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Antioxidant Potential of Hydro-methanolic Extract of *Prasium majus* L: An *in vitro* Study

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Abstract: Phytochemicals are extensively found at different levels in many medicinal plants. To investigate the phenolic compound content and *in vitro* antioxidant activity of hydro-methanolic extract from *Prasium majus* L. (Lamiaceae). The present investigation comprises, estimation of total polyphenol, flavonoid, tannin, *in vitro* antioxidant assays such as total antioxidant capacity, DPPH, ABTS, β-carotene and ferric reducing power. *P. majus* exhibited 64.25 mg GAE g^{-1} extract of polyphenol phenol content and better scavenging activity of DPPH (IC_{50} = 7.95 μg mL^{-1}), $ABTS^{\bullet+}$ (IC_{50} = 373.78 μg mL^{-1}) and β-carotene (IC_{50} = 122.56 μg mL^{-1}). Our results clearly demonstrated that hydro-methanolic extract *P. majus* has antioxidant capacity. Therefore is a valuable source of natural antioxidants.

Key words: *Prasium majus*, antioxidant, extract, phenolic classes

INTRODUCTION

Lipid peroxidation is one of the major causes of deterioration in foods that results in the formation of potentially toxic compounds. Synthetic antioxidants, such as Butylated Hydroxyanisole (BHA), Butylated Hydroxytoluene (BHT), Tert-butyl Hydroquinone (TBHQ) and Propylgallate (PG), are widespread food additives used to preserve against deterioration; however, their use is increasingly restricted, due to their potential health risks and toxicity.

There is currently an upsurge of interest in phytochemicals as potential new sources of natural antioxidants. The goal is to use them in foods and pharmaceutical preparations to replace synthetic antioxidants (Wong *et al*., 2006; Aluko *et al*., 2013). The antioxidant activity of phenolic compounds is mainly due to their redox properties, which allow them to act as reducing agents, hydrogen donators and singlet oxygen quenchers (Rice-Evans *et al*., 1995). However, there are still many plant species that were not evaluated with respect to their antioxidant potential (Kumbhare *et al*., 2012).

The family of Lamiaceae consists of about 233 genera and 6900 species worldwide (Lee *et al*., 2011). Many species of the Lamiaceae family are considered of high importance because of their uses in medicine, culinary and cosmetics. *Prasium majus* L. is a wildflower species belonging to this family. An infusion of the leaves and flowers of the plant is used for gastrointestinal diseases. In this paper we screen the hydro-methanolic extract for their free radical scavenging and antioxidant activity. That should complement to their therapeutic value and improve the popularization of the specie.

MATERIAL AND METHODS

Preparation of plant extract: *P. majus* was harvested in April 2012 from northwest of Algeria (Tlemcen). It was identified in the Laboratory of Natural Products, Department of Biology, University of Tlemcen, Algeria. Voucher specimen was deposited at the Herbarium of the Laboratory. The powder of plant dried (2 g) was first extracted by 25 mL hexane to remove lipids and chlorophylls. After centrifugation, the pellet was resuspended in 25 mL methanol/water (80:20, v:v) for 24 h.

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The obtained extract was filtered through a Whatman N° 4 filter paper and evaporated at 45°C under reduced pressure, re-dissolved in methanol at a concentration of 1 mg mL^{-1} and stored at 4°C for further use (Trabelsi *et al*., 2010).

Quantification of phenolic classes

Total polyphenol quantification: Total polyphenol content of the plant extract was determined using Folin-Ciocalteu reagent (FC) (Dewanto *et al*., 2002), using gallic acid as a standard. An aliquot (250 µL) of diluted sample extract was added to 0.5 mL of distilled water and 0.125 mL of the FC. The mixture was shaken and allowed to stand for 6 min, before addition of 1.25 mL of 7% Na_2CO_3. The solution was then adjusted with distilled water to a final volume of 3 mL and mixed thoroughly and held in dark for 90 min at ambient temperature. After incubation, the absorbance at 760 nm was recorded. Total polyphenol content of plant parts was expressed as mg of gallic acid equivalents per gram of dry weight (mg GAE.g^{-1}DW) through the calibration curve (0-400 µg mL^{-1}range).

