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Isolation and structure determination of nematicidal iridoid sweroside from *Alstonia scholaris*

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The methanol extract of aerial parts of *Alstonia scholaris* (sapthaparna) was found to possess good nematicidal activity against the plant parasitic nematode, *Meloidogyne incognita.* The active nematicidal compound was isolated from the methanol extract by column chromatographic techniques and purified by TLC. Chemical structure was determined by chemical and spectroscopic methods to be that of a sweroside. This is the first report of its isolation from *A. scholaris*. Among other pure compounds: 4, 6 and 7, it showed maximum mortality of 71, 89 and 91%, respectively, after 24 h, while after 48 h, compounds 4, 6 and 7 showed 88, 74 and 76% mortality, respectively. The plant is of economic importance having nematicidal value. These results showed that the compounds isolated from *A. scholaris* have potent nematicidal activity and may be responsible for the antinematicidal activity of whole plant.

Key words: *Meloidogyne incognita, Alstonia scholaris*, iridoid, Apocynaceae, triterpenoids, nematicide.

INTRODUCTION

The context of our previous communication on phyto-chemical studies of the ethanolic extract of flowers of *Alstonia scholaris* of Pakistan origin, have resulted in the isolation of six triterpenoids, one of the oleanane type, alstoprenyol and four of the ursane type, alstoprenylene, α-amyrin, β-amyrin and 3 β-hydroxy-24-nor-urs-4,12,28-triene triterpene. Now we reported the isolation of sweroside (1), *α*-amyrin acetate (2) (Mahato and Sen, 1997), *β*-sitosterol (3), ursolic acid (4) (Brieskorn and Hofmann, 1962), *β*-sitosterol-*3-O-β-D* glucopyranoside (5), lupeol (6), lupeol acetate (7), 3*β*-acetate-24-nor-urs-4,12-diene ester triterpene (8), stigma sterol (9) and 20 (30)-ursa-ene-3-ol (10) Figure 1 (Block et al., 1998) from the aerial parts of this plant. This study discusses and evolves methods for obtaining the plant extracts/pure compounds and its usages in order to act as a nema-ticidal agent. A bioassay guided isolation of the extract, fractions and pure compounds were subjected to nemati-cidal activity at different concentrations in comparison with *Azadirachta indica.* The crude alcoholic extract of aerial parts showed 95% and methanol fraction showed 80% mortality rate after 48 h at 1.0% concentration against *Meloidogyne incognita*.

Plant-parasitic nematodes constitute one of the most important pest groups of the economic crops, especially in the developed and developing countries of the world.

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The use of plants and plant products is one of the promising methods for nematode control. They are cheap, easy to apply, produce no pollution hazards and have the capacity to structurally and nutritionally improve the soil health. In view of these facts, investigations have been undertaken by various groups of scientists (Gommers, 1981; Qamar et al., 1995; Nogueira et al., 1996) which showed an effective control of root-knot nematodes. In the present article, studies on the nematicidal activity of the alcoholic, methanolic, chloroform, ethyl acetate, pet. ether extract, fractions and pure compounds isolated from the air-dried aerial parts of *A. scholaris* are described.

The genus *Alstonia* comprises about twelve species, seven of them are listed in Bentham's *Flora of Australia*. *A. scholaris* Linn. R. Br. belongs to the family Apocynaceae (Kirtikar and Basu, 1980), grows throughout India, in deciduous and evergreen forests, and also in plains (Nadkarni, 1976). It is also widely distributed in the Asia-Pacific region from India, Sri Lanka through mainland South-East Asia and Southern China, throughout Malaysia to Northern Australia and Solomon Islands. The timber is a non-durable hardwood, suitable for light indoor construction purposes, pulp and paper production. The wood has been used for school blackboards, hence the name 'scholaris'. They are milk bearing shrubs or trees, with large, entire, generally whorled leaves, and terminal cymes of white flowers.

