OPEM

Oriental Pharmacy and Experimental Medicine 2003 **3(3)**, 157-162

Screening the extracts of the seeds of *Achillea millefolium*, *Angelica sylvestris* and *Phleum pratense* for antibacterial, antioxidant activities and general toxicity

Satyajit Dey Sarker[1,*], Elaine Eynon[1], Katharine Fok[1], Yashodharan Kumarasamy[1], Eaván Marie Murphy[1], Lutfun Nahar[2], Ehab Mohammed Shaeen[1], Nichola Mary Shaw[1] and Munachonga Siakalima[1]

[1]*Phytopharmaceutical Research Laboratory, School of Pharmacy, The Robert Gordon University, Schoolhill, Aberdeen AB10 1FR, Scotland, UK;* [2]*Japp Laboratory, Department of Chemistry, University of Aberdeen, Meston Walk, Old Aberdeen, Aberdeen AB24 3UE, Scotland, UK*

SUMMARY

Various extracts of higher plants have been used in traditional medicine systems for centuries. While tropical and sub-tropical plants have received considerable attention from the researchers for evaluation of their bioactivity, temeperate plants have always been neglected somewhat. Similarly, seeds of the plants have not been considered seriously compared to other plant parts, e.g. leaves, stems, roots, flowers, etc. as a potential source for biologically active compounds. As part of our on-going evaluation of the extracts of the seeds of temperate plants, especially from Scotland, for biological activity, *Achillea millefolium*, *Angelica sylvestris* and *Phleum pratense* have been chosen for the present study. Both *A. millefolium* and *A. sylvestris* are well known for their traditional medicinal uses in Europe and also in the orient, but there is no report on any medicinal properties of *P. pratense* available to date. Extracts of the seeds of these plants have been assessed for their antioxidant and antibacterial potential and also for general toxicity. Both DCM and MeOH extracts of *A. millefolium* showed the most significant broad spectrum antibacterial activity among the three plants and inhibited the growth of almost all test strains of bacteria. The DCM extracts of all three species were active against methicillin resistant *Staphylococcus aureus* (MRSA) and *Citrobacter freundii* (MIC=6.25×10^{-1} mg/mL). While the MeOH extracts of *A. millefolium* and *P. pratense* were active against *C. freundii*, that of *P. pratense* was also active against MRSA. The MeOH extract of *A. sylvestris* did not show any antibacterial activity against any of the eight bacterial strains at test concentrations. The MeOH extract of *P. pratense* showed the most prominent antioxidant activity (IC_{50}=145 μg/mL) and there was no antioxidant activity observed with the DCM extract of *A. millefolium*. The DCM extract of *P. pratense* was the most toxic (LC_{50}=20 μg/mL) among the extracts.

Key words: *Achillea millefolium*; *Angelica sylvestris*; *Phleum pratense*; Asteraceae; Poaceae; Resazurin; DPPH; Brine shrimp lethality assay

INTRODUCTION

Achellea millefolium L. (common name: Yarrow) is an erect perennial strongly scented far-creeping stoloniferous herb, native to meadows and pastures, grassy banks, hedgerows and waysides of the British Isles, southern Europe and Western Asia, and introduced in North America, Australia and New Zealand (Clapham *et al.*, 1952; GRIN database, 2003). *Angelica sylvestris* L. (common name: Wild angelica) is a stout nearly glabrous British perennial that grows in fens, damp meadows and woods and widely distributed in many other countries in Europe and Asia Minor (Clapham *et al.*, 1952; GRIN database, 2003). Both *A. millefollium* and *A. sylvestris* belong to the family Asteraceae (alt. Compositae). *Phleum pratense* L. (Common name: Timothy grass) is a stout erect perennial of the

*Correspondence: Tel: +44-1224-262547; Fax: +44-1224-262555; E-mail: s.sarker@rgu.ac.uk

family Poaceae. This species is native to the British Isles and distributed widely in several other countries (Clapham *et al.*, 1952; GRIN database, 2003).

A. millefolium has been used medicinally for the treatment of various diseases from early times, and still in use in Austria and Switzerland as 'Herba Millefolii' or 'Flores Millefolii' (Clapham *et al.*, 1952). The seeds of *A. sylvestris* have been used traditionally to make flavoured wine, to stimulate appetite after long illnesses, to treat anorexia nervosa, anaemia, migraine, vertigo, influenza, bronchitis and general dizziness (Howard, 1987). *A. sylvestris* has also been used as an expectorant and to relieve coughs, colds, sore throat flatulence and indigestion. The roots of *A. sylvestris* can be candied and eaten to treat various infections and the herb can be used to treat cystitis and as a urinary antiseptic (Howard, 1987; Potterton, 1997). It is claimed that *A. sylvestris* can induce diuresis, treat muscular cramps, and prevent headaches and rheumatic pain. Despite established traditional medicinal uses of *A. sylvestris* in european and oriental medicine, there is still little documented pharmacological data available for this species. While the grass family (Poaceae), that *P. pratense* belongs to, has many ethnomedical and folk medicinal uses throughout the world (ISI database, 2003), to our knowledge, the species *P. pratense* and the genus *Phleum* have no recorded medicinal uses.

