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Evaluation of phenolic contents and antioxidant properties of the leaves and flowers of *Phlomis biloba* Desf.

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Abstract

The present work was undertaken to evaluate the antioxidant potentials (β-carotene-linoleic acid system and 2, 2-diphenyl-1-picrylhydrazyl, DPPH, free radical scavenging assay) and bioactive components of leaves and flowers of *Phlomis biloba* Desf. Results show that the leaf extract yielded higher total phenolics (153.46 ± 1.36 µg GAE/mg extract) and flavonoid contents (53.84 ± 0.24 µg QE/mg extract) as compared to the flower extract which yielded 81.33 ± 2.29 µg GAE/mg extract and 14.86 ± 0.21 µg QE/mg extract, respectively. However, the flower extract surpassed the leaf extract in flavonol content (06.22 ± 0.05 vs. 03.39 ± 0.06 µg RE/mg extract). The leaf extract had the strongest antioxidant potential as evaluated by DPPH (47.78 ± 1.12 mg/mL) and β-carotene bleaching assays (76.18 ± 0.98%) with high correlation to flavonoid contents. The present work introduced *P. biloba* as a potential bio-resource for antioxidants which can be further utilised in food applications.

Introduction

The species belonging to *Lamiaceae* family have been used since antiquity for both medicinal and culinary purposes especially as spices and herbal tea. Most of their genera have been announced as precious sources of various bioactive compounds (Trivellini *et al*., 2016). The genus *Phlomis* L. is one of their valuable genera which includes over 100 perennial herbs, shrubs and sub-shrubs species, with distribution's zone starting from the Mediterranean zone to Central Asia and China (Sarikurkcu *et al*., 2015).

As mentioned by Dioscorides in "*De Materia Medica*", *Phlomis* species were used as folk remedies for respiratory tract disorders or wound healing since ancient civilisations (Couladis *et al*., 2000). Some *Phlomis* species were regarded as antitussive, tonic, sedative, carminative, astringent agents, and can also be recommended in the treatment of gastrointestinal complications (Sarkhail *et al*., 2010) as well as prophylactics against liver, kidney, bone and cardiovascular diseases (Jabeen *et al*., 2013).

Numerous in vitro investigations have demonstrated the curative properties that *Phlomis* extracts could offer through diverse bioactivities such as antibacterial (Couladis *et al*., 2000; Limem-Ben Amor *et al*., 2008; Dellai *et al*., 2009; Limem-Ben Amor *et al*., 2009), antimutagenic (Uysal *et al*., 2016), antiulcerogenic (Gürbüz *et al*., 2003; Dellai *et al*., 2009; Limem-Ben Amor *et al*., 2009), antigenotoxicity (Dellai *et al*., 2009; Limem-Ben Amor *et al*., 2009), antioxidant (Dellai *et al*., 2009; Limem-Ben Amor *et al*., 2009; Sarikurkcu *et al*., 2015), immunosuppressive and anti-inflammatory (Algieri *et al*., 2013). These favourable effects are accredited to a mixture of bioactive components such as phenylpropanoids, irridoids, diterpenoids, phenylethanoids, alkaloids (Kumar *et al*., 1992) and flavonoids (Kumar *et al*., 1992; Kabouche *et al*., 2005).

Phlomis biloba Desf. (synonym *P. crinita*) is characterised by a golden yellow corolla of flowers with opposite, ovate and tomentose leaves (Bentham, 1832). This plant is widely used as a traditional remedy and drink infusion by the local communities. Previous studies have investigated the phytochemical screening and biological activities of many *Phlomis* species. However, very few reports mentioned the *P. biloba* (Limem-Ben Amor *et al*., 2008; Dellai *et al*., 2009; Limem-Ben Amor *et al*., 2009). Furthermore, there are no published data available on phenolic

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amounts, flavonol contents and detailed antioxidant features of this species.

The aim of the present work was therefore to evaluate the total phenolic, flavonoid and flavonol contents as well as the antioxidant properties of *P. biloba* between leaf and flower extracts so as to unravel their potencies as functional food.