The plant *A. scholaris* has been used in different systems of traditional medication for the treatment of human diseases and ailments. It is reported to contain various alkaloids, flavonoids and phenolic acids. The bark contains alkaloids including ditaine, echitenine, echitamine (ditamine) and echitamidine together with triterpenes *β*-amyrin and lupeol. It has been reported as antimicrobial, antiamoebic, antidiarrhoeal, antiplasmodial, hepatoprotective, immunomodulatory, anti-cancer, antiasthmatic, free radical scavenging, antioxidant, analgesic, anti-inflammatory, anti-ulcer, anti-fertility, anaemia, chronic diarrhea, dysentery, menstrual disorders, malarial fever, colic and acute arthritis and wound healing activities (Dhar et al., 1977; Patil et al., 1999; Fell, 1954; Gandhi and Vinayak, 1990; Keawpradub et al., 1999). There are also reports available on the traditional use of this plant for its cardiotonic, anti-diabetic and anti-arthritic properties. The bark yields a tonic and antiseptic medicine. A con-centrated decoction of the trunk bark is used as a wash in furunculosis and impetigo, and as a gargle in dental caries. The bark, leaves and milky exudates of *A. scholaris* are used in India (Kirtikar and Basu, 1980; Nadkarni, 1976; The Wealth of India, Raw Materials, 2004; Pawan et al., 2011; Abhijit, 2011). Many isolated constituents from *A. scholaris* lack information on pharmacological activities (Patil et al., 1999; Fell, 1954; Gandhi and Vinayak, 1990; Keawpradub et al., 1999; Khan et al., 2003; Atta-ur-Rahman et al., 2002).

Keeping in view the pharmacological significance of the plant, phytochemical studies were undertaken on the constituents of the aerial parts of the plant in this laboratory two years earlier, which resulted in the isolation and characterization of various pentacyclic triterpenoids (Sultana and Muhammed, 2010; Sultana et al., 2012). In the present study, a bio-activity directed isolation of the ethanolic extract showing nematicidal activity was undertaken which resulted in the isolation of an active iridoid and triterpenoid compounds. These compounds were identified as sweroside (1) and *α*-amyrine acetate (2) (1), *β*-sitosterol (3), ursolic acid (4) (2), *β*-sitosterol-*3-O-β-D* glucopyranoside (5), lupeol (6), lupeol acetate (7) through spectral studies (Sultana and Muhammed, 2010). This is the first report on the nematicidal activity (Sultana et al., 2010a, b) of sweroside and any part of *A. scholaris*.

METHODS AND MATERIALS

General experimental procedures

The mass spectra were recorded on a Jeol HX-110 instrument. The ^{1}H and ^{13}C-NMR spectra were recorded in $CDCl_3$ at 500/400 and 125/75 MHz, respectively, on a Bruker AM-500, 400 NMR spectrometer. The UV and IR spectra were recorded on Shimadzu UV-240 and JASCO A-320 spectrophotometers, respectively. Optical rotations were measured on a polatronic D Polarimeter. The purity of the compounds was checked on TLC (Si-gel, Merck PF_{254}, 0.25 mm thickness). Melting points were determined in glass capillary tubes using a Buchi 535 and a Gallenkamp 30/MF-370 melting point apparatus.

Plant material

The aerial parts of *A. scholaris* (5 kg) were collected from the university campus, Kashmir, Pakistan, in October 2006. A voucher specimen (AKUH # 58106) was deposited in the Herbarium of Department of Botany, University of Azad Kashmir, Pakistan.