As part of our on-going evaluation of traditional medicinal plants for their biological activities (Kumarasamy *et al.*, 2002a; Kumarasamy *et al.*, 2003), we now report on the antibacterial and antioxidant activities and general toxicity of the extracts of *A. millefolium*, *A. sylvestris* and *P. pratense*.

MATERIALS AND METHODS

Plant materials

The seeds of *Achillea millefolium* (Cat no. 400622), *Angelica sylvestris* (Cat no. 15057) and *Phleum pratense* (Cat no. 15231) were purchased from B & T World Seeds, Sarl, France and voucher specimens, respectively, PH100 001, PH100 002 and PH100 003 were deposited in the herbarium of the Department of Plant and Soil Science, University of Aberdeen, Aberdeen.

Extraction

Ground seeds (~100 g) of *A. millefolium*, *A. sylvestris* and *P. pratense* were Soxhlet-extracted sequentially using solvents (1.1 L each) of increasing polarity, n-hexane, dichloromethane (DCM) and methanol (MeOH). Solvent was evaporated from the extracts using a rotary evaporator at a temperature not exceeding 50°C.

Preparation of the extract solutions for bioassays

The DCM and MeOH extracts (0.025 g) were dissolved in 5 ml DMSO to obtain stock solutions of 5 mg/mL concentration.

Antibacterial assay

Antibacterial activity of the extracts was tested against 8 species of Gram-positive and Gram-negative pathogenic bacteria (Table 1). The bacterial cultures used were from the properly identified and

Table 1. Antibacterial activity of the DCM and MeOH extracts of the seeds of *A. millefolium*, *A. sylvestris* and *P. pratense*

Bacterial species	MIC (mg/mL)						
	DCM			MeOH			Ciproflox-acin
	AM	AS	PP	AM	AS	PP	
Citrobacter freundii NCTC 9750	6.25×10^{-1}	6.25×10^{-1}	6.25×10^{-1}	6.25×10^{-1}	-	6.25×10^{-1}	2.44×10^{-5}
Enterococcus faecalis NCIMB 775	6.25×10^{-1}	-	-	-	-	-	3.91×10^{-4}
Escherichia coli NCIMB 8110	6.25×10^{-1}	-	-	6.25×10^{-1}	-	-	1.95×10^{-4}
Escherichia coli NCIMB 4174	-	-	-	-	-	-	2.5×10^{-6}
Lactobacillus plantarum NCIMB 6376	6.25×10^{-1}	-	-	6.25×10^{-1}	-	-	2.44×10^{-5}
Salmonella goldcoast NCTC 13175	3.12×10^{-1}	-	-	6.25×10^{-1}	-	-	2.5×10^{-5}
Staphylococcus aureus NCTC 10788	3.12×10^{-1}	-	3.12×10^{-1}	6.25×10^{-1}	-	-	9.76×10^{-5}
Staphylococcus aureus NCTC 11940 (MRSA)	6.25×10^{-1}	6.25×10^{-1}	6.25×10^{-1}	-	-	6.25×10^{-1}	4.88×10^{-5}

AM=*Achillea millefolium*; AS=*Angelica sylvestris*; PP=*Phleum pratense*; -=No inhibition of growth at the highest concentration (5 mg/mL) tested. There was no inhibition of growth observed with the negative control DMSO.

appropriately maintained stock cultures from the Microbiological Research Laboratory, School of Pharmacy, The Robert Gordon University. The antibacterial test was performed using the 96 well microplate-based broth dilution method using resazurin solution (Lorian, 1996; Drummond and Waigh, 2000) as an indicator of bacterial growth. All tests were performed in triplicate.

