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Phenolic content and antioxidant activity of hydromethanolic and aqueous extracts of aerial parts of *Phlomis crinita*

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ABSTRACT

Phlomis crinita Cav. is a plant species of the family Lamiaceae including more than 100 perennial herbs, shrubs, and sub-shrubs species native to the Mediterranean, Central Asia, and India. This species is commonly a good natural source of various secondary metabolites. Therefore, the present study was conducted to determine phenolic content and antioxidant activity of hydromethanolic (PC ME) and aqueous extracts (PC AQE) of aerial parts of *P. crinita*. Total polyphenols, flavonoids and tannins were quantified, respectively by the methods of Folin-Ciocalteu reagent, aluminum trichloride ($AlCl_3$) and Bate-Smith method. The *in vitro* antioxidant activity was assessed using DPPH and ABTS•+ radical scavenging, β-carotene-linoleic acid, reducing power and ferrous ion chelating activity assays. PC ME showed high level of tannins (132,13 ±0.68 µg TAE/mg extract) and total phenolic content (82.71±0.79 µg GAE/mg extract), in addition a marked inhibiting oxidation activity of β-carotene/ linoleic acid (74.10%) was observed. Results showed also a higher iron-chelating activity of PC ME (0.20 mg/mL) compared to PC AQE (0.046 mg/mL). The plant extracts revealed a significant antioxidant activity as evidenced by the DPPH and ABTS radical scavenging activity (IC_{50} = 0.103 mg/mL for PC ME and 0.144 mg/mL for PC AQE) for DPPH assay and (IC_{50} = 0.0130 mg/mL for PC ME and 0.0187 mg/mL for PC AQE), as well as the PC ME exhibits higher reducing power (IC_{50} =0.288 mg/mL) than PC AQE (0.296 mg/mL). As a result, *P. crinita* is suggested as a promising and effective therapeutic medicinal plant for the treatment of several diseases.

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Introduction

Oxidative stress is a process that occurs at excessive production/generation of reactive oxygen species (ROS) in our cells and tissues, leading consequently to oxidation of the main cell macromolecules, abnormal gene expression, and cell death (Holze et al., 2017; Sevindik et al., 2017; Korkmaz et al., 2021). Additionally, the ROS at low concentrations in the cell contribute to various metabolic pathways or regulate various physiological functions as a response to biotic and environmental stresses (Pham-Huy et al., 2008; Del Río, 2015; Kına et al., 2021; Akgül et al., 2022). Further, medicinal plants are valuably used in traditional medicine to treat some illnesses and diseases, as well as their secondary metabolites are used in the production of several drugs and drug derivatives (Jamshidi-Kia et al., 2018; Pehlivan et al., 2021; Uysal et al., 2021). The beneficial effects of medicinal plants against oxidative stress resulting in increased oxidative injuries and diseases are undoubtedly due to the richness in major bioactive molecules, in particular phenolic compounds (phenolic acids, flavonoids, and tannins). (Sharma et al., 2013). *P. crinita* is a large species of Lamiaceae family, including more than 100 perennial herbs, shrubs, and sub-shrubs species native to the Mediterranean, Central Asia, and India (Mathiesen et al., 2011; Sarikurkcu et al., 2015). Also, the species is considered as an efficient natural source of various

secondary metabolites, and a powerful antioxidant plant species owing to its bioactive molecules, including iridoids, diterpenoids, phenylpropanoids, phenylethanoids, alkaloids phenolic acids, and flavonoids (Sarkhail et al., 2014; Kabouche et al., 2005). *P.* species have been previously used in the Jaén region (Spain) to treat some health conditions, such as veterinary diarrhea, smooth muscle pains (Amor et al., 2009), and bacterial infections (Couladis et al., 2000), and similarly, the Algerian people have used this species to heal lesions and burns (Amor et al., 2009). Accordingly, the antidiabetic activity (Sarkhail et al., 2007). antiulcerogenic (Amor et al., 2009) vascular (Ismailoglu et al., 2002) and antioxidant activity (Merouane et al., 2019) of *P. crinita* have been well documented. In this regard, this work was undertaken to determine the phenolic contents, gastroprotective and antioxidant properties of two different extracts of *P. crinita.*

Materials and methods

Plant Material

The flowering aerial part of *P. crinita* was collected from Jijel city (North-East of Algeria) .After the botanical identification of the plant ,the areal part was washed by tap water to get rid of impurities and dust, dried in the shade at room temperature, and then ground into a mortar to form a fine powder.

