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BIOACTIVITY OF *CENTAUREA PERSICA* BOISS. (ASTERACEAE)

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Abstract – The free-radical-scavenging property, antibacterial activity and brine shrimp toxicity of petroleum ether (PE), dichloromethane (DCM) and methanol (MeOH) extracts of *Centaurea persica,* a Turkish medicinal plant, were assessed using the 2,2-diphenyl-1-picryl-hydrazyl (DPPH) assay, the resazurin microtiter plate based assay, and the brine shrimp lethality assay, respectively. Additionally, the disc diffusion assay was also used to assess antibacterial activity. Only the MeOH extract of *C. persica* exhibited a significant free-radical-scavenging property in the DPPH assay, with an RC_{50} value of 0.025 mg/mL. However, in the TLC-based qualitative assay, petroleum ether and DCM extracts showed an extremely low level of free-radical-scavenging property. Among the solid-phase extraction fractions of the MeOH extract, the fractions eluted with 60% and 80% MeOH in water exhibited the highest level of free-radical-scavenging activity (RC_{50} = 0.010 and 0.015 mg/mL, respectively). While DCM extract showed reasonable antibacterial activity against five out of the nine test strains both in the disc diffusion assay and in the resazurin assay, the MeOH extract was highly active against both *Escherichia coli* and ampicillin-resistant *E. coli* strains. Among the solid-phase extraction fractions of the MeOH extract, fractions eluted with 80% MeOH in water and 100% MeOH displayed significant antibacterial potencies against both *E. coli* species. None of the extracts showed any significant toxicity towards brine shrimps (LD_{50} = >1.00 mg/mL).

Key words: Centaurea persica, Asteraceae, free-radical-scavenging activity, antioxidant activity, antibacterial activity, resazurin assay, DPPH, brine shrimp lethality assay

INTRODUCTION

Centaurea persica Boiss., one of the 114 endemic species to Turkey (Wagenitz, 1975), belongs to the genus *Centaurea* L. that comprises 500-600 species of herbaceous thistles and thistle-like flowering plants of the family Asteraceae (*alt.* Compositae) (Uysal et al., 2005). Several species of the genus *Centaurea* are well known for their traditional medicinal uses for the treatment of a number of ailments including bacterial infections, cancers, diabetes, diarrhea, fever, hypertension, malaria, rheumatism and tumors (Sarker et al., 1997; Kargioglu et al., 2010). A variety of secondary metabolites, belonging to the classes of alkaloids, flavonoids, lignans, sesquiterpenes and simple phenolics have been reported from different species of this genus to date (Grange et al., 2009). To the best of our knowledge, apart from a rather brief report on the chemical composition of *C. persica* (Sanz et al., 1990), no other reports on any thorough phytochemical or bioactivity studies on *C. persica* are available to date.

As a part of our ongoing phytochemical and bioactivity studies on the genus *Centaurea* (Kumarasamy et al., 2003; Middleton et al., 2003; Sarker et al., 2005; 2007a; Shoeb et al., 2005; 2006; 2007a-e; Granger et al., 2009), we now report on the free-radical-scavenging activity, antibacterial property and brine shrimp toxicity of the extracts of the aerial parts of *C. persica* growing in Turkey.

MATERIALS AND METHODS

Plant materials

The aerial parts of *Centaurea persica* Boiss. were collected from Antalya province, Elmali district, Turkey (dry slopes, 1.100 m above the sea level) during May-June 2003. A voucher specimen (Gokturk-CP-0301) has been maintained at the Herbarium of the Biology Department of Akdeniz University, Turkey.

Extraction of plant materials

The shade-dried and ground aerial parts of *C. persica* (55 g) were Soxhlet-extracted successively with petroleum ether (PE), dichloromethane (DCM) and methanol (MeOH). The extracts were dried using a rotary evaporator at a temperature not exceeding 45°C.

Solid-phase extraction: fractionation of the MeOH extract

The dried MeOH extract (2 g) was re-suspended in 15 mL of 20% MeOH in water, and fractionated by solid-phase extraction columns (Strata, 20g C_{18} Silica), eluted with 40%, 60%, 80% MeOH in water and 100% MeOH (250 mL each fraction) resulting in four fractions. The fractions were dried using a rotary evaporator at a temperature not exceeding 45° C.

Free-radical-scavenging assay

2,2-Diphenyl-1-picrylhydrazyl (DPPH), molecular formula $C_{18}H_{12}N_5O_6$, was obtained from Fluka Chemie AG, Bucks. Quercetin and Trolox® were obtained from Avocado Research Chemicals Ltd, Shore road, Heysham, Lancs. The method used by Takao et al. (1994) was adopted with suitable modifications as outlined by Kumarasamy et al. (2002). DPPH (8 mg) was dissolved in MeOH (100 mL) to obtain a concentration of 80 μg/mL.