Extraction and isolation

The aerial parts of *A. scholaris* (5 kg dry weight) were extracted with EtOH (50 L). The EtOH extract was concentrated to a gum (822 g), dissolved in distilled water and extracted thoroughly with petrol ether (25 L). The petrol ether-soluble portion was evaporated under reduced pressure to yield a gum (66.92 g) which was chromategraphed on a silica gel column (Merck, 70–230 mesh, 2025.01 g). The elution of the column was initiated with petrol ether. The combined column sub-fractions 1-8 (5.91 g) obtained by elution with 5:95 ethyl acetate-petrol ether, which showed similar TLC behavior upon spraying with ceric sulfate reagent, were combined and again subjected to CC using silica gel (type 60, 70–230 mesh, 200.10 g), and the column was eluted with petrol ether-ethyl acetate (99:1). The subfractions 6-30 (1.86 g), which showed similar TLC behavior were combined and further purified on preparative TLC plates using a solvent system of petrol ether-ethyl acetate (98:2) to afford pure compound 7 (19.5 mg). The fractions obtained on elution of the column with *n*-hexane-ethyl acetate (10: 90) were checked by TLC. Fractions 7-18 showing similar behavior on TLC were combined and further purified by preparative TLC (Merck PF254, 0.2 mm) using

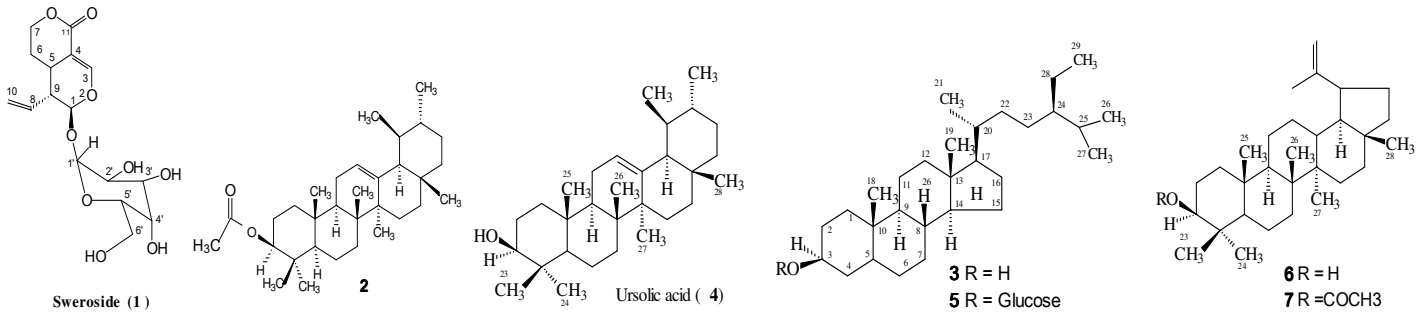

Figure 1. Triterpenes from *Alstonia scholaris*

$CHCl_3$ as eluent to afford pure compound 2 (28 mg). Elution of the major column which was loaded with 66.92 g of petrol ether-soluble material was eluted with 30% ethyl acetate-petrol ether to yield an impure mixture (7.83 g). This mixture was again subjected to CC (silica gel, 70–230 mesh, 60.20 g). The sub-fractions 6–30 (1.86 g), which showed similar TLC behavior, were combined and further purified on preparative TLC plates using a solvent system of petrol ether-ethyl acetate (90:10) to afford pure compound 3 (19.5 mg). The fractions obtained with 30:70 ethyl acetate-petrol ether yielded an impure compound 4, which was further purified by preparative TLC using a solvent system of petrol ether-ethyl acetate (70:30) to obtain pure 4 (20 mg). Fractions 99–145 (1.91 g) obtained by elution with petrol ether-ethyl acetate (35:65, 500 mL each) were collected and again subjected to CC (70–230 mesh, 60.20 gm). The sub-fractions 7–18 (0.92 g, 500 mL each) obtained with 80:20 petrol ether-ethyl acetate showed similar TLC behavior (ceric sulfate-active) and were combined and further purified on preparative TLC (Merck PF254, 0.2 mm) using petrol ether-ethyl acetate 70:30 as eluent to obtain pure 5 (28 mg, *R*f = 0.1). The remaining aqueous layer was extracted with methanol. The methanol soluble portion was dried as a crude mixture which was chromatographed on a Si-gel column (Merck, 70-230 mesh, 30.28 g). Elution of this column with 95% $CHCl_3$: MeOH (5L) yielded an impure mixture (1 g, Fr 5-15) 500 ml each containing compound 1, this mixture was chromatographed on a SiO_2 gel column (2 x70 cm, Merck. 70-230 mesh, 322 g) and eluted with $CHCl_3$ (100%), followed by 2:8 MeOH : $CHCl_3$ to afford 30 fractions (5-10) afford 1.

