Iranian Journal of Pharmaceutical Research (2005) 2: 81-86
Received: May 2004
Accepted: February 2005

Original Article

Antioxidant, Antibacterial Activities and General Toxicity of *Alnus glutinosa*, *Fraxinus excelsior* and *Papaver rhoeas*

Patricia Middleton[a], Fiona Stewart[a], Salem Al-Qahtani[a], Paula Egan[a], Ciara O'Rourke[a], Aysha Abdulrahman[a], Maureen Byres[a], Moira Middleton[a], Yashodharan Kumarasamy[a], Mohammad Shoeb[a], Lutfun Nahar[b], Abbas Delazar[c] and Satyajit Dey Sarker[d]*

[a]*School of Pharmacy, The Robert Gordon University, Aberdeen, Scotland, UK.* [b]*School of Life Sciences, The Robert Gordon University, Aberdeen, Scotland, UK.* [c]*School of Pharmacy, Tabriz University of Medical Sciences, Tabriz, Iran.* [d]*School of Biomedical Sciences, University of Ulster, Coleraine, Londonderry, Northern Ireland, UK*

Abstract

Alnus glutinosa, *Fraxinus excelsior* and *Papaver rhoeas* have long been used in folkloric medicine for the treatment of various ailments. As part of our continuing screening of plant extracts for biological activities, the extracts of *A. glutinosa*, *F. excelsior* and *P. rhoeas* have been screened for their antioxidant and antibacterial activities, as well as their general toxicity towards brine shrimps. Among the extracts, the methanol (MeOH) extract of *F. excelsior* displayed the highest level of antioxidant activity (RC_{50}=1.35x10^{-2} mg/mL) and the dichloromethane (DCM) extract of *P. rhoeas* was the most toxic extract towards brine shrimps (LD_{50}=2.4x10^{-2} mg/mL). The *n*-hexane and DCM extracts of *F. excelsior* and the MeOH extract of *A. glutinosa* were active (MIC values were within 1.25x10^{-1} and 1.00 mg/mL) against all 8 bacterial species tested, including methicillin-resistant *Staphylococcus aureus* (MRSA).

Keywords: *Alnus glutinosa*; *Fraxinus excelsior*; *Papavar rhoeas; 2, 2-Diphenyl-1-picrylhydrazyl (DPPH); Antibacterial; Brine shrimp lethality assay.*

Introduction

Alnus glutinosa (L.) Gaertn. (Family: Betulaceae), commonly known as 'black alder' or 'european alder', native to a number of countries in northern Africa, temperate Asia and Europe, is one of the *ca.* 30 species of trees and shrubs of the genus *Alnus* (1, 2). Various types of plant secondary metabolites including anthraquinones, phenolic glycosides, flavonol glycoside, terpenoids, xanthones, etc. have previously been reported from the barks, buds, leaves and pollens of *A. glutinosa* (3, 4). The decoction of *A. glutinosa* barks has been used to treat swelling, inflammation and rheumatism (5). It has also been used as an astringent, bitter, emetic and hemostatic, and for the treatment of sore throat and pharyngitis (6). *Fraxinus excelsior* L. (Family: Oleaceae), commonly known as 'ash' or 'European ash', is an anemophilous tree native to the countries of temperate Asia and Europe, including Scotland (2, 7, 8). To date, various classes of compounds including benzoquinones, coumarins, flavonoids, phenylethanoids, secoiridoid glucosides, indole

* Corresponding author:
E-mail: s.sarker@ulster.ac.uk

derivatives and simple phenolics have been reported from *F. excelsior* (3, 9, 10). The barks of *F. excelsior* have long been used as antipyretic (11). From the beginning of the 20th century, the leaves of this plant have been recommended in prescriptions for the treatment of fever or rheumatism (12). The alcoholic extract of *F. excelsior* barks possesses an anti-inflammatory property similar to diclofenac (12, 13). The leaf tea is popular in Europe as a mild purgative and is often used for rheumatism, while the bark is effective against intestinal worms (11). Other medicinal uses of this plant include its use in the treatment of arteriosclerosis, hypercholesterolemia, jaundice and kidney problems (14). *Papaver rhoeas* L. (family: Papaveraceae) is commonly known as 'corn poppy' and found wild in various parts of Europe, northern Africa, western Asia and Indian subcontinent (2, 15-17). The medicinal uses of *P. rhoeas* are somewhat unclear. However, as early as the 11th century, Arab physicians used this plant as a cough remedy (18). This plant is claimed to be useful in the treatment of respiratory problems, asthma, cough, loss of voice, hay fever, insomnia, and intestinal and urinary irritations (18, 19). Previous phytochemical investigations on this plant have revealed the presence of mainly various alkaloids (3, 9, 20-22). As part of our on-going screening of plant extracts for biological activities (23-30), we now report on the antioxidant, antibacterial activities, and general toxicity of *A. glutinosa* and *P. rhoeas extracts obtained from their seeds*, and that of of *F. excelsior* leaves .

