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Comparative assessment of antioxidant and cholinesterase inhibitory properties of the marigold extracts from *Calendula arvensis* L. and *Calendula officinalis* L.

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ABSTRACT

Calendula sp. is an important medicinal and industrial plant with various bioactivities. In this study, we examined enzyme inhibitory effects of the *n*-hexane, dichloromethane, acetone, ethyl acetate, methanol, and water extracts of the leaf and flowers of *Calendula arvensis* L. and *C. officinalis* L. against acetylcholinesterase (AChE) and butyrylcholinesterase (BChE). The extracts were screened for their antioxidant activity using 2,2-diphenyl-1-picrylhydrazyl (DPPH), ferric ion-chelating capacity and ferric-reducing antioxidant power (FRAP) assays at 250, 500, and 1000 μg mL^{-1}. Total phenol and flavonoid quantification of the extracts was achieved using Folin–Ciocalteau and $AlCl_3$ reagents, respectively. The ethyl acetate extract of *C. arvensis* flowers was the most active in AChE inhibition assay (31.24 ± 1.29%), while the *n*-hexane extract of *C. officinalis* leaves exerted the highest ferric ion-chelating capacity (74.27 ± 2.25%). Thin layer chromatographic analysis indicated presence of flavonoid and triterpene derivatives mainly in the extracts.

1. Introduction

Calendula L. (Asteraceae), usually known as "marigold", is a reputed medicinal plant with ornamental properties. The yellow or orange-colored flowers are used as food dye, spice, and tea as well as tincture, ointment or cosmetic cream. Although the genus *Calendula* is usually indigenous to the southern European region including Italy, Malta, Greece, Turkey, Portugal, and Spain (Rejšková et al., 2010), it is nowadays cultivated in many temperate regions of the world depending on its commercial value. Since *C. officinalis* is grown in northern parts of Africa, it is also named as "African marigold" (Muley et al., 2009). Recently, *C. officinalis* L. has become quite important in phytotherapy due to its healing effects against dermatological diseases (Bedi and Shenefelt, 2002; Leach, 2008; Fronza et al., 2009). The plant has been reported to contain mainly carotenoids, flavonoids, phenolic acids, and triterpenes (Vidal-Ollivier et al., 1989; Piccaglia et al., 1997; Wojciak-Kosior et al., 2003; Kishimoto et al., 2005). The flower *per se*, flower extract, flower essential oil, and seed oil of *C. officinalis* are cosmetic ingredients and the plant has been presented as a new source for cosmetic industry (Avramova et al., 1988). *C. officinalis* have been found to be usually safe (Andersen et al., 2010), although very rare occurrence of contact dermatitis due to Compositae (Asteraceae) plants should be taken into account (Reider et al., 2001; Paulsen, 2002). On the other hand, marigold was shown to be a promising dye plant for obtaining natural colors (Piccaglia and Venturi, 1998; Guinot et al., 2008).

According to ancient records (Keville, 1991), the *Calendula* flowers were used as a symbol of remembrance and believed to gives great forces of warmth and benign compassion to the human soul, especially helping to balance the active and receptive modes of communication. Besides, the Calendula essential oil has been reported to be used in care of the elderly (Buckle, 2003). Taking this information on *Calendula* into account, in this study, we aimed to examine inhibitory activity of the *n*-hexane, dichloromethane, acetone, ethyl acetate, methanol, and water extracts of the leaf and flowers of *Calendula arvensis* and *C. officinalis* L. against acetylcholinesterase (AChE) and butyrylcholinesterase (BChE), which are the key enzymes for the treatment of Alzheimer's disease and, lately, Down syndrome (Giacobini, 2004; Nieoullon, 2010). Since neurodegeneration is strongly associated with oxidative damage (Mariani et al., 2005), antioxidant activity of the extracts was tested by 2,2-diphenyl-1-picrylhydrazyl (DPPH), ferric ion-chelating capacity, and ferric-reducing antioxidant power (FRAP) assays at 250, 500, and 1000 μg mL^{-1}. Total phenol and flavonoid contents of the extracts were calculated spectrophotometrically

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Table 1
Yields (w/w, %) of the leaf and flower extracts of *Calendula arvensis* and *Calendula officinalis*.

Extract type	Plant part	Extract yield (w/w, %)	
		Calendula arvensis	*Calendula officinalis*
n-Hexane	Leaf	1.33	3.19
	Flower	8.01	8.32
Dichloromethane	Leaf	1.96	2.60
	Flower	5.39	5.09
Acetone	Leaf	3.84	3.31
	Flower	5.77	5.70
Ethyl acetate	Leaf	0.25	0.34
	Flower	0.39	0.71
Methanol	Leaf	21.70	15.03
	Flower	20.23	15.27
Water	Leaf	12.75	26.63
	Flower	21.38	12.99

using Folin–Ciocalteau and $AlCl_3$ reagents, respectively. Thin layer chromatographic (TLC) analysis was also carried out in the extracts.

2. Materials and methods

2.1. Plant materials

The sample of *C. arvensis* was collected from the vicinity of Bodrum town in Mugla province, while the sample of *C. officinalis* was collected from Bartin province (Turkey) in 2008. The plant samples were identified by Prof. Dr. Gulnur Toker from Department of Pharmacognosy, Faculty of Pharmacy, Gazi University, Ankara (Turkey) and the voucher specimens of *C. arvensis* (GUE 2971) and *C. officinalis* (GUE 2972) are preserved at the Herbarium of Faculty of Pharmacy, Gazi University, Ankara (Turkey).

