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Impact of removal of micro and nano sized particles on the phenolic content and antioxidant activity: Study on aqueous and methanolic leaves extracts of *Phlomis crinita*

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ABSTRACT

Due to the excellent ethnopharmacological properties of *Phlomis crinita*, it is widely used as natural remedy in the Mediterranean region. This study inspects the effect of micro and nano sized particles, contained in aqueous and hydromethanolic leaves extracts, on the antioxidant activities, phenolic and flavonoid contents. The macro particles were removed from extracts via filtration whereas the micro and nano particles were isolated through speed centrifugation. Antioxidant activities were screened in vitro by β-carotene-linoleic acid and 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging assays.

The results showed that removal of microparticles don't affect the properties of extracts, whereas the nanoparticles contributed much in the content of total phenolics and antioxidant potencies of both extracts. The highest impact of nanoparticles removal on antioxidant activity was observed in methanolic extract losing 72% of β-carotene bleaching inhibition and four-doubled its IC_{50} index. These findings suggest that nano sized particles are the nature's store of bioactive constituents in herbal extracts, therefore their retention is highly recommended to preserve the totality of antioxidant capacities.

1. Introduction

Phenolic compounds play a crucial role in the pharmacological properties of herbal extracts. This class is considered as the most group of plant secondary metabolites studied during the last decade with more than 301,483 data and their antioxidant activities have been scientifically reported (Elsevier, 2017). Moreover, these compounds participate potentially in the enzyme inhibitory (Sarikurkcu et al., 2015), anti-inflammatory (Mohajer et al., 2005), antimicrobial (Bahadori et al., 2015), antiamoebic (Tepe et al., 2011), antiviral (Wang et al., 2009), anti-aging (Paredes-Lopez et al., 2010), cardio-protective, neuroprotective and anti-carcinogenic (Crozier et al., 2009) activities of plant-based remedies.

The genus *Phlomis* L., one of the largest genuses of *Lamioidae* sub-family (*Lamiaceae*), with more than 100 species with distribution's zone starting from the Mediterranean region to the central Asia and China (Sarikurkcu et al., 2015). The species of this genus have been mentioned for their phytotherapeutic uses since the years of Dioscorides (Couladis et al., 2000). They are used essentially as traditional remedy for ulcer, inflammation, hemorrhoids and healing of wounds (Couladis et al., 2000; Kırmızıbekmez et al., 2005; Limem-Ben Amor et al., 2009). They are also recommended in the treatment of gastrointestinal complains (Sarkhail et al., 2010), and as prophylactics against liver, kidney, bone and cardiovascular diseases (Jabeen et al., 2013).

Several studies have mentioned *Phlomis* species as a good natural source of various bioactive compounds such as phenylpropanoids, iridoids, diterpenoids, phenylethanoids, alkaloids (Kumar et al., 1992) and phenolic compounds dominated by phenolic acids and flavonoids (Kabouche et al., 2005; Sarikurkcu et al., 2015). This chemical mixture offer to the species a various beneficial bio-activities (Algieri et al., 2013; Dellai et al., 2009; Gürbüz et al., 2003; Limem-Ben Amor et al., 2009; Sarikurkcu et al., 2015).

Despite the numerous investigations realized on the screening of phenolic compounds and antioxidant activity of *Phlomis* species, all of them have been focused only on the extracts, where the dosages were performed in the preparations obtained by maceration in water or organic solvents followed by filtration. The current study looks differently to the extracts, which contain microparticles and nanoparticules escaping the filtration step. To the best of our knowledge, there is no report carried out on the studying of the effect of removal of micro and nanoparticles from extracts on the phenolic contents and antioxidant activity of *Lamiaceae* species.

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Therefore, the current study was designed to investigate and compare the importance of micro and nanoparticles in the phenolic content and antioxidant activity of aqueous and methanolic extracts prepared from leaves of *Phlomis crinita* widely used as wound healing in Algeria.