Qualitative assay

Test sample solutions were applied on a TLC plate and sprayed with DPPH solution using an atomizer. It was allowed to develop for 30 min. The color changes were noted.

Quantitative assay

The *n*-hexane and DCM extract were dissolved in DCM, and the MeOH extract in MeOH to obtain the test concentration 10 mg/mL. Dilutions were made to obtain concentrations of $5x10^{-2}$, $5x10^{-3}$, $5x10^{-4}$, $5x10^{-5}$, $5x10^{-6}$, $5x10^{-7}$, $5x10^{-8}$, $5x10^{-9}$, $5x10^{-10}$ mg/mL. Diluted solutions (1.00 mL each) were mixed with DPPH (1.00 mL) and allowed to stand for half an hour for any reaction to occur. The UV absorbance was recorded at 517 nm. The experiment was performed in triplicate and the average absorption was noted for each concentration. The same procedure was followed for the positive controls, quercetin and Trolox® (1 mg/mL in MeOH).

Brine shrimp lethality (BSL) assay

Brine shrimp eggs were purchased from Water Life, Middlesex, UK. The bioassay was conducted following the procedure published previously (Meyer et al., 1982). LD_{50}s were determined from the 24 h counts using the Probit analysis method (Finney, 1971). Percentage mortalities were adjusted relative to the natural mortality rate of the control, following Abbots formula P = (Pi – C)/(1 – C), where P denotes the observed nonzero mortality rate and C represents the mortality rate of the control.

Antibacterial assay

The antibacterial activity of the extracts were assessed against nine bacterial strains, *Bacillus cereus*

(ATCC 11778), *Bacillus subtilis* (NCTC 10400), *Staphylococcus aureus* (NCTC 1803), *Escherichia coli* (ATCC 8739), ampicillin-resistant *Escherichia coli* (NCTC 10418), *Salmonella typhii 4* (ATCC 6539), and three strains of *Pseudomonas aeruginosa* (PA01 NCCB2452, and two clinical isolates PA26 and PA64), obtained from the culture collection of the Institute of Biomedical Sciences Research, University of Ulster. Active cultures were generated by inoculating a loop-full of culture in separate 100 mL nutrient broths and incubating on a shaker at 37°C overnight. The cells were harvested by centrifuging at 4000 rpm for 5 min, washed with normal saline, spun at 4000 rpm for 5 min again and diluted in normal saline to obtain 5 x 10^5 cfu/mL.

Disc diffusion assay

The conventional disc diffusion method (Bauer et al., 1966; Cruickshank, 1968) was employed for the initial assessment of antibacterial potential of the extracts. Sterile 6.0 mm diameter blank discs (BBL, Cocksville, USA) were impregnated with test substances at a dose of 500 µg/disc. These discs, along with the positive control discs (ciprofloxacin, 10 µg/disc) and negative control discs, were placed on Petri dishes containing a suitable agar medium seeded with the test organisms using a sterile transfer loop and kept at 4°C to facilitate maximum diffusion. The plates were kept in an incubator (37°C) to allow the growth of the bacteria. The antibacterial activities of the test agents were determined by measuring the diameter of the zone of inhibition in terms of millimeter.

Resazurin microtiter assay

The recently published 96-well microtiter assay (Sarker et al., 2007b) using resazurin as the indicator of cell growth, was employed for the determination of the minimum inhibitory concentration (MIC) of the active extracts.

Assessment of bacteriostatic/bactericidal property

The agar plate was seeded with the mixture from the well, which was just before the well of the MIC, using a sterile transfer loop and kept at 4°C to facilitate maximum diffusion. The plates were kept in an incubator (37°C) to allow the growth of the bacteria. Any bacterial growth would indicate the bacteriostatic property of the extract, and no growth would be an indicator of bactericidal activity (Genest et al., 2008).

RESULTS

The free-radical-scavenging property, antibacterial activity and brine shrimp toxicity of PE, DCM and MeOH extracts of *Centaurea persica,* an endemic Turkish medicinal plant, were assessed using the 2,2-diphenyl-1-picryl-hydrazyl (DPPH) assay, the resazurin microtiter plate based assay, and the brine shrimp lethality assay, respectively. In addition, the disc diffusion assay was also used to assess antibacterial activity.