Extraction Procedure

The PC ME of the plant was prepared as described elsewhere by Marrkham (1982) with slight modification. Briefly, the dried powder of *P. crinita* was extracted at room temperature with methanol (85%) for 72 hours. The resulted mixture was filtered and evaporated at 45°C to form methanolic extracts. Whilst, the aqueous extract was prepared according to the method previously described by Kandil and collaborators (1994). Here a 100 g of plant material powder was boiled in 1 L of distilled water for 4 hours, and then filtered and dried at 45°C to form an aqueous extract. The dried ME and AQE extracts were screened for their pharmacological properties.

Determination of Total Polyphenols

The total phenolic compounds in plant extracts were determined by the Folin-Ciocalteu (FC) method with slight modifications (Sánchez-Rangel et al., 2013). Briefly, 100 µl of each extract or standard (gallic acid) was mixed with a 500 µl folin-ciocalteu reagent (diluted 10 times). After 4 minutes, 400 µl of 7.5% sodium carbonate solution (NA2Co3) was added, and then the resulted mixture was incubated for 1 hour and a half at the laboratory temperature. The mixture absorbance was measured against blank at 765 nm, and the total polyphenols content was expressed as gallic acid equivalents (mg GA/g DW).

Determination of Total Flavonoids Contents

The total flavonoid contents in plant extracts were quantified by AlCl3 method (Bahorun et al., 1996). Briefly, 1 mL of AlCl3 solution (2% dissolved in methanol) was mixed with 1 mL of the extract samples and incubated at room temperature for 10 min prior to the absorbance reading at 430 nm. The results were expressed as quercetin equivalent per gram of dry plant extract weight (mg QE/g DW).

Tannins Quantification

The total tannins content in plant extracts was quantified by the method of Bate smith (1973) based on the precipitation of hemoglobin from fresh bovine blood by tannins. In brief, a volume of diluted blood solution (optical density = 1.6) with the same volume of plant extract was mixed and dissolved in distilled water. After 20 min of incubation, the mixture was centrifuged (400 rpm for 10 d) and then subjected to spectrophotometric measurement at 576 nm. The amount of tannins was expressed as tannic acid equivalents (mg tannic acid/g dried extract).

Antioxidant Activity Assays

DPPH radical scavenging assay

The scavenging effect of the plant extracts on the DPPH free radical scavenging ability was spectrophotometrically determined as described elsewhere (Burits and Bucar, 2000). In this method, a volume of 50 µL was taken from plant extracts of various concentrations, and mixed with 1250 µL of methanolic solution of DPPH (0.004%). Of note, the butylated hydroxytoluene (BHT) was used as a positive control. All mixtures were incubated for 30 minutes in darkness at room temperature prior to spectrophotometric measurement at 517 nm in comparison with the negative control. The free radical scavenging activity of the extracts is expressed in percent % and calculated as follow:

$$\text{Scavenging activity (\%)} = (AC - AS) \times 100 / AC$$

Where Ac and As are the absorbances of the DPPH solution without extract and the test sample respectively. The antioxidant activity is expressed in terms of IC50 (mg/mL).