2.2. Preparation of the extracts

The leaf and flower parts of *C. arvensis* and *C. officinalis* were separated by hand, air-dried at room temperature, and powdered in a mechanical grinder. Each plant part was weighed accurately in a digital balance (Shimadzu AW320), extracted sequentially with *n*-hexane, dichloromethane, acetone, ethyl acetate, methanol, and distilled water at room temperature by shaking with hand occasionally. Each solvent extraction took 2 days. Then, the filtrated solvents were evaporated *in vacuo* until dryness using rotary evaporator (Büchi, Switzerland). The extract yields (w/w) are given in Table 1.

2.3. Determination of total phenol and flavonoid contents in the extracts

Total phenol contents of the extracts were determined in accordance with Folin–Ciocalteau's method (Singleton and Rossi, 1965). In brief, a number of dilutions of gallic acid (Sigma–Aldrich, Steinheim, Germany) were obtained to prepare a calibration curve. The extracts and gallic acid dilutions (100 μL) were mixed with 750 μL of Folin–Ciocalteau's reagent (Sigma–Aldrich, Steinheim, Germany) and 600 μL of sodium carbonate (Emir Kimya, Turkey) in test tubes. The tubes were then vortexed and incubated at 40 °C for 30 min. Afterward, absorption was measured at 760 nm at a Unico 4802 UV–visible double beam spectrophotometer (Dayton, NJ, USA). Total flavonoid content of the extracts was calculated by aluminum chloride colorimetric method (Woisky and Salatino, 1998). To sum up, a number of dilutions of quercetin (Sigma, St. Louis, MO, USA) were obtained to prepare a calibration curve. Then, the extracts and quercetin dilutions (500 μL) were mixed with 95% ethanol (1500 μL), aluminum chloride reagent (100 μL), and sodium acetate (100 μL) (Emir Kimya, Turkey) as well as distilled water (2800 μL). Following incubation for 30 min at room temperature, absorbance of the reaction mixtures was measured at wavelength of 415 nm with a Unico 4802 UV–visible double beam spectrophotometer (USA). The total phenol and flavonoid contents of the extracts were expressed as gallic acid and quercetin equivalents (mg/g extract), respectively.

2.4. Ferric-reducing antioxidant power assay (FRAP)

The ferric-reducing power of the extracts was tested using the assay of Oyaizu (1986). Different concentrations of the extracts dissolved in ethanol (80%) were mixed with 2500 μL of phosphate buffer (pH 6.6) and 2500 μL of potassium ferricyanide. Later, the mixture was incubated at 50 °C for 20 min and, then, trichloroacetic acid (10%, 1250 μL) was added. After the mixture was shaken vigorously, this solution was mixed with distilled water (1250 μL) and ferric chloride (0.1%, 250 μL). After 30 min of incubation, absorbance was read at 700 nm using a Unico 4802 UV–visible double beam spectrophotometer (Dayton, NJ, USA) against ethanol (80%) as blank. Analyses were achieved in triplicates. Increased absorbance of the reaction meant increased reducing power and compared to that of chlorogenic acid as the reference.

2.5. DPPH radical scavenging activity

The stable 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity was determined by the method of Blois (1958). The samples and references dissolved in ethanol (80%) were mixed with DPPH solution (1.5×10^{-4} M). Remaining DPPH amount was measured at 520 nm using a Unico 4802 UV–visible double beam spectrophotometer against ethanol (80%) as blank. Gallic acid was employed as the reference. Inhibition of DPPH in percent ($I\%$) was calculated as given below:

$$I\% = \left[\frac{A_{\text{blank}} - A_{\text{sample}}}{A_{\text{blank}}}\right] \times 100,$$

where A_{blank} is the absorbance of the control reaction (containing all reagents except the test sample), and A_{sample} is the absorbance of the extracts/reference. Analyses were run in triplicates and the results were expressed as average values with S.E.M. (standard error mean).

2.6. Ferrous ion-chelating effect

The ferrous ion-chelating effect of the all extracts and reference was estimated by the method of Chua et al. (2008). Briefly, various dilutions of the extracts dissolved in ethanol (80%) were incubated with 2 mM $FeCl_2$ solution (200 μL). The reaction was initiated by the addition of 800 μL of 5 mM ferrozine (Sigma, St. Louis, MO, USA) into the mixture and left standing at ambient temperature for 10 min. The absorbance of the reaction mixture was measured at 562 nm using a Unico 4802 UV–visible double beam spectrophotometer (USA) against ethanol (80%) as blank. The ratio of inhibition of ferrozine-Fe^{2+} complex formation was calculated as follows:

$$I\% = \left[\frac{A_{\text{blank}} - A_{\text{sample}}}{A_{\text{blank}}}\right] \times 100,$$

where A_{blank} is the absorbance of the control reaction (containing only $FeCl_2$ and ferrozine), and A_{sample} is the absorbance of the extracts/reference. Analyses were run in triplicates and the results were expressed as average values with S.E.M.