Among the extracts, only the MeOH extract of *C. persica* exhibited considerable free-radical-scavenging properties, with an RC_{50} value of 0.025 mg/mL (Table 1). However, in the TLC-based qualitative assay, petroleum ether and DCM extracts showed an extremely low level of free-radical-scavenging property. Among the solid-phase extraction fractions of the MeOH extract, the fractions eluted with 60% and 80% MeOH in water demonstrated the highest level of free-radical-scavenging activity (RC_{50} = 0.010 an 0.015 mg/mL, respectively) (Table 1). While the DCM extract showed reasonable antibacterial activity against five out of the nine test strains, both in the disc diffusion assay (zones of inhibition ranging from 10-14 mm) and in the resazurin assay (MIC ranging from 1.25 to 2.50 mg/mL), the MeOH extract was highly active against both *Escherichia coli* and ampicillin-resistant *E. coli* strains (Table 2). Among the solid-phase extraction fractions of the MeOH extract, fractions eluted with 80% MeOH in water and 100% MeOH displayed significant antibacterial potencies against both *E. coli* species (Table 2). None of the extracts showed any significant toxicity towards brine shrimps (LD_{50} = >1.00 mg/mL) (Table 1).

Table 1. Free-radical-scavenging activity and brine shrimp toxicity of the extracts and solid-phase fractions of *Centaurea persica*.

Extracts	Free-radical-scavenging activity[a]		Brine shrimp toxicity[b] LD_{50} in mg/mL
	Qualitative	Quantitative (RC_{50} in mg/mL)	
Petroleum ether	+	>10.00	>1.00
DCM	+	>10.00	>1.00
MeOH	+	0.025	>1.00
Solid-phase fractions[c]			
30% MeOH in water	+	0.055	NP
60% MeOH in water	+	0.010	NP
80% MeOH in water	+	0.015	NP
100% MeOH	+	0.100	NP
Quercetin	+	2.00×10^{-3}	NP
Trolox®	+	2.60×10^{-3}	NP
Podophylotoxin	NP	NP	2.80×10^{-3}

[a] Determined by the DPPH assay.
[b] Determined by the brine shrimp lethality assay.
[c] Solid-phase fractions of the MeOH extract.
+ = Activity; NP = Not performed

DISCUSSION AND CONCLUSION

Apart from environmental sources, free radicals are also formed because of the normal metabolism of aerobic cells. Free radicals may adversely interact with biological systems causing cytotoxicity (Elmereed et al., 2005). In fact, overproduction of free radicals has been implicated in various chronic diseases, such as cancer, atherosclerosis, diabetes and inflammatory diseases and in aging. Therefore, an external supply of free-radical-scavengers is often necessary to maintain good health and prevent diseases. In the quantitative DPPH assay, the MeOH extract of *C. persica* was the most active among the extracts, and showed considerable free-radical-scavenging activity with an RC_{50} value of 0.025 mg/mL (Table 1) compared to that of the positive controls, Quercetin and Trolox® (RC_{50} = 2.0×10^{-3} and 2.60×10^{-3} mg/mL, respectively). Although the PE and the DCM extracts displayed weak positive response in the qualitative DPPH assay, the RC_{50} value was >10.0 mg/mL in the quantitative assay. The results obtained in this study (Table 1) indicated that the significant free-radical scavenging activities were associated with the polar extract, e.g. MeOH. This suggested that the compounds responsible for the free-radical scavenging activities of this plant were possibly phenolic compounds, which are of common occurrence within the species of the genus *Centaurea* (Granger et al., 2009; ISI Web of Knowledge Database, 2011). The MeOH extract was fractionated using the solid-phase extraction technique, and the fractions were tested in the DPPH assay. All solid-phase extraction fractions showed varying levels of free-radical-scavenging activities, with 60% and 80% MeOH in water fractions being the most active (Table 1). Although the free-radical-scavenging activity of the MeOH extract and its fractions was more than 10-fold lower than the positive controls, this was quite usual, as often the crude extracts tend to produce lower activities than the purified single compound. The positive control is a pure compound whereas the extracts and fractions are mixtures of several compounds (Granger et al., 2009). Therefore, it could be assumed that isolation and purification of active constituents from the MeOH extract would lead to free-radical scavengers with comparable activity to that of Quercetin or Trolox®. The free-radical-scavenging activity of *C. persica,* as noted in this present study, corresponds well with the previously reported free-radical-scav-

Table 2. Antibacterial activity of the extracts and solid-phase fractions of *Centaurea persica.*

