tube prepared by replacing the extract with methanol. The same protocol was followed for the standard solution of Quercetin and the calibration graph was construed. All tests were realized in triplicate. The flavonoids content was calculated from standard calibration curve constructed with concentrations of Quercetin ranging from 10 to 60 µg/mL and expressed as µg Quercetin equivalents/mg of extract (µg QE/mg).

2,2-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging assay

The DPPH assay was conducted according to the conventional method.[17] In brief, 0.5 ml of extracts prepared at 25, 50, 75, 100, 125, 150, 175 and 200 µg/ml was added to 01 ml of methanolic DPPH solution prepared at 0.1 mM. The mixture was homogenized vigorously and stored in dark at room temperature for 30 min. Readings were performed using a spectrophotometer (Optizen 2120, Mecasys Co. Ltd., Korea) at 517 nm. Inhibition of DPPH free radicals in percent (I %) was calculated from the following formula:

$$I(\%) = \left(\frac{A_0 - A_S}{A_0}\right) \times 100$$

Where A_0 is the absorbance of the DPPH solution without extract and A_S is the absorbance of the test sample. The antioxidant activity was expressed in term of IC_{50} (µg/ml) defined as the concentration of test sample which reduced the initial DPPH concentration by 50%. All samples were prepared in three independent experiments.

ß-Carotene-linoleic acid assay

In this test, the antioxidant activity was evaluated by indirectly measuring the inhibition of volatile organic compounds and conjugated diene hydroperoxides resulting from linoleic acid oxidation.[18] One ml of ß-carotene solution in chloroform (0.5 mg/10ml) was mixed with 25 µl of linoleic acid and 200 mg of Tween 20. After complete evaporation of the chloroform using a vacuum evaporator at 40 ºC, 50 ml of distilled water (aerated with oxygen for 1 h) was added with vigorous shaking, 2.5 ml of this emulsion was dispersed in test tubes and 350 µl of the extracts or butylated hydroxyl toluene (BHT) prepared at 2 g/l was added. The mixture was stored in dark at room temperature for 72 h. Readings were performed against blank (consisting of 350 µl methanol in 2.5 ml of distilled water) using a spectrophotometer (Optizen 2120, Mecasys Co. Ltd., Korea) at 490 nm. Total antioxidant activity was expressed in term of percentage inhibition relative to the activity of BHT and calculated following next formula:

$$\%\, Inhibition = (A_S/A_C) \times 100$$

Where A_S is the absorbance of test sample after incubation period, and A_C is the absorbance of BHT at the moment of preparation (t=0). Tests were carried out performing three replicate measurements.

Statistical analysis

All results were presented as mean value ± standard deviation of three repetitions. The data were subjected to analysis of variance (ANOVA) and statistical significance between mean values was assessed by Turkey's test at the confidence

level of 95%. Pearson's correlation coefficients were calculated to reveal the relationship between assays. Calculations and figures were accomplished using statistical package for the social sciences 16.0 software for Windows (SPSS Inc., Chicago, IL, USA).

Results

Bioactive content

The results shown in Table 1 correspond to the yield and phenolic and flavonoid contents of hydromethanolic extracts obtained from leaves and flowers of three populations of *P. crinita*.

The hydromethanolic extracts of *P. crinita* were obtained with a yield ranging from 21.66 to 26.56% for leaves and 22.26 to 30.08% (w/w) for flowers part.

The obtained data indicated superiority of leaves extracts than flowers part in all populations ($p<0.05$) decreasing in the following order: Ouled benabdelkader>Medjadja> El-Nakhla (Table 1).

Table 1. Yield and bioactive contents of hydromethanolic extracts of three *Phlomis crinita*'s populations

Population	Part	Yield (% w/w)	Phenolic content (µg EGA/mg)	Flavonoid content (µg QE/mg)
Medjadja	leaves	24.80	112.91± 1.18^{a}	42.72± 0.53^{a}
	flowers	26.22	68.67± 1.16^{b}	15.85± 0.40^{b}
El-Nakhla	leaves	24.72	104.34± 1.68^{c}	32.77± 0.72^{c}
	flowers	22.66	59.99± 1.62^{d}	8.09± 0.83^{d}
Ouledbenabdelkader	leaves	21.66	117.96± 1.70^{e}	38.16± 0.38^{e}
	flowers	30.08	86.66± 0.86^{f}	13.89± 0.34^{f}

w/w: weight of extract/weight of sample, **µg EGA/mg:** µg gallic acid equivalents/mg of extract, **µg QE/mg:** µg quercetin equivalents/mg of crude extract, **IC_{50}:** The sample concentration providing 50% DPPH free radical scavenging, **a-f:** values (mean ± standard deviation, n=3) in the same column sharing different letters are significantly different ($p < 0.05$).

