OPEM
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Oriental Pharmacy and Experimental Medicine 2010 **10(4)**, 304-309
DOI 10.3742/OPEM.2010.10.4.304

Assessment of free-radical-scavenging and antibacterial activities, and brine shrimp toxicity of *Scutellaria pinnatifida* (Lamiaceae)

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Received for publication March 22, 2010; accepted December 6, 2010

SUMMARY

Scutellaria pinnatifida A. Hamilt. (Lamiaceae) is an endemic Turkish herb. This plant is also endemic to Iran, and grows abundantly in other central and western Asian countries. Several species of the *Scutellaria* are known for their traditional uses in the treatment of hypertension, arteriosclerosis, inflammatory diseases, hepatitis, allergy, cancer and diarrhoea. Free-radical-scavenging property, antibacterial activity and brine shrimp toxicity of the *n*-hexane, dichloromethane (DCM) and methanol (MeOH) extracts of *S. pinnatifida* were assessed using the 2,2-diphenyl-1-picryl-hydrazyl (DPPH) assay, the resazurin microtitre plate based assay, and the brine shrimp lethality assay, respectively. The DCM and MeOH extracts exhibited free-radical-scavenging property, with the RC_{50} values of 0.362 and 0.127 mg/ml, respectively. Among the solid-phase extraction fractions of the MeOH extract, the 50% aqueous-MeOH fraction showed the highest level of free-radical-scavenging activity (RC_{50} = 0.039 mg/ml). While the DCM extract showed low level of antibacterial activity against *Bacillus subtilis* and ampicillin-resistant *Escherichia coli*, the MeOH extract was active against *B. cereus*, *B. subtilis*, *E. coli* and ampicillin-resistant *E. coli*. However, the minimum inhibitory concentrations (MIC) of the MeOH extract against these bacterial strains were >10 mg/ml. None of the extracts showed any significant toxicity towards brine shrimps (LD_{50} = > 1.00 mg/ml).

Key words: *Scutellaria pinnatifida*; Lamiaceae; free-radical-scavenging activity; antibacterial; brine shrimp lethality; Turkish medicinal plant

INTRODUCTION

Scutellaria pinnatifida A. Hamilt. (tribe: Scutellaroideae; family: Lamiaceae) is one of the fifteen endemic Turkish herbs of the genus *Scutellaria* that comprises ca. 300 species (Ghannadi and Mehregan, 2003). This plant is also endemic to Iran, and grows widely in other central and western Asian countries (Mozaffarian, 1996; Ghannadi and Mehregan, 2003). A number of species of the genus *Scutellaria* are well known for their traditional medicinal uses in the treatment of hypertension, arteriosclerosis, inflammatory diseases, hepatitis, allergy, cancer and diarrhoea (Mozaffarian, 1996). *Scutellaria pinnatifida*

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is known to produce essential oils, the composition of which has recently been studied (Ghannadi and Mehregan, 2003; Masoudi *et al*., 2009). To the best of our knowledge, no bioactivity studies have ever been conducted with this species. As part of our on-going studies on plants from the Turkish flora (Sarker *et al*., 2005, 2007; Shoeb *et al*., 2005, 2006; 2007a-e), we now report on the free-radical-scavenging activity, antibacterial property and brine shrimp toxicity of the extracts and fractions of the aerial parts of *S. pinnatifida*.

METERIALS AND METHODS

Plant materials

The aerial parts of *Scutellaria pinnatifida* A. Hamilt. were collected from Antalya province, Elmali district, Turkey (dry slopes, 1.100 m above the sea level) during May 2003. A voucher specimen (no; Gokturk 5098) has been maintained at the Herbarium of the Biology Department of Akdeniz University, Turkey.

Extraction

The dried and ground aerial parts of *S. pinnatifida* (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

A portion of the dried MeOH extract (1 g) was re-suspended in 10 ml of 20% MeOH in water, and fractionated using solid-phase extraction cartridge (Strata, 10g, C_{18} Silica), under vacuum, eluted with 20%, 50%, 80% MeOH in water and 100% MeOH (200 ml each fraction) resulting in four fractions from the MeOH extract. The fractions were dried using a rotary evaporator at a temperature not exceeding 45°C.