Preparation of bacterial species

The bacterial cultures were prepared by incubating a single colony overnight in nutrient agar at 37°C. For each of the bacterial species used, 35 g of the bacterial culture was weighed using aseptic techniques into two plastic centrifuge tubes. The containers were covered with laboratory parafilm. The bacterial suspension was then spun down using a centrifuge at 4000 rpm for 10 min. The pellets were resuspended in normal saline (20 mL). The bacterial culture was then centrifuged again at 4000 rpm for another 5 min. This step was repeated twice in order to obtain a clean bacterial culture for the purpose of the bioassay. The supernatant was discarded and the pellets in each of the centrifuge tube was then resuspended in 5 mL of normal saline. The two bacterial suspensions of the same bacteria were then added aseptically to a sterile universal bottle achieving a total volume of 10 mL. The optical density was then measured at a wavelength of 500 nm using a CE 272 Linear Readout Ultraviolet Spectrophotometer and serial dilutions were performed to obtain an optical density in the range of 0.5 to 1.0. The actual values were noted and the cell forming units were calculated using equations from previously provided viability graphs for the particular bacterial species (Richards and Xing, 1993; Kumarasamy *et al.*, 2002a). The bacterial solution was diluted accordingly in order to obtain a concentration of 5×10^5 cfu/mL.

Preparation of Resazurin solution

One tablet of resazurin was dissolved in 40 mL sterile distilled water to obtain standard resazurin solution.

Preparation of 96 well plates and assay

The top of the 96 well plates was labelled appropriately. Ciprofloxacin, a well known antibiotic, was used as positive control. Normal saline, resazurin solution and dimethyl sulphoxide (DMSO) were used as negative controls. A 100 mL of the dichloromethane (DCM) and methanol (MeOH) extracts in DMSO, ciprofloxacin, normal saline and resazurin solution were pipetted into the first row. The two extracts were added to two columns each while the controls were added to one column each. Normal saline (50 mL) was added to the rows 2 to 11. Using fresh sterile pipette tips, 50 mL of the contents of the first row was transferred to the second row. Serial dilutions were carried out until all the wells contained 50 mL of either extracts or controls in descending concentrations. Resazurin solution (10 mL) was added which was followed by the addition of 30 ml of triple strength broth (or triple strength glucose in the case of *Enterococcus faecalis*) to each of the wells. Finally, 10 ml of bacterial solution of concentration 5×10^5 cfu/mL was added to all the wells starting with row 12. The plates were then wrapped with clingfilm to prevent bacterial dehydration and then incubated overnight for 18 hours at 37°C. The presence of bacterial growth was indicated by colour changes from purple to pink.

Antioxidant assay

2,2-Diphenyl-1-picrylhydrazyl (DPPH), molecular formula $C_{18}H_{12}N_5O_6$, was obtained from Fluka Chemie AG, Bucks. Quercetin was obtained from Avocado Research Chemicals Ltd, Shore road, Heysham, Lancs. The method used by Takao *et al.* (1994) was adopted with suitable modifications to our particular circumstance (Kumarasamy *et al.*, 2002b). DPPH (4 mg) was dissolved in MeOH (50 mL) to obtain a concentration of 80 mg/mL.

Qualitative

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

Quantitative

Stock solutions (5 mg/mL) of the plant extracts were prepared in MeOH. Serial dilutions were carried out to obtain concentrations of 5×10^{-1}, 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 mL each) were mixed with DPPH (1 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 standard (quercetin).

Brine shrimp lethality assay for general toxicity

The method of Meyer *et al.* (1982) was adopted to study the general toxicity of the compound. Waterlife® brand brine shrimp (*Artemia salina*) eggs were purchased from The Waterlife Research Industries, Bath Road, Longford, Middlesex, UK. The eggs were hatched in a conical flask containing brine shrimp medium (300 mL). 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 was left on. The nauplii hatched within 48 h. The extracts were dissolved in DMSO to obtain a concentration of 5 mg/mL. These were serially diluted ten-times. Solution of each concentration (1 mL) was transferred into clean sterile universal vials with a pipette and aerated seawater (20 mL) was added. About 10-15 nauplii were transferred into each vial with a pipette. A check count was performed. The number alive after 24 h was noted. The mortality endpoint of this bioassay was defined as the absence of controlled forward motion during 30 sec of observation. The experiment was carried out in triplicate and the average values were noted. The controls used were DMSO normal saline, and podophyllotoxin (3 mg/mL). Abbots formula was used to correct the values, i.e., $P=Pi-C/1-C$, where P denotes the observed non-zero mortality rate and C represents the mortality rate of the DMSO control.