Experimental

General

All solvents were purchased from Fischer Scientific Ltd., Loughborough, England. 2, 2-Diphenyl-1-picrylhydrazyl (DPPH), molecular formula $C_{18}H_{12}N_5O_6$, was obtained from Fluka Chemie AG, u. k. Quercetin was obtained from Avocado Research Chemicals Ltd, Heysham, u.k. Resazurin tablets were purchased from BDH Laboratory Supplies, Poole, England. Waterlife® brand brine shrimp (*Artemia salina*) eggs were purchased from The Waterlife Research Industries, middlesex, UK. Podophyllotoxin was obtained from Sigma-Aldrich, Dorset, UK.

Plant materials

The seeds of *Alnus glutinosa* (Cat no. 225), *Papaver rhoeas* (Cat no. 13928) and the leaves of *Fraxinus excelsior* (Cat no. 402314) were purchased from B & T World Seeds, Sarl, France and voucher specimens, respectively, PH00171103-2-SDS, PH00171103-3-SDS and PH00171103-1-SDS were deposited in the herbarium of the Department of Plant and Soil Science, University of Aberdeen, Aberdeen.

Extraction

Ground seeds (~100 g) of *A.glutinosa* and *P. rhoeas,* and leaves (~100 g) of *F. excelsior* 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 *n*-hexane, DCM and MeOH extracts (0.025g) were dissolved in 5 mL DMSO (or MeOH) to obtain stock solutions of 5 mg/mL concentration.

DPPH assay

The DPPH assay was used to determine the free radical scavenging (antioxidant) activity. The method used by Takao *et al.* (31) was adopted with suitable modifications (23, 24, 27). DPPH (4 mg) was dissolved in MeOH (50 mL) to obtain a concentration of 80 µg/ml.

Qualitative assay

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 to white) were noted.

Quantitative assay

Stock solutions (5 mg/mL) of the plant extracts were prepared in MeOH. Serial dilutions were carried out to obtain concentrations of $5x10^{-1}$, $5x10^{-2}$, $5x10^{-3}$, $5x10^{-4}$, $5x10^{-5}$, $5x10^{-6}$ $5x10^{-7}$, $5x10^{-8}$, $5x10^{-9}$ and $5x10^{-10}$ mg/mL.

Diluted solutions (1 mL each) were mixed with DPPH (1 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 control (quercetin).

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 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 methods and the resazurin solution as an indicator of bacterial growth (24, 32). 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, following the procedure described by Sarker *et al.* (24). The bacterial solution was diluted in order to obtain a concentration of 5 x 10^5 cfu/mL.

Preparation of resazurin solution

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

Preparation of 96 well plates and assay

The top 96 well plates were prepared and the assays were performed according to the method described by Sarker et al. (24). Norfloxacin, a well-known antibiotic, was used as the positive control. Normal saline, resazurin solution and dimethyl sulphoxide (DMSO) were used as negative controls. The presence of bacterial growth was indicated by colour changes from purple to pink.

Brine shrimp lethality assay

The method of Meyer *et al.* (33) was adopted to study the general toxicity of the extracts (24). Briefly, the brine shrimp 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, and the nauplii hatched within 48 h. The stock solution of each extract (5 mg/mL) was 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 µg/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

Antioxidant activity

The DPPH assay (23, 24, 31) was used to determine the antioxidant potential of the extracts of *A. glutinosa*, *P. rhoeas* and *F. excelsior*. The DPPH contains an odd electron which becomes paired off in the presence of antioxidant compounds. In the stable free radical form DPPH is purple and when in contact with antioxidant compounds, it becomes yellow (34, 35). This resulting decolourisation is stoichiometric with respect to the concentration of antioxidant. In the qualitative DPPH assay, while the *n*-hexane and DCM extracts of *A. glutinosa* showed extremely low levels of antioxidant activity evident from faint white spots against a pink background on the TLC plate, the MeOH extract displayed a quite significant antioxidant property. In the quantitative assay (Table 2), the RC_{50} value of the *n*-hexane and DCM extract could not be determined within the test concentrations (5 mg/mL being the concentration of stock solutions). The RC_{50} value of the MeOH extract was found to be 1.27 x 10^{-1} mg/mL. The