Flavonoid quantification: Total flavonoid content was measured using a colorimetric assay (Dewanto *et al*., 2002). An aliquot (250 µL) of diluted sample or standard solution of catechin was added to 75 µL of $NaNO_2$ solution (7%) and mixed for 6 min, before adding 150 µL $AlCl_3$ (10%). After 5 min, 0.5 mL of NaOH solution (1M) was added. The final volume was adjusted to 2.5 mL, thoroughly mixed and the absorbance of the mixture was determined at 510 nm. Total flavonoid content was expressed as mg catechin equivalent per gram of dry weight (mg CEf.g^{-1}DW), through the calibration curve of catechin (0-400 µg mL^{-1}range).

Tannin quantification: The tannin content was measured using a colorimetric assay (Sun *et al*., 1998). Briefly, 50 µL of diluted extract solution was mixed with 3 mL of 4% vanillin-methanol solution and 1.5 mL of concentrated hydrochloric acid and the mixture was allowed to stand for 15 min. Absorbance was read at 510 nm against the blank (water). Tannin content was expressed as mg Catechin Equivalents (CE) per gram of dry weight (mg CEt.g^{-1}DW), through the calibration curve of catechin. The calibration curve range was 0-400 µg mL^{-1}.

Determination of antioxidant activities: There are several methods for determination of antioxidant activities. The chemical complexity of extract, often a mixture of dozens of compounds with different functional groups, polarity and chemical behaviour, could lead to scattered results, depending on the test employed. Therefore, an approach with multiple assays for evaluating the antioxidant potential of extract would be more informative and even necessary. In this study, mainly five methods, evaluation of total antioxidant capacity, DPPH (1,1-diphenyl-2-picryl hydrazyl), ABTS (2,2'-azino-bis-3-ethyl benzthiazoline-6-sulphonic acid), β-carotene bleaching method and ferric reducing power, were successively measured for hydro-methanolic extract of *P. majus* L.

Total antioxidant capacity: This assay is based on the reduction of Molybdene (VI) to Molybdene (V) by the sample extract, which produces a green phosphomolybdenum (V) complex under acidic pH conditions (Prieto *et al*., 1999). An aliquot (0.1 mL) of hydro-methanolic extract was combined to 1 mL of reagent solution (0.6 M sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The tubes were incubated in a thermal block at 95°C for 90 min. After, the mixture had cooled to room temperature. The absorbance of each solution was measured at 695 nm against a blank. The antioxidant capacity was expressed as mg gallic acid equivalent per gram dry weight (mg GAE.g^{-1}DW). The calibration curve of gallic acid range was 0-400 µg mL^{-1}.

DPPH assay: The ability of the corresponding extracts to donate hydrogen atoms or electrons was measured from the bleaching of purple coloured methanol solution of DPPH (Masuda *et al*., 1999). Fifty microlitre of various concentrations (1-100 µg mL^{-1}) of hydro-methanolic extract was added to 1950 µL of 6.34×10^{-5} M DPPH radical solution in methanol. The mixture was shaken vigorously and allowed to stand for 30 min in the dark. The absorbance of the resulting solution was measured at 517 nm and Butylated Hydroxytoluene (BHT) was used as a positive control (standard). Inhibition of DPPH radical was calculated as follows:

$$\text{DPPH scavenging effect (\%)} = \frac{A_0 - A_1}{A_0} \times 100 \quad (1)$$

where, A_0 and A_1 are the absorbance at 30 min of the control and the sample, respectively.

The antiradical activity was expressed as IC_{50} (µg mL^{-1}), the extract dose required to cause a 50% decrease of the absorbance at 517 nm. A lower IC_{50} value corresponds to a higher antioxidant activity.