As can be seen from Table 1, the leaf extracts exhibited the highest amounts of total flavonoid attaining 42.72±0.53 µg QE/mg in the population of Medjadja followed by Ouled benabdelkader and El-Nakhla respectively with significance variation at $p < 0.05$. In the other hand, the flowers displayed the lowest contents of total flavonoids with significance differences among three populations ($p<0.05$) and respecting the same order of leaves.

Antioxidant activity

In the current study, hydromethanolic extracts of *P. crinita* were subjected to a screening for their possible antioxidant potential by two tests, namely ß-carotene-linoleic acid system and DPPH free radical scavenging assay to better appreciate this beneficial bioactivity. The results are presented in Figures 1 and 2.

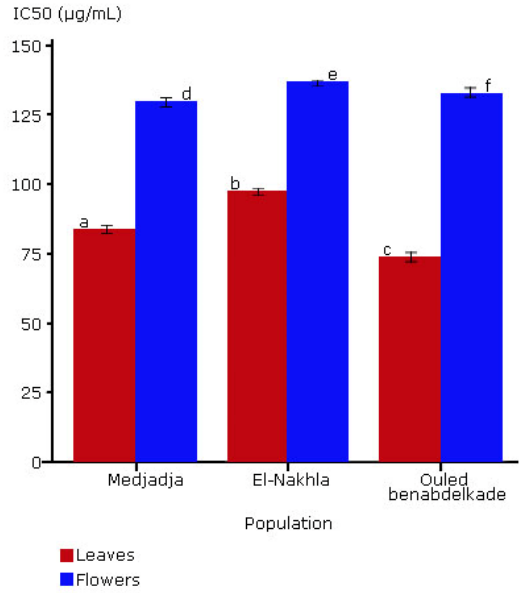

Fig. 1. Antiradical activity of aerial parts of *P. crinita* by DPPH free radical scavenging. IC_{50}: the half inhibitory concentration.

As summarized in Figure 1, there were significant differences ($p<0.05$) between IC_{50} values (inversely proportional to the antiradical efficacy) of parts used for extraction and populations. The leaves showed more DPPH free radical scavenging effectiveness than flowers in three populations. Ouled benabdelkader population had the strongest antiradical activity followed by Medjadja and El-Nakhla respectively.

In the ß-carotene bleaching model, ß-carotene loses rapidly its yellow coloration in the absence of antioxidants such as phenolic compounds that inhibit the oxidation of ß-carotene by neutralizing the free radicals formed within the system. Figure 2 shows the percentage inhibition of ß-carotene bleaching, the highest potency was recorded by Medjadja and El-Nakhla populations without significance difference ($p>0.05$) for leaves whereas the flowers effects were similar ($p>0.05$) in three populations.

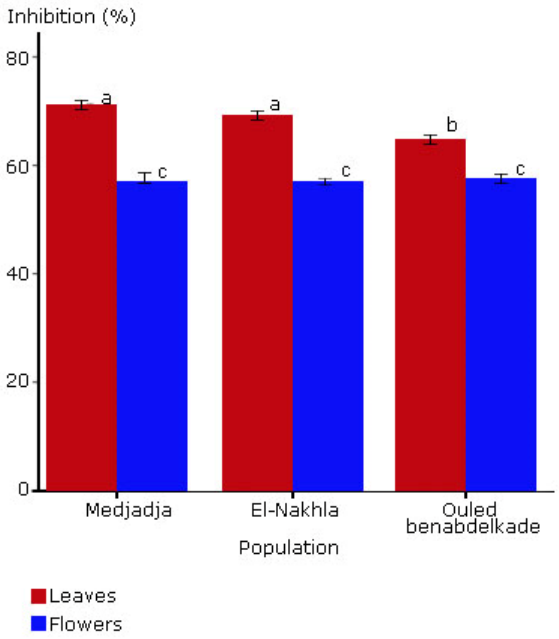

Fig. 2. Antioxidant activity of aerial parts of *P. crinita* by ß-carotene bleaching method.

