Arch. Biol. Sci., Belgrade, 61 (3), 447-452, 2009 DOI:10.2298/ABS0903447G

BIOACTIVITY OF EXTRACTS OF *CENTAUREA POLYCLADA* DC. (ASTERACEAE)

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Abstract — The free radical-scavenging property, antibacterial activity, and brine shrimp toxicity of n-hexane, dichloromethane (DCM), and methanol (MeOH) extracts of *Centaurea polyclada*, an endemic Turkish species, 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. The DCM and MeOH extracts of *C. polyclada* exhibited free radical-scavenging ability with RC50 values 1.17 and 0.015 mg/mL, respectively. Among solid-phase extraction fractions of the MeOH extract, the fraction eluted with 60% MeOH in water demonstrated the highest level of free radical-scavenging activity (RC50 = 0.016 mg/mL). Only the DCM extract showed considerable antibacterial activity against all nine test strains except *Escherichia coli*, with MIC ranging from 1.25 to 2.50 mg/mL. This antibacterial activity pattern was also observed with solid-phase extraction fractions of the DCM extract with varied potencies. None of the extracts showed any significant toxicity towards brine shrimps (LD50 = >1.00 mg/mL).

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

UDC 582.998.1:615.451.1

INTRODUCTION

Centaurea polyclada DC., species endemic to Turkey, belongs to the genus *Centaurea* L., which comprises about 500 species of herbaceous thistles and thistle-like flowering plants of the family Asteraceae (alt. Compositae) (Uysal et al., 2005). It is a perennial species with a height of 25-60 cm and branches in the upper part of the stem. This species grows in soil poor in calcium, medium in phosphorus, and rich in organic matter content. Many species of this genus are well-known for their traditional medicinal uses for the treatment of a number of ailments, including diabetes, diarrhoea, rheumatism, malaria, hypertension, tumor, and cancer (Sarker et al., 1997). A variety of secondary metabolites (belonging to the classes of alkaloids, lignans, flavonoids, sesquiterpenes, and simple phenolics) have been reported from different species of this genus. However, to the best of our knowledge, no phytochemical or bioactivity studies on *C. polyclada* are available to date.

As a part of our continuing studies on the *Centaurea* species endemic to Turkey (Sarker et al., 2005, 2007b; Shoeb et al., 2005, 2006, 2007a-e), we now present data on the free radical-scavenging property, antibacterial activity, and brine shrimp toxicity of extracts of the aerial parts of *C. polyclada.*

MATERIALS AND METHODS

Plant materials

The aerial parts of *Centaurea polyclada* DC. were

collected from Turkey (Çanakkale: Biga, Karabiga, opposite the castles, 30 m, slopes), during July-August 2000, and a voucher specimen (CHN-9) has been kept in the Çanakkale Onsekiz Mart University Herbarium (CNH).

Extraction of plant materials

The dried and ground aerial parts of *C. polyclada* (120 g) were Soxhlet-extracted with *n*-hexane, dichloromethane (DCM), and methanol (MeOH), successively. 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 DCM and MeOH extracts (1 g each) were re-suspended in 10 mL of 20% MeOH in water, fractionated by solid-phase extraction columns (Strata, 10g C_{18} Silica), and eluted with 40, 60, and 80% MeOH in water and 100% MeOH (200 mL each fraction), resulting in four fractions from each extract. 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 (Kumarasamy et al., 2002). DPPH (4 mg) was dissolved in MeOH (50 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 (purple on white) were noted.

Quantitative assay: The *n*-hexane and DCM extracts were dissolved in DCM, and the MeOH extract in MeOH to obtain a test concentration of 10 mg/mL. Dilutions were made to obtain concentrations of 5×10^{-2}, 5×10^{-3}, 5×10^{-4}, 5×10^{-5}, 5×10^{-6}, 5×10^{-7}, 5×10^{-8}, 5×10^{-9}, and 5×10^{-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). Values of LD_{50} were determined from 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

Antibacterial activity of the extracts was assessed against nine bacterial strains, viz., *Bacillus cereus* (ATCC 11778), *Bacillus subtilis* (NCTC 10400), *Staphylococcus aureus* (NCTC 1803), *Escherichia coli* (ATCC 8739), ampicillin-resistant *Escherichia coli* (NCTC 10418), *Salmonella typhi 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 5×10^5 cfu/mL.