Free-radical-scavenging assay: the 2,2-Diphenyl-1-picrylhydrazyl (DPPH) 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® (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) 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, 2007). DPPH (4 mg) was dissolved in MeOH (50 ml) to obtain a concentration of 80 mg/ml.

Qualitative analysis: Test sample solutions were applied on a TLC plate and sprayed with the DPPH solution using an atomiser. It was allowed to develop for 30 min. The colour changes (purple on white) were noted.

Quantitative analysis: The n-hexane and the DCM extracts 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 5×10^{-2}, 5×10^{-3}, 5×10^{-4}, 5×10^{-5}, 5×10^{-6}, 5×10^{-7}, 5×10^{-8}, 5×10^{-9}, 5×10^{-10} mg/ml. Diluted solutions (1.00 ml each) were mixed with DPPH (1.00 ml) and allowed to stand for 30 min 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). The RC_{50} value, which is the concentration of the test material that reduces 50% of the free radical concentration, was calculated as mg/ml.

Antibacterial activity

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 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.

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 disks (ciprofloxacin, 10 μg/disc) and negative control disks were placed on Petri dishes containing a suitable agar medium seeded with the test organisms using 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 millimetre.

Resazurin microtitre assay: The recently published 96-well microtitre assay (Sarker *et al*., 2007b; Genest *et al*., 2008) 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 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).

Brine shrimp lethality assay

Shrimp eggs were purchased from The Pet Shop, Kittybrewster Shopping Complex, Aberdeen, UK. The bioassay was conducted following the procedure described by Meyer *et al.* (1982). The eggs were hatched in a conical flask containing 300 ml artificial seawater. The flasks were well aerated with the aid of an air pump, and kept in a water bath at 29 - 30°C. A bright light source was left on and the nauplii hatched within 48 h. The extracts were dissolved in 2% aq. DMSO to obtain a concentration of 1 mg/ml. These were serially diluted to obtain seven different concentrations. A solution of each concentration (1 ml) was transferred into clean sterile universal vials with pipette, and aerated sea-water (9 ml) was added. About 10 nauplii were transferred into each vial with pipette. A check count was performed and the number alive after 24 h was noted. LD_{50} values were determined 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. Podophylotoxin, a well known cytotoxic lignan, was used as positive control.

RESULTS AND DISCUSSION

The DPPH assay is a convenient way of assessing the antioxidant potential of plant extracts, chromatographic fractions and any isolated compounds. The DPPH°, purple in colour, is a stable free radical. An antioxidant (AH) can donate an electron to DPPH° resulting in a colour change that can be measured quantitatively from the absorbance reading at 517 nm. In the qualitative DPPH assay all extracts showed the presence of free-radical-scavenging (antioxidant) activity indicated by the yellowish white spots against purple background on Silica plate. In the quantitative assay, both the DCM and the MeOH extracts *S. pinnatifida* displayed moderate levels of antioxidant activity with the RC_{50} values of 0.362 and 0.127 mg/ml, respectively (Table 1),

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

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	+	0.362	>1.00
MeOH	+	0.127	>1.00
Solid-phase fractions[c]			
20% MeOH in water	+	0.128	NP
50% MeOH in water	+	0.039	NP
80% MeOH in water	+	1.280	NP
100% MeOH	+	10.00	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

compared to that of the positive controls, quercetin and Trolox® (RC_{50} = 2.0×10^{-3} and 2.60×10^{-3} mg/ml, respectively). Clearly, the MeOH extract was about 3-fold more potent than the DCM extract as a free-radical-scavenger. Although the *n*-hexane extract showed 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 free-radical-scavenging activities were associated with the polar and medium polarity extracts, e.g., DCM and MeOH extracts. This also suggested that the compounds responsible for the free-radical scavenging activities of this plant could possibly be phenolic compounds with various chemical structures, e.g., flavonoids and their glycosides, tannins and simple phenolics, which are of common occurrence within the species of the genus *Scutellaria* (Mozaffarian, 1996; ISI Database, 2010).