Materials and methods

Chemicals

HPLC-grade methanol was purchased from VWR International (Leuven, Belgium). HPLC-grade chloroform, anhydrous sodium carbonate, aluminium chloride, sodium acetate, gallic acid, quercetin, 2,2-diphenyl-1-picrylhydrazyl, α-tocopherol, β-carotene and linoleic acid were purchased from Sigma-Aldrich (Germany). Folin-Ciocalteu's phenol reagent and butylated hydroxyl toluene (BHT) were purchased from Sigma Chemical Co. (USA). Ascorbic acid was purchased from Biochem Chemopharma (France).

Plant materials

Aerial parts (leaves and flowers) of *P. biloba* were harvested during the flowering season in May 2016 from Abou El-Hassen region (36˚25′25″ N, 01˚11′39″ E) located in the north central part of Chlef province, Algeria. Leaves and flowers were separately dried under shade at room temperature, and finely powdered.

Preparation of extracts

The extraction was performed according to Das *et al*. (2013). Briefly, 500 mg *P. biloba* leaf and flower powder were separately 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 No. 1 filter paper, and the residue was rinsed twice with 10 mL 80% methanol, and pooled with the initial filtrate. The filtrate was then concentrated using rotary vacuum evaporator (Büchi, Switzerland) at 40°C, followed by dry freezing at -20°C (Christ Alpha 1-4 LD plus, Germany) to obtain crude extract which was kept at 4°C until further investigation.

Assay for total phenolics

The total phenolic content (TPC) was determined by the spectrophotometric method given by Singleton *et al*. (1999), involving Folin-Ciocalteu reagent and gallic acid as standard with slight modifications. The reaction mixture was prepared by mixing 0.1 mL extract (2 mg/mL) and 2.5 mL 10% Folin-Ciocalteu's reagent in water. After 10 min, 2.5 mL 7.5% NaHCO3 was added. The mixture was incubated in the 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. All tests were carried out in triplicate. The TPC was calculated from the standard calibration curve constructed with various concentrations of gallic acid, and expressed as µg GAE/mg extract.

Assay for total flavonoids

The determination of total flavonoid content (TFC) was based on the method described by Tepe *et al*. (2011) with slight modifications. Briefly, 0.5 mL extract (2 mg/mL) was mixed with 1 mL 2% aluminium chloride ($AlCl_3$) dissolved in 0.5 mL methanol. The mixture was allowed to stand for 1 h in the dark at room temperature, and measured spectrophotometrically (Optizen 2120, Mecasys Co. Ltd., Korea) at 420 nm against blank. The same protocol was followed for the standard solution of quercetin, and the calibration line was constructed. All tests were done in triplicate. The TFC was calculated from the standard calibration curve and expressed as µg QE/mg extract.

Assay for total flavonols

The determination of total flavonols (TFOLC) in hydromethanolic extracts of *P. biloba* was performed following the method described by Ali Haimoud *et al*. (2016). The mixture consisted of 0.2 mL extract (2 mg/mL), 0.5 mL 2% $AlCl_3$ dissolved in methanol, 0.5 mL sodium acetate (50 g/L) and 0.3 mL methanol. The absorbance of samples and blank was read by UV-Vis spectrophotometer (Optizen 2120, Mecasys Co. Ltd., Korea) at 440 nm following incubation for 2.5 h at 20°C. The experiment was performed in triplicate. The TFOLC was calculated from the standard calibration curve constructed with various concentrations of rutin, and expressed as µg RE/mg extract.

DPPH free radical scavenging assay

The ability of *P. biloba*'s extracts and several standard compounds (ascorbic acid, α-tocopherol and BHT) to quench free radicals was measured from the bleaching of purple colour of DPPH solution. The DPPH assay was conducted according to Muid *et al*. (2013). Briefly, 0.5 mL extract at various concentrations (20-160 µg/mL) was added to 1 mL methanolic DPPH solution prepared at 0.1 mM. The mixture was homogenised and incubated in the dark at room temperature for 30 min. The same procedure was repeated for ascorbic acid, α-tocopherol and

BHT. UV-Vis readings were performed using a spectrophotometer (Optizen 2120) at 517 nm. The inhibition of DPPH free radicals in percent (I%) was calculated using the following equation:

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

where A_0 was the absorbance of the DPPH solution without extract and A_S was 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.