ABTS assay: ABTS radical-scavenging activity of extracts was determined according to Re *et al*. (1999). The $ABTS^{•+}$ cation radical was produced by the reaction between 5 mL of 14 mM $ABTS^{•+}$ solution and 5 mL of 4.9 mM potassium persulfate ($K_2S_2O_8$) solution, stored in

the dark at room temperature for 16 h. Before use, this solution was diluted with methanol to get an absorbance of 0.700±0.020 at 734 nm. In a final volume of 1 mL, the reaction mixture comprised 950 μL of $ABTS^{•+}$ solution and 50 μL of the hydro-methanolic extract at various concentrations (20-200 μg mL^{-1}). It was homogenised and its absorbance was recorded at 734 nm. Methanol blanks were run in each assay and all measurements were done after at least 6 min. Similarly, the reaction mixture of standards group was obtained by mixing 950 μL of $ABTS^{•+}$ solution and 50 μL of BHT and Trolox. As for the antiradical activity, $ABTS^{•+}$ scavenging ability was expressed as IC_{50} (μg mL^{-1}). The inhibition percentage of $ABTS^{•+}$ radical was calculated using the following formula (2):

$$ABTS^{•+} \text{ scavenging effect (\%)} = \frac{A_0 - A_1}{A_0} \times 100 \quad (2)$$

where, A_0 and A_1 have the same meaning as in Eq. 1.

β-carotene bleaching test: A modification of the method described by Koleva *et al.* (2002) was employed. The β-carotene (2 mg) was dissolved in 20 mL chloroform and to 4 mL of this solution, linoleic acid (40 mg) and Tween 40 (400 mg) were added. Chloroform was evaporated under vacuum at 40°C and 100 mL of oxygenated ultra-pure water was added, then the emulsion was vigorously shaken. Sample extract and standards group (BHA and BHT) were prepared in ethanol. An aliquot (150 μL) of the β-carotene/linoleic acid emulsion was distributed in each of the wells of 96-well microtitre plates and 10 μL of various concentrations (20-200 μg mL^{-1}) of hydro-methanolic extract was added. The microtitre plates were incubated at 50°C for 120 min and the absorbance was measured using a model EAR 400 microtitre reader (Labsystems Multiskan MS) at 470 nm. Readings of all samples were performed immediately (t = 0 min) and after 120 min of incubation. The antioxidant activity of the extract was evaluated in term of β-carotene blanching using the following formula (3):

$$\beta\text{-carotene bleaching inhibition (\%)} = \frac{S - C_{120}}{C_0 - C_{120}} \times 100 \quad (3)$$

where, C_0 and C_{120} are the absorbance values of the control at 0 and 120 min, respectively and S is the sample absorbance at 120 min. The results were expressed as IC_{50} values (μg mL^{-1}).

Iron reducing power: The capacity of plant extracts to reduce Fe^{3+} was assessed by the method of Oyaizu (1986). One milliliter of various concentrations (50-500 μg mL^{-1}) of hydro-methanolic extract was mixed with 2.5 mL of sodium phosphate buffer (0.2 M, pH 6.6) and 2.5 mL of 1% potassium ferricyanide and the mixture was incubated at 50°C for 20 min. After that, 2.5 mL of 10% trichloroacetic acid were added and the mixture was centrifuged at 650×g for 10 min. The upper layer fraction (2.5 mL) was mixed with 2.5 mL of distilled water and 0.5 mL of ferric chloride and thoroughly mixed. The absorbance was measured at 700 nm and ascorbic acid was used as a positive control. A higher absorbance indicates a higher reducing power. EC_{50} value (μg mL^{-1}) is the effective concentration giving an absorbance of 0.5 for reducing power and was obtained from linear regression analysis.

Statistical protocol: Values shown in tables were Means±Standard Deviations of three parallel measurements. The IC_{50} values were calculated from linear regression analysis.