Table 1. Antibacterial activity of the extracts obtained from the seeds of *A. glutinosa* (AG), *P. rhoeas* (PR) and the leaves of *F. excelsior* (FE)

Bacterial species	MIC (mg/mL)									
	n-Hexane			DCM			MeOH			Positive control (Norfloxacin)
	AG	PR	FE	AG	PR	FE	AG	PR	FE	3.91×10^{-4}
Citrobacter freundii NCTC 9750	2.00	-	0.250	2.00	-	0.250	0.500	-	1.00	9.77×10^{-5}
Escherichia coli NCIMB 8110	2.00	-	0.250	2.00	-	0.250	0.125	-	2.00	1.56×10^{-3}
Escherichia coli NCIMB 4174	2.00	-	0.250	2.00	-	0.250	0.500	-	-	1.56×10^{-3}
Klebsiella aerogenes NCTC 9528	-	-	0.250	-	-	0.250	0.250	-	2.00	1.56×10^{-3}
Lactobacillus plantarum NCIMB 6376	-	-	0.250	-	-	0.250	0.500	-	2.00	1.56×10^{-3}
Pseudomonas aeruginosa NCTC 6750	2.00	-	0.250	2.00	-	0.250	0.500	-	2.00	1.56×10^{-3}
Staphylococcus aureus NCTC 10788	2.00	-	0.125	2.00	-	0.125	0.250	-	2.00	3.90×10^{-4}
Staphylococcus aureus NCTC 11940 (MRSA)	-	-	0.500	-	-	0.500	1.00	-	-	3.13×10^{-3}

- = No inhibition of growth at the highest concentration (5 mg/mL) tested There was no significant inhibition of growth observed with the negative control DMSO

antioxidant property ($RC_{50} = 1.35 \times 10^{-2}$) of the MeOH extract of *F. excelsior* was the most potent of all extracts among the three plants. While all three *F. excelsior* extracts showed a significant activity in the qualitative DPPH assay, the RC_{50} value for the *n*-hexane and DCM extracts could not be determined due to the interference from the high amounts of chlorophyll present in these extracts: this is because chlorophyll also absorbs light significantly at the wavelength of 517 nm, which was used to determine the RC_{50} values spectrophotometrically. None of the *P. rhoeas* extracts exhibited any antioxidant activity, either in the qualitative or quantitative DPPH assay.

Antibacterial activity

The micro-plate based serial dilution checkerboard method is one of the most convenient assays for determining antibacterial activity quantitatively (MIC determination). The convenience of this method can even be enhanced significantly by incorporating resazurin as an indicator of cell growth. The extracts of *P.rhoeas* did not show any antibacterial activity, at test concentrations, against any of the 8 bacterial species (Table 1). Among the three extracts of *A. glutinosa*, the MeOH extract was found to be active against all bacterial species including MRSA; the most potent activity was against *E. coli* (8110) with an MIC value of 1.25×10^{-1} mg/mL. Despite the high MIC value against MRSA (1.00 mg/mL), this finding could be considered significant, at least qualitatively, because this activity was not due to a purified compound, but to a crude extract. The *n*-hexane and DCM extracts of *F. excelsior* were also active against all bacterial species tested (MIC values within the range of 1.25×10^{-1} to 1.00 mg/mL). Like the MeOH extract of *A. glutonosa*, the *n*-hexane and DCM extracts of *F. excelsior* were active against methicillin-resistant *Staphylococcus aureus* (MRSA), but with a lower MIC value (5.0×10^{-1} mg/mL).

General toxicity

Brine shrimp lethality assay is a convenient method for general screening for toxicity of the extracts or compounds towards brine shrimp,

Table 2. Antioxidant activity and brine shrimp toxicity of the seeds of *A. glutinosa* (AG), *P. rhoeas* (PR) and the leaves of *F. excelsior* (FE)

Assay	mg/mL									Quercetin/ podophyllotoxin* (mg/mL)
	n-Hexane			DCM			MeOH			
	AG	PR	FE	AG	PR	FE	AG	PR	FE	
Antioxidant activity (RC_{50})	-	-	NO	-	-	NO	1.27×10^{-1}	-	1.35×10^{-2}	2.88×10^{-5}
Brine shrimp toxicity (LC_{50})	5.29×10^{-1}	ND	2.6×10^{-2}	8.3×10^{-1}	2.4×10^{-2}	7.0×10^{-2}	1.29×10^{-1}	2.6×10^{-2}	8.71×10^{-1}	2.4×10^{-3}