Disk diffusion assay: Conventional disk diffusion procedure (Bauer et al., 1966; Cruickshank, 1968) was employed for the initial assessment of antibacterial potential of the extracts. Sterile blank disks 6.0 mm in diameter (BBL, Cocksville, USA) were impregnated with test substances at a dose of 500 µg/disk. These disks, along with the posi-

tive control disks (ciprofloxacin, 10 µg/disk) and negative control disks, were placed on Petri dishes containing a suitable agar medium seeded with the test organisms using a sterile transfer loop and kept at 4ºC to facilitate maximum diffusion. The plates were kept in an incubator (37ºC) to allow 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 millimeters.

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

Assessment of bacteriostatic/bactericidal activity: An agar plate was seeded with the mixture from the well just before the well of the MIC using a sterile transfer loop and kept at 4ºC to facilitate maximum diffusion. The plates were kept in an incubator (37ºC) to allow growth of the bacteria. Any bacterial growth would indicate bacteriostatic activity 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 the *n*-hexane, DCM, and MeOH extracts of *Centaurea polyclada*, an endemic Turkish species, 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. The DCM and MeOH extracts of *C. polyclada* exhibited free radical-scavenging activity with RC_{50} values 1.17 and 0.015 mg/mL, respectively (Table 1). Among the solid-phase extraction fractions of the MeOH extract, the fraction eluted with 60% MeOH in water demonstrated the highest level of free radical-scavenging activity (RC_{50} = 0.016 mg/mL) (Table 1). Only the DCM extract showed considerable antibacterial activity against all nine test strains except *Escherichia coli*, with MIC ranging from 1.25 to 2.50 mg/mL (Table 2). This antibacterial activity pattern was also observed with solid-phase extraction fractions of the DCM extract with varied potencies (Table 2). None of the extracts showed any significant toxicity towards brine shrimps (LD_{50} = >1.00 mg/mL) (Table 1).

DISCUSSION AND CONCLUSION

In the quantitative DPPH assay, the MeOH extract of *C. polyclada* was the most active among the extracts and displayed considerable antioxidant activity with an RC_{50} value of 0.015 mg/mL (Table 1) compared to that of the positive controls, quercetin and Trolox® (RC_{50} = 2.0 x 10^{-3} and 2.60 x 10^{-3} mg/mL, respective-

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

Extracts	Antioxidant activity[a]		Brine shrimp toxicity[b] LD_{50} in mg/mL
	Qualitative	Quantitative (RC_{50} in mg/mL)	
n-Hexane	+	>10.00	>1.00
DCM	+	1.17	>1.00
MeOH	+	0.015	>1.00
Solid-phase fractions[c]			
40% MeOH in water	+	0.018	NP
60% MeOH in water	+	0.016	NP
80% MeOH in water	+	0.023	NP
100% MeOH	+	0.022	NP
Quercetin	+	2.00 x 10^{-3}	NP
Trolox®	+	2.60 x 10^{-3}	NP
Podophylotoxin	NP	NP	2.80 x 10^{-3}

Table 2. Antibacterial activity of the extracts and solid-phase fractions of *Centaurea polyclada*. Abbreviations: 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 typhi* 4 (ATCC 6539); PA = *Pseudomonas aeruginosa* (PA01 NCCB2452 and two clinical isolates, PA26 and PA64); NP = not performed. (a) Solid-phase fractions of the DCM extract.