2. Materials and methods

2.1. Chemicals

HPLC grade methanol was purchased from VWR International (Leuven, Belgium), HPLC grade chloroform, aluminum chloride, Gallic acid, Quercetin, 2,2-diphenyl-1-picrylhydrazyl (DPPH), β-carotene and Linoleic acid were purchased from Sigma–Aldrich (Steinheim, Germany). Folin- Ciocalteu's phenol reagent and butylated hydroxyl toluene (BHT) were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

2.2. Plant material

Leaves of *P. crinita* were harvested during flowering season in May 2017 from Sidi Amer region (36°10′56″ N, 01°18′27″ E) located in Chlef province, Algeria. The taxonomic identification of this species was proved by botanist Mr. Abdeldjebbar Rouabhi in the department of Life and Natural Sciences, Chlef University. The voucher specimen has been deposited at the Herbarium of Laboratory of Natural Bio-Resources, Chlef University (Voucher No: LBRN 2017/003). Leaves were separately dried under shade at 29 ± 2 °C and relative humidity of 12–60%, then finely powdered and kept in airtight bottles at 4 °C until use.

2.3. Preparation of extracts

In order to prepare the initial extracts, 0.5 g of fine powder of *P. crinita* leaves was macerated separately in 100 mL of 80% methanol and distilled water at room temperature under continuous shaking (WIS-10, Daihan Scientific co. Ltd., Korea) for 24 h. The samples were then filtered by Whatman No. 1 filter paper and coded PM_0. Extracts yield were calculated as 34.46% and 14.04% (w/w) for methanolic and aqueous extracts respectively.

The particles suspended in the initial extracts (PM_0) were separated via differential centrifugation according to Gopal et al. (2016) with modifications (with temperature control at 4 °C) at 11929 × *g* for 5 min to eradicate microparticles resulting in methanolic and aqueous extracts without microparticles coded PM_1, followed by centrifugation at 47718 × *g* for 4 h to remove nanoparticles, the obtained supernatants were coded PM_2. All the six extracts were stored at 4 °C until further investigations. The codes attributed to extracts will be used in following sections.

2.4. Assay for total phenolic contents

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 of each extract prepared at 2 mg mL^{-1} 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 amount was expressed as µg gallic acid equivalents/mg of dry matter (µg AGE/mg DM) based on calibration line constructed from standard solution of gallic acid.

2.5. 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. In brief, 0.5 mL of each extract prepared at 2 mgmL^{-1} was mixed with 1 mL of 2% aluminum chloride ($AlCl_3$) dissolved in 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 flavonoid content was calculated from standard calibration curve constructed with various concentrations of quercetin and expressed as µg quercetin equivalents/mg of dry matter (µg QE/mg DM).

2.6. Antioxidant activity

2.6.1. DPPH free radical scavenging assay

The ability of *P. crinita*'s extracts and standard BHT to quench free radicals was measured from the bleaching of purple color of DPPH solution. The DPPH assay was conducted according to Muid et al. (2013). 0.5 mL of each extract at various concentrations (20–160 µg mL^{-1}) was added to 1 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. The same procedure was repeated for the standard BHT. UV–vis readings were performed using a spectrophotometer Optizen 2120 at 517 nm. Inhibition of DPPH free radicals in percent (I%) was calculated from the following formulae:

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

Where A_0 and A_S symbolize the absorbance of the DPPH solution without extract and test sample respectively. The antioxidant activity was expressed in term of IC_{50} (µg DM mL^{-1}) defined as the concentration of test sample which reduced the initial DPPH concentration by 50%. All samples were prepared in three independent experiments.

2.6.2. β-Carotene–linoleic acid assay

In this test, antioxidant activity was evaluated by indirectly measuring the inhibition of volatile organic compounds and conjugated diene hydroperoxides resulting from linoleic acid oxidation (Dapkevicius et al., 1998). One mL of β-carotene solution in chloroform (0.5 mg in 10 mL) 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 °C, 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 with 350 µL of the extracts or standard prepared at 2 mg mL^{-1}. The mixture was stored in dark at room temperature for 72 h. UV–vis readings were performed against blank (consisting of 350 µL methanol in 2.5 mL of emulsion) using a spectrophotometer Optizen 2120 at 490 nm. Total antioxidant activity was expressed in term of percentage inhibition relative to the control BHT calculated in following way:

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

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

2.7. 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