β-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 (Merouane *et al*., 2014). Briefly, 1 mL β-carotene solution in chloroform (0.5 mg/mL) was mixed with 25 µL linoleic acid and 200 mg Tween 20. After complete evaporation of the chloroform using a vacuum evaporator at 40°C, 50 mL distilled water (aerated with oxygen for 1 h) was added with vigorous shaking. Next, 2.5 mL of this emulsion was dispersed in test tubes, and 350 µL extracts/standards prepared at 2 g/L was added. The mixture was incubated in the dark at room temperature for 72 h. UV-Vis readings were performed against blank (consisting of 350 µL methanol in 2.5 mL distilled water) using a spectrophotometer (Optizen 2120) at 490 nm. The total antioxidant activity was expressed in term of percentage inhibition relative to the control BHT calculated using the following equation:

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

where A_S was the absorbance of test sample after incubation period, and A_C was the absorbance of control BHT at the moment of preparation ($t = 0$).

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 Tukey's test at 95% confidence level. Pearson's correlation coefficients were calculated to reveal the relationship between antioxidant activity and phytochemical contents. Calculations and Figures were accomplished using statistical package for the social sciences 16.0 software for Windows (SPSS Inc., Chicago, IL, USA).

Results and discussion

Aqueous-methanolic extract yields

The yield of aqueous-methanolic (hydromethanolic) extracts from flowers and leaves of *P. biloba* on dry basis were 25.46% and 20.82% (w/w), respectively. These findings are higher than extraction with pure methanol (9.1%) for flowers (Dellai *et al*., 2009) and 8.12% for leaves (Limem-Ben Amor *et al*., 2009). Aqueous-methanol solvent has in fact been shown to be able to dissolve majority compounds (Do *et al*., 2014).

Total phenolic contents

The determination of concentrations and biological activities of polyphenols in medicinal plants helps to better elucidate their potencies as functional foods and/or therapeutic agents. It is well known that most of therapeutic applications of these plants are attributed to the phenolic compounds and their different classes that are considered as primary factor against various diseases related to reactive oxygen species (ROS) (Zhang and Wang, 2009). Hence, TPC, TFC and TFOLC were determined in the hydromethanolic extracts of *P. biloba* leaves and flowers, and the results are shown in Figure 1. As seen from Figure 1, the TPC values of leaf extract was significantly ($p < 0.05$) higher (153.46 ± 1.36 µg GAE/mg) as compared to flower extract (81.33 ± 2.29µg GAE/mg). Although different plant parts have shown different values of TPC, the leaves usually yield the highest (Mahdi-Pour *et al*., 2012). In comparison to other members of this genus, the methanolic and water extracts of *P. armeniaca* yielded 55.22 ± 1.95 and 54.39 ± 2.77 µg GAE/mg, respectively (Sarikurkcu *et al*., 2015). In another study, *P. nissolii* yielded 50.83 ± 1.11 µg GAE/mg for methanolic extract and 50.90 ± 0.47 µg GAE/mg in water extract, while *P. pungens* var. pungens yielded 41.10 ± 2.69 and 57.68 ± 1.52 µg GAE/mg in methanolic and water extracts, respectively (Sarikurkcu *et al*., 2014). It has been mentioned in previous investigations that chlorogenic, rosmarinic and benzoic acids are the main phenolic compounds in *Phlomis* species (Zhang and Wang, 2009; Sarikurkcu *et al*., 2014; Sarikurkcu *et al*., 2015). These species showed remarkable variability in term of TPC. The present work revealed the TPC of *P. biloba* with clear variation between leaves and flowers. The phenolic compounds naturally contained in plants are the most researched natural components (Cheynier *et al*., 2013). Several studies have investigated their impact on human health and stated that intake of phenolics-rich foods might help in the prevention of various

illnesses such as diabetes, cardiovascular diseases, neurodegenerative diseases and cancers as well as symptoms related to aging and menopause (Vauzour *et al*., 2010).

Total flavonoid contents

The TFC results are also shown in Figure 1. It is apparent that TFC exhibited similar trend to TPC in which the leaf extract yielded higher TFC (53.84 ± 0.24 µg QE/mg) as compared to the flower extract (14.86 ± 0.21 µg QE/mg). Previous studies conducted on methanolic extracts obtained from leaves (Limem-Ben Amor *et al*., 2009) and flowers (Dellai *et al*., 2009) have shown the presence of flavonoids in *P. biloba*. Kabouche *et al*. (2005) have also isolated four flavonoids from aerial parts of this species. According to the literature, the qualitative chemical investigation of *Phlomis* species reported that glycosylated apigenin, luteolin, naringenin, eriodictyol and chryseriol are the most frequently found flavonoids (Zhang and Wang, 2009; Li *et al*., 2010; Sarikurkcu *et al*., 2015). The importance of flavonoids in foods and herbal extracts appears in their protective effects against a variety of diseases related to ROS through their capacity to transfer free radical electrons, chelate metal catalysts, activate antioxidant enzymes, reduce α-tocopherol radicals and inhibit oxidation (Lin and Weng, 2006).