Extracts	Antibacterial activity																	
	Disk 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
n-Hexane	-	-	-	-	-	-	-	-	-	NP	NP	NP	NP	NP	NP	NP	NP	NP
DCM	10	8	-	14	12	11	13	10	13	1.25	2.50	NP	2.50	2.50	2.50	1.25	2.50	1.25
MeOH	-	-	-	-	-	-	-	-	-	NP	NP	NP	NP	NP	NP	NP	NP	NP
Solid-phase fractions[a]																		
40% MeOH in water	NP	NP	NP	NP	NP	NP	NP	NP	NP	0.63	1.25	NP	1.25	1.25	0.63	0.63	1.25	0.63
60% MeOH in water	NP	NP	NP	NP	NP	NP	NP	NP	NP	0.12	0.63	NP	>10.0	>10.0	0.63	0.63	0.48	0.48
80% MeOH in water	NP	NP	NP	NP	NP	NP	NP	NP	NP	3.74	5.00	NP	>10.0	>10.0	2.50	2.50	7.50	2.50
100% MeOH	NP	NP	NP	NP	NP	NP	NP	NP	NP	5.00	10.0	NP	>10.0	>10.0	>10.0	5.00	>10.0	>10.0
Ciprofloxacin	33	33	32	36	30	32	32	32	32	2.5×10^{-7}		NP	2.5×10^{-7}	2.5×10^{-8}		2.5×10^{-7}		

ly). Clearly, the MeOH extract was about 100-fold more potent than the DCM extract as a free radical scavenger. Although the *n*-hexane extract showed a 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) indicate that significant free radical-scavenging activities were associated with the polar extract, e.g., MeOH. This suggests that the compounds responsible for the free radical-scavenging activities of this plant are possibly phenolic compounds, which are of common occurrence within species of the genus *Centaurea* (ISI Web of Knowledge Database, 2009). The MeOH extract was fractionated using solid-phase extraction technique, and the fractions were tested in the DPPH assay. All fractions showed quite similar levels of free radical-scavenging activities (Table 1) were comparable to that of the MeOH extract. Although the free radical-scavenging activity of the MeOH extract and its fractions was about 10-fold lower than the positive controls, this was nothing unusual, since crude extracts often 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. The compounds that were actually responsible for the free radical-scavenging activities were present in much lower concentrations than those of the crude extracts. It could therefore be assumed that isolation and purification of active constituents from the MeOH extract would lead to free radical scavengers with activity comparable to that of quercetin or Trolox®. The free radical-scavenging property of *C. polyclada* observed in this present study is in line with the previously reported free radical-scavenging property of a number of other *Centaurea* species (ISI Web of Knowledge Database, 2009).

The conventional disk diffusion assay, which is quite useful for assessing preliminary antibacterial potency of antibacterial compounds or extracts, was applied to evaluate the antibacterial property of *n*-hexane, DCM, and MeOH extracts of *C. polyclada* (Table 2). Only the DCM extract displayed antibacterial activities against all nine test strains of bacteria except *E. coli*, with zones of inhibition in the range of 0.8-14 mm. The most notable was its activity against three strains of *Pseudomonas aeruginosa* (PA01 NCCB2452 and two clinical isolates, PA26 and PA64), and the most potent activity was observed against ampicillin-resistant *E. coli*.

The MIC values of the extracts were determined by the resazurin microtiter assay (Sarker et al., 2007a).

The MIC values for the DCM extract of *C. polyclada* were in the range of 1.25 to 2.5 mg/mL (Table 2). All solid-phase extraction fractions of this extract also demonstrated a good level of antibacterial activities; in particular, the fraction eluted with 60% MeOH in water displayed significant antibacterial activity against the clinical isolates of *Pseudomonas aeruginosa*, with MIC values ranging from 0.48 to 0.63 mg/mL. The active extract and fractions were found to be bacteriostatic rather than bactericidal. The antibacterial activity of *C. polyclada* was mainly due to medium polarity compounds present in the DCM extract. A few other species of the genus *Centaurea* have previously been shown to possess antimicrobial properties (ISI Web of Knowledge Database, 2009).

The brine shrimp lethality assay (BSL) has been used routinely in primary screening of both crude extracts and isolated compounds to assess toxicity towards brine shrimps, which could also give an indication of possible cytotoxicity of the test materials (Meyer et al., 1982). It has been shown that cytotoxic compounds usually exhibit 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. polyclada* showed any significant toxicity (LD_{50} = >1.00 mg/mL) towards brine shrimps in the BSL assay (Table 1). The LD_{50} value of one positive control, podophylotoxin, was 2.80×10^{-3} mg/mL.

As none of these plants were particularly toxic to brine shrimps, indicating a 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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