RESULTS AND DISCUSSION

The antibacterial activity of the extracts was determined by a modified micro-plate-based broth dilution assay (also known as Checkerboard assay) using resazurin as an indicator of bacterial growth (Lorian, 1996; Drummond and Waigh, 2000). Use of resazurin indicator enabled the determination of MIC values more accurately and easily than any other antibacterial assays, as inhibition of bacterial growth could be determined by the presence of blue colour of resazurin as opposed to pink colour indicating the presence of bacterial growth. Among the extracts of *A. millefolium*, *A. sylvestris* and *Phleum pratense*, both DCM and MeOH extracts of *A. millefolium* showed the most significant broad spectrum antibacterial activity and inhibited the growth of almost all test strains of bacteria (Table 1). The most remarkable finding of this present work is the observed antibacterial activity of the DCM extracts of all three plants against methicillin resistant *Staphylococcus aureus* (MIC=6.25×10^{-1} mg/mL). While the DCM extracts of all three species were also active against *Citrobacter freundii* (MIC=6.25×10^{-1} mg/mL), that of *P. pratense* showed activity against methicillin sensitive *Staphylococcus aureus* (MIC= 3.12×10^{-1}). The MIC values obtained for these extracts are certainly quite high compared to that of the positive control ciprofloxacin, but considering the fact that plant extracts contain hundreds of compounds, this result could be used as a valuable qualitative indication of the anti-MRSA potential of these extracts and the presence of anti-MRSA compound (s) in these extracts. Apart from the MeOH extract of *P. pratense*, no other MeOH extract was active against MRSA which indicated that the active components present in these extracts are of medium polarity because of their presence in DCM extracts. The MeOH extract of *A. sylvestris* was totally inactive against all test organisms at test concentrations. Previous phytochemical investigations on *A. sylvestris* revealed the presence of various non-polar or medium-polar furanocoumarins (ISI database, 2003) which might have contributed to the antibacterial activity of the DCM extract.

The DPPH antioxidant assay is based on the ability of 2,2-diphenyl-1-picryl-hydrazyl (DPPH), a stable free radical, to decolourise in the presence of antioxidants. The DPPH radical contains an odd electron which is responsible for the absorbance at 517 nm and also for visible deep purple colour. When DPPH accepts an electron donated by an antioxidant compound, the DPPH is decolourised which can be quantitatively measured from the changes in absorbance. In the TLC-based qualitative antioxidant assay using DPPH spray, all extracts, except the n-hexane and DCM extracts of *A. millefolium*, showed considerable free radical scavenging properties indicated by the presence of a yellow/white spot on a purple background on the TLC plates. The n-hexane and DCM extracts of *A. millefolium* showed

Table 2. Antioxidant activity and brine shrimp toxicity of the DCM and MeOH extracts of the seeds of *A. millefolium*, *A. sylvestris* and *P. pratense*

Assay	μg/mL						Quercetin/ Podophyllotoxin*
	DCM			MeOH			
	AM	AS	PP	AM	AS	PP	
Antioxidant activity (IC_{50})	-	3320	476	1990	1220	145	0.0288
Brine shrimp toxicity (LC_{50})	144	500	20	89	250	45	2.4

*Quercetin and podophyllotoxin were used as positive controls, respectively, for antioxidant and brine shrimp toxicity assays. AM=*Achillea millefolium*; AS=*Angelica sylvestris*; PP=*Phleum pratense*; - =no activity at test concentrations.

very weak antioxidant activity in this qualitative assay. The MeOH extract of *P. pratense* showed the most prominent anti-oxidant activity (IC_{50}=145 μg/mL) among the extracts and there was no antioxidant activity observed with the DCM extract of *A. millefolium*. The level antioxidant activity observed with the extracts of *A. sylvestris* was very low (Table 2).

The general toxicity of the extracts was assessed by a simple and low-cost assay using brine shrimp lethality as an indicator of toxicity. The brine shrimp lethality assay is not specific to any pharmacological activity but can certainly provide an indication of toxicity. Anticancer compounds show high level of toxicity to brine shrimps, however, it is important to note that not all compounds/ extracts that show toxicity in this assay can be considered as a potential source of anticancer drugs. The extracts of *A. sylvestris* showed very low level of general toxicity in the brine shrimp lethality assay (Table 2). Very low level of toxicity observed with both DCM (LC_{50}=500 μg/mL) and MeOH extracts of *A. sylvestris* (LC_{50}=250 μg/mL) could possibly assure its safety in relation to its use in traditional medicine preparations. The DCM extract of *P. pratense* was the most toxic (LC_{50}=20 μg/mL) among the extracts and also its MeOH extract showed prominent general toxicity towards brine shrimps ((LC_{50}=45 μg/mL). The toxicity level of the extracts of *A. millefolium* was not found to be very high (Table 2), especially compared to that of podophyllotoxin.

CONCLUSIONS

From the results obtained in the present study, it can be concluded that among the extracts of *A. millefolium*, *A. sylvestris* and *Phleum pratense*, the extracts of *A. millefolium* showed quite broad-spectrum antibacterial activity, all three plants were active against MRSA, and the extracts of *Phleum pratense*, which was very toxic to brine shrimp, might have some potential of exhibiting potent cytotoxic property.

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