*Quercetin and podophyllotoxin were used as positive controls, respectively, for antioxidant and brine shrimp toxicity assays
- = no activity at test concentrations
NO = The RC_{50} value could not be obtained due to the interference from high amounts of chlorophyll present in the extracts
ND = Could not be done due to extremely oily nature of the extract

and it can give an indication regarding possible cytotoxicity of the test samples. All cytotoxic compounds show positive results in this assay, but not necessarily all extracts or compounds that show a positive result in this assay are cytotoxic. An LD_{50} value of <1 mg/mL is considered to be significant, and the lower the value the higher is the toxicity of the test sample (13). Apart from the *n*-hexane extract of *P. rhoease*, which was too oily to disperse in the brine shrimp medium, all extracts were tested for general toxicity using the brine shrimp lethality assay. High levels of toxicity were observed with the *n*-hexane and DCM extracts of *F. excelsior* (LD_{50} = $2.6x10^{-2}$ and $7.0x10^{-2}$ mg/mL, respectively), and the DCM and MeOH extracts of *P. rhoeas* (LD_{50} = $2.4x10^{-2}$ and $2.6 x10^{-2}$ mg/mL, respectively). All three extracts of *A. glutinosa* showed low levels of toxicity towards brine shrimps (LD_{50} values were in the range of $1.29x10^{-1}$ to $8.30x10^{-1}$ mg/mL).

The antibacterial, antioxidant activities and general toxicities of various extracts of *A. glutinosa, P. rhoeas* and *F. excelsior,* found in this study, may explain some of the traditional medicinal uses of these plants. The anti-MRSA activity of the MeOH extract of *A. glutinosa* and the *n*-hexane and DCM extracts of *F. excelsior* could be of particular interest in relation to the isolation and identification of new 'lead' compounds for the development of anti-MRSA drugs.

References

(1) Mitchell A and Wilkinson J. Parey's Buch der Baume (The Trees of Britain and Northern Europe, 1982), 3rd edition, Blackwell Wissenschafts-Verlag (1997) 150-288

(2) GRIN database. USDA, ARS, National Genetic Resources Program, Germplasm Resources Information Network - (GRIN), National Germplasm Resources Laboratory, Beltsville, Maryland. Available on-line at: http://www.ars-grin.gov/npgs/tax/ (2004)

(3) DNP CD-ROM. *Dictionary of Natural Products*. Version 9:2, Chapman & Hall/ CRC, Florida (2001)

(4) *Phytochemical and Ethnobotanical Database*. USDA-ARS-NGRL, Beltsville Agricultural Research Center, Beltsville, Maryland, USA. Available on line at- http://www.ars-grin.gov/cgi-bin/duke/farmacy2.pl (2004)

(5) Grieve M. *A Modern Herbal Alder*. Available on-line at: http://www.botanical.com/ botanical/mgmh/a/alder019.html (2004)

(6) Holistic Online Database. *Herb Information*. Available on-line at: http://www.holistic-online.com/Herbal0Med/Herbs/h90.htm (2004)

(7) *Flora Celtica database*. The Royal Botanic Garden Edinburgh, Edinburgh, UK. Available on-line at, http://193.62.154.38/celtica/dbase/searchform.html (2004)

(8) *Flora Europaea*. The Royal Botanic Garden Edinburgh, Edinburgh, UK. Available on-line at, http://www.rbge.org.uk/rbge/web/search/index.jsp (2004)

(9) *ISI Database*. Institute for Scientific Information, UK. Available on-line through Web of Science at: http://wos.mimas.ac.uk/ (2004)

(10) *PubMed Database*. U.S. National Library of Medicine, 8600 Rockville Pike, Bethesda. Available on-line at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?CMD=search&DB=PubMed (2004)

(11) Lust J. *The Herb Book*. Benedict Lust Publications, USA (1974) 298-300

(12) von Kruedener S, Schneider W and Elstner EF. Effects of extracts from *Populus tremula* L., *Solidago virgaurea* L. and *Fraxinus excelsior* L. on various myeloperoxidase systems. *Arzneimittelforschung.* (1996) 46: 809-814

(13) Meyer B, Schneider W and Elstner EF. Antioxidant properties of alcoholic extracts from *Fraxinus excelsior*, *Populus tremula* and *Solidago virgaurea*. *Arzneimittelforschung.* (1995) 45: 174-176

(14) Kenner D and Requena Y. *Botanical Medicine: A European Professional Perspective*. Paradigm Publications, USA (1996) 89-183

(15) Gubruz I, Ustun O, Yesilada E, Sezik E and Kutsal O. Antiulcerogenic activity of some plants used as folk remedy in Turkey. *J. Ethnopharmacol.* (2003) 88: 93-97