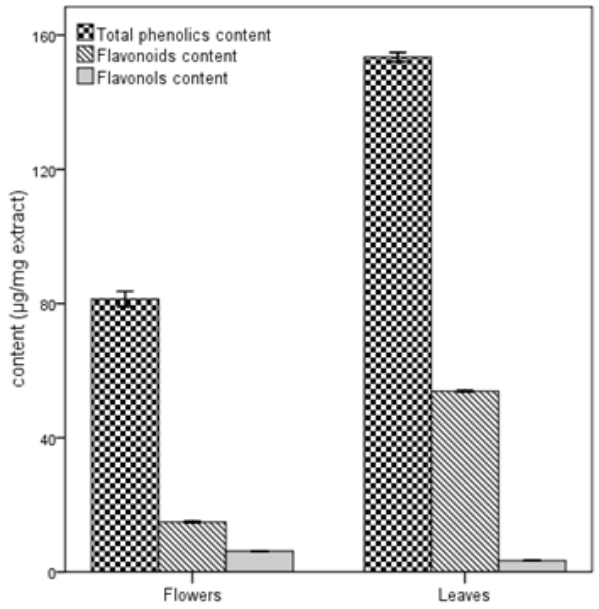

Figure 1. The bioactive components of *Phlomis biloba* aerial plant parts

Total flavonol contents

The values of TFOLC in leaf and flower extracts of *P. biloba* are also shown in Figure 1. Contrary to TPC and TFC results, the TFOLC values in the hydromethanolic extract of leaves (03.39 ± 0.06 µg RE/mg) was significantly ($p < 0.05$) lower than that of flowers (06.22 ± 0.05 µg RE/mg). Despite its wide distribution in medicinal plants, the flavonol fraction represents relatively minor concentrations in the phenolic mixture (El Gharras, 2009). According to a study conducted by Sarikurkcu *et al*. (2015), *P. armeniaca* yielded 1.20 ± 0.05 µg catechin equivalents/mg in methanolic extract. In another investigation by Sarikurkcu *et al*. (2014), the TFOLC of various extracts obtained from *P. nissolii* and *P. pungens* fluctuated between 0.27 ± 0.04 to 2.88 ± 0.06 and 0.33 ± 0.05 to 3.49 ± 0.06 catechin equivalents/mg, respectively.

Antioxidant activity

The in vitro methods involved in the estimation of antioxidant potential of plant extracts are diverse, and differed in term of targeted oxidants which include free radicals and non-radical forms. Therefore, the use of multiple assays is recommended to cover various mechanisms of antioxidant action. In the present work, we have inspected the antioxidant potential of *P. biloba* through two complementary and universally-employed tests, namely DPPH free radical scavenging activity and β-carotene-linoleic acid system.

DPPH free radical scavenging

The free radical scavenging abilities of the standards and extracts analysed are listed in Figure 2. The strongest anti-radical activity was recorded in leaf extract showing an IC_{50} of 47.78 ± 1.12 µg/mL, whereas the flower extract exhibited 90.85 ± 1.04 µg/mL. These findings indicate that the extracts obtained from *P. biloba* could significantly quench free radicals. However, none of the extracts displayed activity as strong as standard compounds: ascorbic acid (03.82 ± 0.09 µg/mL), α-tocopherol (07.43 ± 0.04 µg/mL) and BHT (17.95 ± 0.09 µg/mL). Even so, natural antioxidants are considered as harmless anti-radical agents to human health when compared to synthetic ones which are viewed to have risks of possible toxicity (Venskutonis *et al*., 2004).