ABTS radical scavenging assay

The antioxidant activity of the plant extracts assayed by ABTS method was according to procedures described by Re et al. (1999) with slight modifications. the working solution was prepared by mixing two stock solutions (mM of ABTS solution and 2.4 mM of potassium persulfate solution) in equal quantities and allowing them to react for 24 h at room temperature in the dark. The solution was mixed and diluted in 1 mL cuvette containing ABTS solution and methanol to obtain a spectrophotometric absorbance of 0.706 ± 0.01 at 734 nm. The Fresh ABTS solution was prepared for each assay. Plant extracts (50 µl) were afterward mixed with 1 mL of the ABTS solution and the absorbance was read at 734 nm after 30 min. The ABTS scavenging capacity of the extracts was compared to that obtained with BHT. ABTS radical scavenging activity is calculated as follows:

$$\text{ABTS (\%)} = \text{Abs control} - \text{Abs sample} / \text{Abs control}$$

Where Abs control is the absorbance of ABTS radical in methanol; Abs sample is the absorbance of ABTS radical solution mixed with sample extract/standard.

All determinations were performed in triplicate (n = 3).

β-carotene bleaching assay

The antioxidant activity in this test was determined by the ability to inhibit the formation of diene conjugated hydroperoxide bonds and volatile organic compounds resulting from the oxidation of linoleic acid (Dapkevicius et al., 1998). This method consists to dissolve 0.5 mg of β-carotène in 1 mL of chloroform, to add 25 µL of linoleic acid and 200 mg of Tween 40. The chloroform was completely evaporated at 40° C, and followed by addition of 100 mL of oxygen-saturated distilled water. After that, the emulsion system was prepared by adding 2.5 mL of the resulted mixture and placed in test tubes containing 350 µl (2 mg/mL) of the prepared samples and incubated at darkness and room temperature. BHA and Gallic acid (GA) were also used as a positive control. The absorbance was measured at 490 nm during 0, 1, 2, 3, 4, 6, 12, and 24 h. The antioxidant activity of the samples was compared to that of BHT and negative control. The activity of relative antioxidant extracts (AA%) is calculated according to the following equation:

$$A\% = (A_S / A_C) \times 100$$

Where Ac and As are the absorbance of BHA at t=0 and test sample respectively.

Ferrous ion chelating activity assay

The Ferrous ion chelating activity of the plant extracts was determined by the method based on the capacity of extract samples to chelate ferrous ion and to inhibit the formation of ferrous ion-ferrozine complex, after long term incubation with $FeCl_2$ (Decker and Welch, 1990). 250 µL of the extracts (in different concentrations) was mixed with 50 µL of $FeCl_2$ (0.6mM in distilled water) and 450 µL of methanol. After 5min of incubation time, 50 µL of ferrozine (5mM) solution was added and the mixture was then stirred for 10 minutes up to the end of the reaction. The absorbance was measured at 562 nm, and the rate of chelation of iron ions is calculated according to the following equation:

$$\text{Chelation (\%)} = [(Ab_C - Ab_S) / Ab_C] \times 100$$

Where Abc is the control absorbance, and Abs is the absorbance of the test sample

Reducing power assay

Then reducing power of the plant extracts was determined by the method previously described (Chung et al., 2005). In brief, 0.1 mL of various methanol-diluted extracts were mixed with the same volume of phosphate buffer (0.2 M, pH=6.6) and 0.1 mL of K_3FeCN_6 (1%). The resulting mixture was incubated at 50° C for 20 min, and 0.25 mL (1%) of acid trichloroacetic was then added to stop the reaction of transformating ferricyanide to ferrocyanide. Thereafter, the mixture was centrifuged at 3000 /minutes for 10 minutes, and 0.25 mL of the supernatant was added to 0.25 mL of distilled water and 0.5mL $FeCl_3$ (0.1%). The absorbance of the resulting solution was measured at 700 nm to quantify the formed ferric ferrocyanide.

Statistical Analysis

Statistical analysis was performed using the Graph Pad Prism (version 6.01 for Windows). All results were displayed as mean ± SD, and were tested by One-way analysis of ANOVA with Tukey post hoc test. $p < 0.05$ was considered significant.