Extracts	Antibacterial activity																	
	Disc diffusion assay (Zone of inhibition in mm)									Resazurin assay (MIC in mg/mL)								
	BC	BS	EC	AEC	SA	ST	PA01	PA26	PA64	BC	BS	EC	AEC	SA	ST	PA01	PA26	PA64
Petroleum ether	-	-	-	-	-	-	-	-	-	NP	NP	NP	NP	NP	NP	NP	NP	NP
DCM	14	12	14	-	12	10	-	-	-	1.25	2.50	1.25	NP	2.50	2.50	NP	NP	NP
MeOH	-	-	22	20	-	-	-	-	-	NP	NP	0.625	0.625	NP	NP	NP	NP	NP
Solid-phase fractions[a]																		
30% MeOH in water	NP	NP	-	-	NP	NP	NP	NP	NP	NP	NP	-	-	NP	NP	NP	NP	NP
60% MeOH in water	NP	NP	-	-	NP	NP	NP	NP	NP	NP	NP	-	-	NP	NP	NP	NP	NP
80% MeOH in water	NP	NP	18	16	NP	NP	NP	NP	NP	NP	NP	1.25	1.25	NP	NP	NP	NP	NP
100% MeOH	NP	NP	26	21	NP	NP	NP	NP	NP	NP	NP	0.312	0.625	NP	NP	NP	NP	NP
Ciprofloxacin	33	33	32	36	30	32	32	32	32			2.5×10^{-7}			2.5×10^{-8}			2.5×10^{-7}

BC = *Bacillus cereus* (ATCC 11778), BS = *Bacillus subtilis* (NCTC 10400), EC = *Escherichia coli* (ATCC 8739), AEC = Ampicillin-resistant *Escherichia coli* (NCTC 10418), SA = *Staphylococcus aureus* (NCTC 1803), ST = *Salmonella typhii* 4 (ATCC 6539) and PA = *Pseudomonas aeruginosa* (PA01 NCCB2452, and two clinical isolates PA26 and PA64), NP = not performed. [a] Solid-phase fractions of the MeOH extract.

enging property of a number of other *Centaurea* species (Granger et al., 2009; ISI Web of Knowledge Database, 2011).

The conventional disc diffusion assay, also known as the agar diffusion assay, which is quite useful in assessing the preliminary antibacterial potency of antibacterial compounds or extracts, was employed to evaluate the antibacterial property of PE, DCM and MeOH extracts of *C. persica,* as well as the solid-phase extraction fractions of the MeOH extract (Table 2). The DCM extract displayed reasonable antibacterial activities against five out of the nine test strains of bacteria, having the most potent activity against *B. cereus* and *E. coli* (Table 2). However, the MeOH extract displayed strong, but very specific, antibacterial activity against *E. coli* and ampicillin-resistant *E. coli* with zones of inhibition of 22 and 20 mm, respectively (Table 2). The antibacterial activity of the MeOH extract against ampicillin-resistant *E. coli* was of particular significance as the isolation of an active principle from this extract may lead to the development of newer antibacterial agents to fight against drug-resistant bacteria. Only the 80% MeOH in water and 100% MeOH solid-phase extraction fractions of the MeOH extract were active against both *E- coli* strains, and fractionation seemed to have improved the activity slightly. For example, the zone of inhibition displayed by the 100% MeOH fraction against *E. coli* was found to be 26 mm.

The extracts and fractions were subjected to the resazurin microtiter assay (Sarker et al., 2007) to determine their minimum inhibitory concentration (MIC) against susceptible bacterial strains (Table 2). The MIC value of the MeOH extract against *E. coli* and ampicillin-resistant *E. coli* was 0.625 mg/mL. As observed in the disc diffusion assay, two of the four solid-phase extraction fractions of this extract also demonstrated a good level of antibacterial activities: in particular, the fraction eluted with 100% MeOH displayed a significant antibacterial activity against both *E. coli* species with MIC values of 0.312 and 0.625 mg/mL (Table 2). The active extract and fractions were found to be bacteriostatic rather than bactericidal. The antibacterial activity of *C. persica* was mainly due to polar compounds present in the MeOH extract. However, the compounds responsible for the significant antibacterial activity were slightly less polar in nature (as they were present in the 100% MeOH solid-phase extraction fraction) when compared to the compounds that showed free-radical-

scavenging properties (as they were present in the 60% MeOH solid-phase extraction fraction). Some *Centaurea* species have previously been shown to possess antimicrobial properties (ISI Web of Knowledge Database, 2011).

The brine shrimp lethality assay (BSL) is a cheap, yet excellent assay for the routine primary screening of crude extracts and fractions as well as isolated compounds to assess toxicity towards brine shrimps. Any observed toxicity towards brine shrimps generally provides an indication of the possible cytotoxicity of the test materials (Meyer et al., 1982). It has been established that cytotoxic compounds usually show good activity in the BSL assay, and this assay is often recommended as a guide for the screening of antitumor and pesticidal compounds because of its simplicity and cost-effectiveness. None of the extracts of *C. persica* displayed any significant toxicity (LD_{50} = >1.00 mg/mL) towards brine shrimps in the BSL assay (Table 1). The LD_{50} value of the positive control, podophylotoxin, was 2.80 x 10^{-3} mg/mL.

As none of these plants displayed any significant toxicity to brine shrimps, indicating an extremely low level of toxicity, this plant could be used as a source of less toxic but potent free-radical-scavengers and antibacterial compounds.

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