(16) Bisset GB and Witchl M. *Herbal Drugs and Phytopharmaceutics, A Handbook of Practice on a Scientific Basis with Reference to German Commission E Monograph*. 2nd edition, Medpharm Scientific, Stuttgart (2001) 23-35

(17) Nicholsan BE, Ary S and Gregory M. *The Oxford Book of Wild Flowers*. Oxford University Press, London (1960)

(18) Howard M. *Traditional Folk Remedies, a Comprehensive Herbal*. Century Hutchinson, London (1987)

(19) Potterton D and Stringer M. *Culpeper's Colour Herbal*. Foulsham, London (1996) 16-24

(20) Kalav YN and Sariyar G. Alkaloids from Turkish *Papaver rhoeas*. *Planta Medica* (1989) 5: 488

(21) Slavik J, Slavikova L and Bochorakova J. Alkaloids from *Papaver rhoeas* var. *chelidoniodes* O. Kuntze, *P. confine* and *P. dubium* L. *Collection of the Czech Chemical Communication* (1989) 54: 2009-2020

(22) El-Masry S, El-Ghazooly MG, Omar AA, Khafagy SM and Philipson JD. Alkaloids from Egyptian *Papaver rhoeas. Planta Medica* (1981) 41: 101-105

(23) Kumarasamy Y, Fergusson M, Nahar L and Sarker SD. Biological activity of moschamindole from *Centaurea moschata. Pharmaceutical Biology* (2002) 40: 307-310

(24) Sarker SD, Eynon E, Fok K, Kumarasamy Y, Murphy EM, Nahar L, Shaheen EM, Shaw NM and Siakalima M. Screening the extracts of the seeds of *Achillea millefolium*, *Angelica sylvestris* and *Phleum pratense* for antibacterial, antioxidant activities and general toxicity. *Oriental Pharmacy and Experimental Medicine* (2003) 3:157-162

(25) Kumarasamy Y, Nahar L, Cox PJ, Jaspars M and Sarker SD. Screening seeds of Scottish plants for antibacterial activity, *J. Ethnopharmacology* (2002) 83: 73-77

(26) Kumarasamy Y, Nahar L, Byres M, Delazar A and Sarker SD. Assessment of biological activities associated with the major constituents of the methanol extract of 'wild carrot' (*Daucus carota* L.) seeds. *J. Herbal Pharmacotherapy* (2005) (in press)

(27) Uddin SJ, Shilpi JA, Delazar A, Nahar L and Sarker SD. Free radical scavenging activity of some Bangladeshi plant extracts. *Oriental Pharmacy and Experimental Medicine* (2004) 4: 185-193

(28) Kumarasamy Y, Byres M, Cox PJ, Delazar A, Jaspars M, Nahar L, Shoeb M and Sarker SD. Isolation, structure elucidation and biological activity of flavone C-glycosides from the seeds of *Alliaria petiolata*. *Chem. Nat. Comp.* (2004) 40: 122-128

(29) Datta BK, Khan TH, Kundu JK, Rashid MA, Datta SK, Nahar L and Sarker SD. Anti-cholinergic, cytotoxic and anti-HIV-1 activities of sesquiterpenes and a flavonoid from *Polygonum viscosum. Pharm. Biol.* (2004) 42: 18-23

(30) Kumarasamy Y, Cox PJ, Jaspars M, Nahar L. and Sarker SD. Bioactivity of secoiridoid glycosides from *Centaurium erythraea*. *Phytomedicine* (2003) 10: 344-347

(31) Takao T, Watanabe N, Yagi I and Sakata K. A simple screening method for antioxidants and isolation of several antioxidants produced by marine bacteria from fish and shellfish. *Biosci. Biotech.Biochem* (1994) 58: 1780-1783

(32) Drummond AJ and Waigh RD. In 'Recent Research Developments in Phytochemistry' vol. 4 (Pandalai SG, ed.), Research Signpost, India (2000) 143-152

(33) Meyer BN, Ferrigni NR, Putnam JE, Jacobson LB, Nicholas DE and McLaughlin JL. Brine shrimp: a convenient general bioassay for active plant constituents. *Planta Medica* (1982) 45: 31-34

(34) Choi CW, Kim SC, Hwang SS, Choi BK, Ahn HJ, Lee MY, Park SH and Kim SK. Antioxidant activity and free radical scavenging capacity between Korean medicinal plants and flavonoids by assay guided comparison. *Plant Science* (2002) 163: 1161-1168

(35) Conforti F, Statti GA, Tundis R, Menichini F and Houghton P. Antioxidant activity of methanolic extract of *Hypericum triquertifolium* Turra aerial part. *Fitoterapia* (2002) 73: 479-483

This article is available online at http://www.ijpr-online.com