β-Carotene/linoleic acid system

In this β-carotene bleaching model, β-carotene discolours rapidly in the absence of antioxidant that could delay the extent of β-carotene destruction by neutralising the free radicals (Ali Haimoud *et*

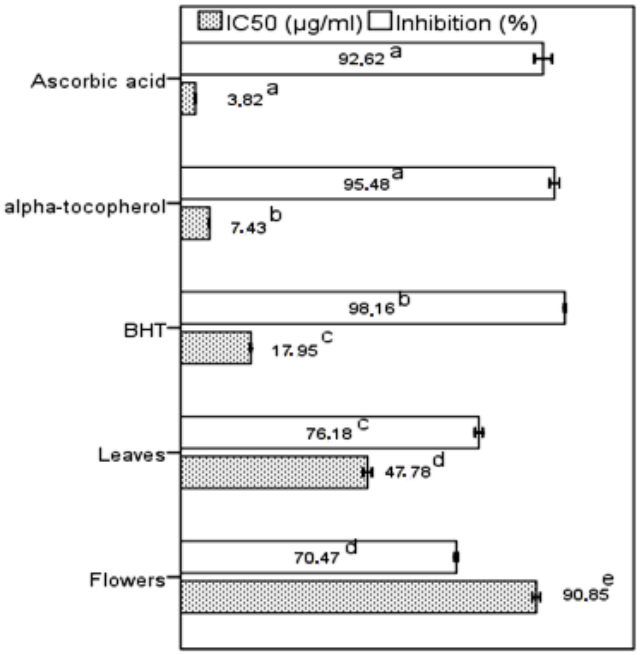

Figure 2.Antioxidant activities of *Phlomis biloba* aerial plant parts and standard compounds as assessed by DPPH free radical scavenging (IC_{50}) and β-carotene bleaching (% inhibition). Different letters in the same assay indicate significant difference at $p < 0.05$.

al., 2016). The effect of hydromethanolic extract obtained from leaves prepared at 2 g/L exhibited the strongest activity than the extract obtained from flowers (76.18 ± 0.98 vs. 70.47 ± 0.48%; Figure 2). These results described the powerful antioxidant potency of *P. biloba* that can scavenge ROS in lipid milieu. The antioxidant activities of synthetic antioxidants were also screened. In general, the inhibition potential of corresponding extracts and standard antioxidants significantly decreased ($p < 0.05$) in the order of BHT (98.16 ± 0.22%) > α-tocopherol (95.48 ± 1.26%) = ascorbic acid (92.62 ± 2.25%) > leaf extract > flower extract. As can be seen from Table 1, the correlation analysis between antioxidant activity and phytochemical amounts revealed that TPC and TFC had a strong positive correlations ($p < 0.01$) with antioxidant activity. The correlations data of TPC were 0.998 and 0.975 with DPPH free radical scavenging effect and inhibition of β-carotene bleaching, respectively. There was also a high significant positive correlation ($p < 0.01$) between TFC and TPC (0.999 and $p < 0.001$). This relationship indicates that flavonoids contribute greatly in this bioactivity together with other phenolic compounds. The strong correlation between phenolic compounds and antioxidant activity of *P. biloba* is in concordance with the data obtained for *P. nissolii*, *P. pungens* (Sarikurkcu *et al*., 2014) and 30 plant extracts of industrial interest (Dudonne *et al*., 2009).

Table 1. Pearson correlation coefficients between assays for *Phlomis biloba*.

	TPC	TFC	TFOLC	DPPH*	CART
TPC	1				
TFC	0.999**	1			
TFOL	-0.998**	-0.999**	1		
DPPH*	0.998**	0.998**	-0.996**	1	
CART	0.975**	0.978**	-0.980**	0.970**	1

TPC: total phenolic content; TFC: total flavonoid content; TFOLC: total flavonol content; DPPH: 2, 2-diphenyl-1-picrylhydrazyl; CART: β-carotene/ linoleic acid.

*The values of DPPH assay were taken as $1/IC_{50}$

**Correlation is significant at $p < 0.01$.

Conclusion

In the present work, *P. biloba* was demonstrated to be a rich source of bioactive compounds offering significant antioxidant potentials. The antioxidant activity was found to correlate with the polyphenolic content. In the future, further investigations should be carried out to explore their potencies in functional food applications. In the motivation to further utilise *P. biloba*, their natural antioxidants could also be characterised and used as alternative natural antioxidants in the food industry in order to promote public health via their protective effects against diseases related to ROS and to satisfy the increasing demand for natural-based products.

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