RESULTS AND DISCUSSION

Total polyphenol, flavonoid and condensed tannin contents: The Gallic acid linear curve was obtained using the formula y = 0.00253x+0.01344 (R^2 = 0.9954). Using this gallic acid linear curve, total polyphenol content of *P. majus* value was 64.25±0.30 mg GAE g^{-1} DW. The total flavonoid content was obtained using the regression calibration curve y = 0.00355x+0.09903 (R^2 = 0.997) with catechin equivalent, the value was 31.56±0.20 mg CEf g^{-1} DW. The total tannin content was obtained using the regression calibration curve y = 0.00076x-0.00760 (R^2 = 0.9948) with catechin equivalent, the value was 7.37±0.20 mg CEt g^{-1} DW.

The findings of the phytochemical screening indicated that extract hydro-méthanolic is rich in polyphenol, flavonoids and tannins which may be responsible for the antioxidative efficacy (Loew and Kaszkin, 2002; Gautam *et al.*, 2010). Due to redox properties, these compounds play an important role in adsorbing and neutralizing free radicals, quenching singlet and triplet oxygen or decomposing peroxides (Zheng and Wang, 2001; Paliwal *et al.*, 2011). It has been also recognized that flavonoids show antioxidant activity through scavenging or chelating process and their effects on human nutrition and health are considerable (Kessler *et al.*, 2003).

Lamiaceae species are known to produce a diverse array of phenolic compounds, these phytochemicals can occur either as aglycones or glycosides. Concerning our specie no references could be found despite the thorough literature survey. Comparing the obtained results with the

previously data published by Stagos *et al.* (2012), on polyphenol contents of 24 species of Lamiaceae, whose contents obtained are between 91-575 mg GAE.g^{-1} DW, we have found that total phenolic contents in our extract is lower.

Recent studies have shown that storage time (Aganga and Mosase, 2001), extrinsic factors (Ksouri *et al*., 2008), genetic (Ebrahimzadeh *et al*., 2008) and physiological factors (Maisuthisakul *et al*., 2007) have a strong influence on the content of polyphenols, which makes comparison difficult.

Antioxidant activity of hydro-methanolic extract: The phenolic compounds are very important plant constituents because of their scavenging ability on free radicals due to their hydroxyl groups (Khomdram and Singh, 2011). Therefore, their content in plants can directly contribute to their antioxidant action. To the best of our knowledge, we were among the first to provide data on the antioxidant effect of the hydro-methanolic extract of *P. majus* by five methods.

Total antioxidant capacity: The Gallic acid linear curve was obtained using the y = 0.3072 lnx - 0.7541 (R^2 = 0.992) and the results are expressed as mg gallic acid equivalent. The result for the total antioxidant capacity of hydro-methanolic extract was 85.12±0.80 mg GAE g^{-1} DW (Table 1).

DPPH radical-scavenging activity: The scavenging ability of hydro-methanolic extract on DPPH radical is represented in Table 2 and compared with BHT as positive control (a synthetic antioxidant). The scavenging activity of the investigated extract varied widely from 12 to 98.5% (IC_{50} = 7.95±0.60 μg mL^{-1}) and in standard from 10.78 to 79.88% (IC_{50} = 15.6±0.45 μg mL^{-1}). The DPPH radical contains an odd electron which is responsible for the absorbance at 517 nm and also for a visible deep purple colour. When DPPH accepts an electron donated by an antioxidant compound, the DPPH is decolorised which can be quantitatively measured from the changes in absorbance (Pal aknkd Mitra, 2010). It was observed that the radical scavenging activity is increasing with the increase of phenolic compound content (Oki *et al*., 2002). The extract was also reported a high concentration between DPPH radical scavenging potential and total polyphenol content (Liu and Ng, 2000; Siriwardhana *et al*., 2003).

Compared with positive control, the hydro-methanolic extract was presented a high antioxidant activity, higher than BHT. That result suggests that *P. majus* possess phenolic compounds that have a high potential to neutralize free radicals. Petersen and Simmonds (2003) who reported the existence of rosmarinic acid-caffeic acid conjugates in lamiaceae plants, this may explain the increased the scavenging ability of DPPH radical.