As the MeOH extract was the most potent extract, it was fractionated using the solid-phase extraction technique, and the fractions were tested in the DPPH assay (Table 1). All fractions, except the fraction eluted with 100% MeOH, displayed free-radical-scavenging activities (Table 1), and the fraction eluted with 50% MeOH in water had the highest level of activity among the fractions. Although the free-radical-scavenging activity of the MeOH extract and its fractions was much lower than that of the positive controls, this was nothing unusual, because often the crude extracts tend to produce lower activities than 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 the concentrations of the crude extracts. Therefore, it could be assumed that isolation and purification of active constituents from the active extract might lead to free-radical scavengers with comparable activity, if not better, to that of quercetin or Trolox®. The free-radical-scavenging property of *S. pinnatifida* observed in this present study is in line with the previously reported free-radical-scavenging property of a number of other *Scutellaria* species (Mozaffarian, 1996; ISI Database, 2010).

The conventional disc diffusion assay (Bauer *et al.*, 1966; Cruickshank, 1968) is quite useful to assess preliminary antibacterial potency of antibacterial compounds or extracts. This assay was used to

Table 2. Antibacterial activity of the extracts and solid-phase fractions of *Scutellaria pinnatifida*

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
n-Hexane	-	-	-	-	-	-	-	-	-	NP	NP	NP	NP	NP	NP	NP	NP	NP
DCM	-	9	-	9	-	-	-	-	-	-	>10.0	-	>10.0	NP	NP	NP	NP	NP
MeOH	10	9	12	15	-	-	-	-	-	>10.0	>10.0	>10.0	>10.0	NP	NP	NP	NP	NP
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}				

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* (PA1; NCCB2452 and clinical strains nos. PA26 and PA64). NP = not performed.

assess the antibacterial property of the *n*-hexane, the DCM and the MeOH extracts of *S. pinnatifida* (Table 2). The *n*-hexane extract did not show any activity at test concentrations, the DCM extract showed low level of activity only against *B. subtilis* and ampicillin-resistant *Escherichia coli*, and the MeOH extract displayed antibacterial activity against four test bacterial strains, e.g., *B. cereus*, *B. subtilis*, ampicillin-resistant *E. coli* and *E. coli* with the zones of inhibition in the range of 09-15 mm.

The resazurin microtitre assay (Sarker *et al.*, 2007b) was employed to determine the minimum inhibitory concentration (MIC) values of the active extracts. However, no MIC could be determined even at the stock concentration of 10 mg/ml. As these extracts showed some degree of antibacterial activity in the disc diffusion assay, but at a much higher doses, e.g., 500 mg/disc, it could be assumed that the MIC of these extracts might be > 10.0 mg/ml. The antibacterial activity of the extracts was found to be bacteriostatic, rather than bactericidal.

The brine shrimp lethality assay (BSL) has been used routinely in the primary screening of plant crude extracts as well as isolated compounds to assess the toxicity towards brine shrimps, which could also give an indication of possible cytotoxicity of the test materials (Meyer *et al.*, 1982). Cytotoxic compounds generally exhibit considerable toxicity towards brine shrimp in the BSL assay, and this assay is often recommended as a guide for the screening of antitumour and pesticidal compounds because of its simplicity and cost-effectiveness. None of the extracts of *S. pinnatifida* displayed any mention-worthy 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 2.80×10^{-3} mg/ml.

ACKNOWLEDGMENTS

This is the first report on any bioactivity of *S. pinnatifida.* The present findings might shade some light on the possible scientific basis of the traditional uses of this plant. As none of the extracts or fractions was particularly toxic to brine shrimps, indicating low level of toxicity, this plant could be used as a source of less toxic antioxidant and antibacterial agents.

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