The correlation analysis between bioactive content and antioxidant activity of leaves and flowers of *P. crinita* is shown in Table 2. The results revealed high significant positive correlation (negative for IC_{50}) between antiradical activity and phenolic content (leaves) and flavonoid content (flowers). There was also a positive correlation between antioxidant assays but not significantly at $p < 0.05$. This finding proves the ability of phenolics compounds from medicinal plants to scavenge free radicals.

Table 2. Pearson's correlation coefficients between assays for leaves and flowers of *P. crinita*

	Leaves				Flowers			
	Phenols	Flavonoids	DPPH	ß-carotene	Phenols	Flavonoids	DPPH	ß-carotene
Phenols	1				1			
Flavonoids	0.657	1			0.557	1		
DPPH	-0.981**	-0.613	1		-0.324	-0.915**	1	
ß-carotene	-0.473	0.252	0.548	1	0.251	0.170	0.903	1

Correlation is significant at the 0.01 level; **DPPH: free radical scavenging; **ß-carotene:** inhibition by ß-carotene bleaching test.

Discussion

With respect to the yield, the solvent mixing water with methanol is the most rentable extract from medicinal plants due to the combination of their individual

solubilisation potential dissolving majority of herbal compounds.[19] Generally, the yield from medicinal plants fluctuates depending on internal (genotype, organ, maturation stage) and external factors (climatic conditions, harvesting time, storage period, origin and extraction method).

To the best of our knowledge, the phenolic and flavonoid compounds of *P. crinita* have not been evaluated in the published literature. However, two reports have evidenced previously the presence of these bioactive components in aerial parts of this species.[4,11]

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[1] and *P. pungens* revealing 41.10±2.69 µg EGA/mg in methanolic extract and 57.68±1.52 µg EGA/mg in water extract.[9]

Habitually, the qualitative features of phenolic compounds in *Phlomis* species consists mainly of phenolic acids commonly chlorogenic, rosmarinic and benzoic acids as well as flavonoids represented essentially by glycosylated apigenin, luteolin, naringenin, eriodictyol and chryseriol,[1,8,9,20,21,22] the beneficial bioactivities of bioactive compounds for human health have been previously elucidated.[23,24]

The findings obtained in antioxidant investigation indicate importance of *P. crinita* as a prosperous source of natural antioxidants. Comparison with other members of same genus confirms its powerful potential than *P. nissolii*, *P. pungens* and *P. armeniaca* from Turkish flora especially in term of DPPH free radical scavenging.[9] The variability between assays, used in the estimation of antioxidant activity, might be attributed to various factors such as nature of phenolic compounds, structure and substitution pattern of hydroxyl groups, mechanisms of antioxidant action in each system and type of radicals targeted.[25]

The results of relationship between phytochemical features and antiradical activity, mentioned in Table 2, indicate that phenolic compounds contribute greatly in the biological potency of leaves whereas flavonoids class is the main actor in the antiradical activity of flowers extracts. Our study is in accordance with previous studies.[9,16,18] The correlation of widely distributed phenolic compounds in medicinal plants with their antioxidant properties has been previously reviewed.[26] The *P. crinita* showed high amounts of bioactive compounds and antioxidant properties, these results confirm the popular use of this species as traditional remedy. It is notable that leaves possess more effective potencies than flowers. Further *in vivo* studies would be required to examine some traditional descriptions of this medicinal plant.

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Conflict of interest

The authors declare that there are no conflicts of interest.

Authors' contribution

Abdelaziz Merouane: Research designing, reviewing and editing the manuscript, helped in statistical analysis, conceiving and supervising the research. Approved the final version of the manuscript.

Sara Fellag: Samples preparation and extraction, antioxidant activity. Approved the final version of the manuscript.

Abdallah Noui: Determination of bioactive content, statistical analysis. Approved the final version of the manuscript.