Results

Phytochemical Analysis

As indicated in table 1, the PC ME revealed high values of total flavonoids content (26.16±0.88 ug QE/mg), since the high tannins content (132, 13 ± 0.68 µg TAE/mg) was noticed in PC AQE. Whereas, the total phenolic content in terms of mg GAE/g of the dry weight of extract decreased in the following order: PC ME> PC AQE.

Antioxidant Activities

DPPH radical scavenging assay

The antioxidant activity of the extracts evidenced by the determination of IC_{50} using DPPH assay reveals a lower antioxidant activity of both extracts as compared with the standard (Figure 1). Hence, their antioxidant effect shows significant difference (P<0.0001,), and their scavenging activity decreases in the order of PC AQE> PC ME > BHT with the corresponding IC_{50} values of 0,144 mg/mL 0,103 mg/mL and 0,007 mg/mL respectively.

ABTS radical scavenging activity

As shown in Figure 2, PC ME exhibited a stronger antioxidant activity than that of the positive control BHA (0.0181 mg/mL). Also, the ABTS radical scavenging antioxidant activity of PC ME (0.130 mg/mL) differs significantly (P<0.0001) and no significantly with that of PC AQE (0.0187 mg/mL).

β -carotene / Linoleic acid

Figure 3 depicts a good inhibitory effect of *P .crinita* extracts on oxidation of β-carotene molecules; the inhibitory capacity of PC ME (74.47%) is lightly higher (P>0.05) than that recorded seen in the presence of gallic acid used as a positive control (64.37%) and PC AQE (61.26%).

Table 1. Contents of total phenolics, flavonoids and tannins in *P. crinita* extracts

	PC ME	PC AQE
Total phenolics[1]	82.71±0.79	81.28±0.13
Total flavonoids[2]	24.09±0.45	26.16±0.88
Total tannins[3]	132.13±0.68	54.86±0.4

1: (ug GAE/mg extract); 2: (ug QE/mg extract); 3: (ug TAE/mg extract)

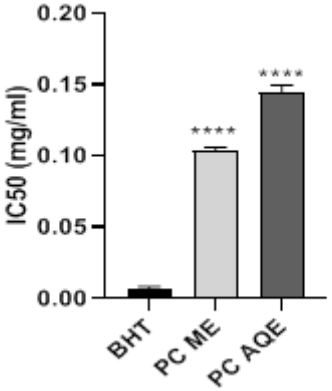

Figure 1. IC50 values of *P. crinita* extracts determined by DPPH assay. Results are provided as mean± SD (n=3); ****P<0.0001 Significant difference *vs* the standard BHT. PC ME: *P crinita* methanol extract, PC AQE: *P crinita* aqueous extract.

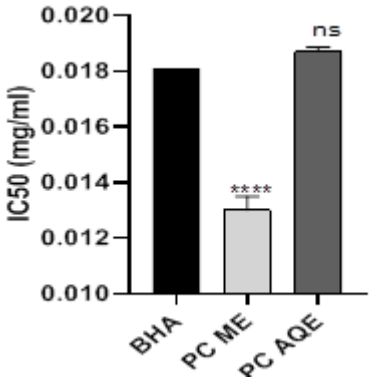

Figure 2. Antioxidant activity in ABTS free radical scavenging assay of plant extracts and the standard BHA.

Data are presented as IC50 means ± SD (n = 3). ****P<0.0001 statistically significant *vs* the standard BHA.

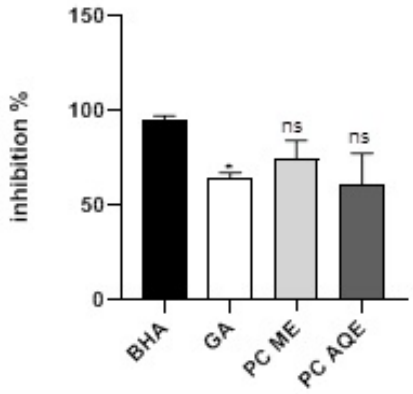

Figure 3. Inhibition percentage of plant extracts assayed by β-carotene test using linoleic acid.