ABTS radical-scavenging activity: The effect of hydro-methanolic extract and standard on $ABTS^{\bullet+}$ cation was compared and shown in the Table 3. The scavenging effect increases with the concentration of standard and samples. At 620 μg mL^{-1}concentration, *P. majus* possessed 73.07% scavenging activity on $ABTS^{\bullet+}$. IC_{50} of *P. majus* (373.78±5.1 μg mL^{-1}) showed lower activity than the standard BHT (73.1±0.85 μg mL^{-1}).

β-carotene bleaching method: The radical scavenging capacity based on β-carotene was determined and the result is shown in Table 4. The percentage of scavenging effect on the β-carotene was increased with the increase in the concentrations of the extract from 125-1000 μg mL^{-1}. The percentages of inhibition were varying from 42.07% (in 125 μg mL^{-1} of the extract) to 70.43% (in 1 mg mL^{-1} of extract). The IC_{50} value of the hydro-methanolic extract was estimated at 122.56±4.3 μg mL^{-1}. The BHT in all concentrations showed higher percentage of inhibition of free radicals than the extract, IC_{50} = 11.5±1.5 μg mL^{-1}, ten times more effective than the extract.

Table 1: Total antioxidant capacity, total polyphenol, flavonoid and condensed tannin contents of hydro-methanolic extract of *P. majus*

	Polyphenol content (mg of GAE g^{-1} DW)	Flavonoid content (mg of CEf g^{-1} DW)	Tannin content (mg of CEt g^{-1} DW)	Total antioxidant capacity (mg of GAE g^{-1} DW)
P. majus	64.25±0.30	31.56±0.20	7.37±0.20	85.12±0.80

Table 2: DPPH activity of hydro-methanolic extract of *P. majus*

Concentration (μg mL^{-1})	Percentage activity (%)	IC_{50} (μg mL^{-1})
1.25	12.00	7.95±0.60
3.75	45.21	
15	91.37	
22.5	98.52	

Table 3: ABTS activity of hydro-methanolic extract of *P. majus*

Concentration (μg mL^{-1})	Percentage activity (%)	IC_{50} (μg mL^{-1})
80	17.82	373.78±5.1
160	28.11	
320	47.32	
620	73.02	

Table 4: β-carotene activity of hydro-methanolic extract of *P. majus*

Concentration (μg mL^{-1})	Percentage activity (%)	IC_{50} (μg mL^{-1})
125	42.07	122.56±4.3
250	56.81	
500	61.48	
1000	70.43	

Table 5: Reducing power activity of hydro-methanolic extract of *P. majus*

Concentration ($\mu g\ mL^{-1}$)	DO	EC_{50} ($\mu g\ mL^{-1}$)
150	0.244	354±4.6
300	0.408	
600	0.903	

Iron reducing power: The reducing properties of antioxidants are generally associated with the presence of reductones, such as BHT and other secondary metabolites. Such reductones exert antioxidant action by breaking the free radical chain by donating hydrogen atoms; Reductones have also been reported to react with certain precursors of peroxide, thus preventing peroxide formation (Xing *et al*., 2005). In the presence of antioxidants in the sample, would result in the reduction of Fe^{3+} to Fe^{2+} by donating an electron. The amount of Fe^{2+} complex can then be monitored by measuring the absorbance at 700 nm. In the present study, this activity also showed with an EC_{50} value of 354±4.6 µg higher than the standard BHT (130±3.5 µg mL^{-1}). Their absorption values increased with increase in concentration, showing that as the concentration of the extract was increased, their ability to reduce Fe^{3+} to Fe^{2+} was also increased (Table 5).

CONCLUSION

The results of the study clearly indicate that the hydro-methanolic extract of *P. majus* possess average antioxidant activity. Hence, it is worthwhile to isolate and elucidate the bioactive principle(s) responsible for the antioxidant activity against DPPH radical. The research is being continued to study the role of different classes of phenols in the antioxidant protection in *P. majus* by means of activity guided fractionation.

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