Samples were assayed after 24h using BHA as a standard, and data are displayed as mean±SD (n = 3). ns: no significant difference ; *P,0.05 Statistically significant PC ME: *P crinita* methanol extract, PC AQE: *P crinita* aqueous extract.

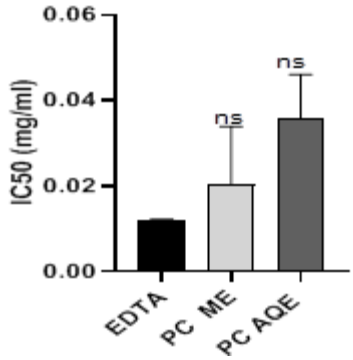

Figure 4. Metal chelating activity *P.crinita* extracts.

Data are presented as mean ±SD (n=3). ns: no significant difference *vs* EDTA as standard. PC ME: *P crinita* methanol extract, PC AQE: *P crinita* aqueous extract.

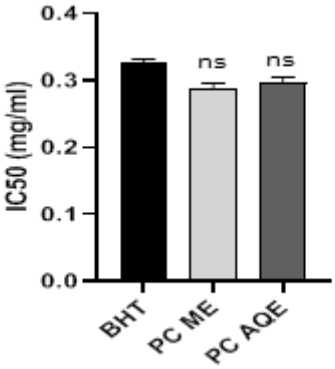

Figure 5. IC50 values of reducing power of P. extracts and BHT.

Values are expressed as mean ± SD (n = 3); ns: no significant difference *vs* the standard BHT. PC ME*: P. crinita* methanol extract, PC AQE: *P. crinita* aqueous extract.

Metal chelating activity

As shown in Figure 4, both of the plant extracts have a strong ability to chelate metals (0.20 mg/mL for PC ME and 0.035 mg/mL for PC AQE), and hence this activity is very close to that of the positive control EDTA (0.011 mg/mL).

Reducing power

Figure 5 depicts a concentration dependent manner variation in the reducing power activity of *P. crinita* activity. In addition, the PC ME exhibits the higher reducing power (IC50 = 0.288 mg/mL) than that of PC AQE (0.296 mg/mL) and BHT (IC_{50} = 0.327 mg/mL).

Discussion

In this study, P.*crinita* of both extracts showed comparable polyphenols level, and this is similar to that found in the study of Merouane and his collaborator (2018) for PC ME (81.80 ± 2.25 µg GAE/mg) and higher for the aqueous extract (47.67 ± 2.08 µg GAE/mg extract). Furthermore, *Phlomis* species was previously reported to contain mainly chlorogenic, rosmarinic, and benzoic acids (Sarikurkcu et al., 2015, 2014), providing to this species remarkable variability in total phenolic content. Our data showed the highest amount of tannins (132.13ug TAE/mg extract) in PC ME as compared to PC AQE, and to that obtained by Dellai et al. (2009) (21.5%). Similarly, these authors have reported a significant quantity of flavonoids in *P. crinita* extracts. Several previous studies have reported that the antioxidant activity is directly correlated with the content of phenolic compounds contained in plant extracts. However, these studies did not involve in this relationship other chemical features of the phenols, including nature and structure. In this regard, a previous study (Cho et al., 2012) showed that the double bond between carbon 2 and 3 atoms connected to the hydroxyl group in cycle C increases the scavenging power of flavonoids, and the catechol ring (O-dihydroxybenzene) increases the scavenging power of flavonoids, and this is evidenced by the content of flavonoids derived from hydroxy-luteolin (the most important flavonoids in the PC ME extract) (Kabouche et al., 2005). Moreover, the antioxidant activity of the plant extracts determined by DPPH free radical scavenging assay was found to be strongly correlated with the content of phenolic components, exactly as reported by some previous studies (Barhé and Tchouya, 2016; Bencheikh et al., 2016). Hence, a potent antioxidant activity was noticed respectively in

methanolic and aqueous extracts of *P. crinita*. These results are greater than those obtained by Merouane et al (2019) (IC_{50} = 1.04 ± 0.02mg/ mL) and three times higher than those of Amor et al (2009) (32 ug/mL). Further, *P.crinita* extracts revealed relatively strong antiradical activity toward the ABTS•+ free radical,. This finding is in agreement with the study of Dellai et al. (2009), reporting high-dose-dependent ABTS•+ radical scavenging activities with an inhibition percentage of ABTS radical formation of 94% in methanolic flower's extracts of *P. crinita*. This activity may be due to the presence of tannins, flavonoids, iridoids, sterols, cardiac glycosides, and anthraquinones in the plant extract. Interestingly, free radicals can be scavenged by antioxidant agents whether through hydrogen atom transfer (HAT) from O-H bond, or single electron transfer–proton transfer (SET–PT) (Košinová et al., 2011) As reported Zhang and Wang (2009), the antioxidant radical scavenging activity of plant extracts is related to the contents in phenolic compounds which may act as free radical scavengers owed to their hydrogen-donating ability and scavenging ability. On top of that, the results showed that the ability to inhibit lipid peroxidation was higher in PC ME (74.47%) than that in PC AQE (61.26%) and the gallic acid. These results are quite similar to some previous result studies (Merouane et al., 2020, 2019), reporting the powerful antioxidant potency of *P. crinita* that can scavenge ROS in the lipid milieu. In this study, the IC50 value of metal chelating assay of PC ME is lower than that of PC AQE, and this is likely due to the high tannins content in PC ME (Bencheikh et al., 2016). As previously reported, the effective metal chelating ability of *Phlomis* genus, including *P. viscosa P. platystegia* and *P. brachyodon* Boiss (Al-Qudah et al., 2018) *P. pungens* (Keser et al., 2012) may be due to the presence of verbascoside (the common phenylethanoid glycosides, in *Phlomis* genus) in their extracts. Also, it was reported (Fernandez et al., 2002) that flavonoids can chelate metals by two major phases; the loss of a hydrogen atom from the hydroxyl groups, and the formation of a stable complex between the oxygen atom and the iron ion. Recently, the polyphenols and flavonoids have been reported to be excellent chelators for iron and copper (Papuc et al., 2017), and noteworthy flavonoids can chelate metals by more than one possible process way. This depends on flavonoid structure, the type of metal ion and pH of the reaction (e.g., in *vivo* conditions: acidic in the stomach and alkaline in intestine). In vitro previous studies (Chirug et al., 2021; Malešev and Kuntić, 2007) have revealed many different dependencies between pH and the flavonoid moieties responsible for metal chelation. As instance in quercetin, the ortho-dihydroxyl group chelates part in Fe^{3+}, Cu^{2+} and Al^{3+} in alkaline solutions, but can create complexes with Fe^{3+} (1:2) in acidic solutions with coordination via the 3–4 or 4–5 site and induce binding of Fe^{3+} to the catechol group in a 1:1 metal/ligand ratio at higher pH. Additionally, the strong correlation between the Fe^{3+} reducing capacity and the inhibition of lipid peroxidation has been previously proved (Yen and Hsieh, 1998). Our findings revealed that the reducing power activity of *P. critina* extracts varies in a concentration-dependent manner, and is close to that of BHT. This activity may be due to its richness in phenolic compounds. Noteworthy, the antioxidant activity was reported to be directly correlated with the reduction power of some plant components, depending on multiple parameters such as the concentration of the metal ion and polyphenol, temperature, pH, and the presence of complexing agents (Ghedadba et al., 2015).

Conclusion

Our study investigating the phytochemical compositions and the antioxidant capacities of two extracts of *P. crinita* using five different in vitro assays showed significant quantities of polyphenols, flavonoids, and tannins, and a significant antioxidant activity which explains the effectiveness of their widespread use in traditional medicine.

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