Nematicidal Activity

Experiments were performed under laboratory conditions at 28 ± 2°C. Fresh egg masses collected from stock culture maintained on tomato root tissues were kept in water for egg hatching. The larvae emerged after 48 h from the egg masses incubated at 30°C and were used as test species for larval mortality studies (Nighat et al., 2011). The movements of the nematodes were checked by touching them with the needle. Stock solutions (30 mg/ml) of the fractions and pure compounds were prepared. To determine the nematicidal effect of the various fractions and the pure compounds, 100 freshly hatched second-stage juveniles were taken in 5 ml of tap water. Freshly hatched second stage juveniles were used, while distilled water with nematode larvae was taken as control.

A measured amount of stock solution was added to make dilutions of 0.5, 0.25, 0.125 and 0.05%. Standard nematicide *A. indica* was taken for comparison and tap water was taken as control. After 48 h of exposure with *Buddleja crispa* fractions and pure compounds, the larvae were counted for mortality and non-mortality under stereoscopic microscope. The death of the nematodes was confirmed by keeping them in tap water for 24 h. The percent mortality was worked out from an average of three replicates.

RESULTS AND DISCUSSION

Hepatoprotective compound sweroside (1) was isolated as a yellowish gummy substance from the methanol extract of aerial parts of *A. scholaris* by column and thin-layer chromatography. Compound 1 ($C_{16}H_{22}O_9$) was identified by comparison of its data with those reported earlier, which was originally isolated as a major constituent from *Centaurium spicatum* and from *Swertia mileensis* (He and Nie, 1980) and this is the first report of its isolation from this plant. The spectral data of compounds 2-7 also compared well with reported compounds, (+)- previously isolated from plants (Mahato and Sen, 1997; Brieskorn and Hofmann, 1962; Block et al., 1998).

A bio-assay guided isolation of the alcoholic extract of the air-dried aerial parts of *A. scholaris* yielded seven nematicidal compounds (1-7) showing nematicidal activity at 0.5, 0.25, 0.125 and 0.05%, respectively. The structures of these constituents were earlier reported (Mahato and Sen, 1997; Brieskorn and Hofmann, 1962; Block et al., 1998; Sultana, 2011) through chemical and spectroscopic methods including one-dimensional (^{1}H-NMR, ^{13}C-NMR, broad band and DEPT) and two-dimensional (COSY-45, NOESY, *J*-resolved, hetero COSY) NMR techniques. The nematicidal activity of the crude alcoholic extract (AS-AS), its fractions (AS-MS, AS-CS and AS-PS) as well as pure compounds (1-7) were tested against a root-knot nematode (*M. incognita*). The direct antinemic action shown by AS and its fractions in the *in vitro* investigation against second-stage juveniles of *M. incognita* is presented in Tables 1 to 2. The crude alcoholic extract (AS) showed 80% mortality at 1.0% concentration after 24 h, and 95% mortality at 1.0% concentration after 48 h, whereas the methanol soluble fraction (AS-MS) showed 80% mortality, and the hexane soluble fraction (AS-PS) showed 40% mortality at the same concentration after 48 h. Conventional nematicide *A. Indica* showed 90% mortality. The alcoholic soluble (AS), methanol soluble (AS - MS), and pet.ether soluble (AS-PS) fractions showed 80, 75

Table 1. Nematicidal activity of different fractions isolated from *A. scholaris* on the larval mortality of *M. incognita* (root-knot nematode).

Fraction	Percent mortality/concentration after 24 h (%)				
	1.0	0.5	0.25	0.125	0.00 (control)
AS-AS	80	72	70	42	02.00
AS-MS	75	65	60	55	00.00
AS-PS	32	30	25	20	03.00
Azadirachta indica	88	85	73	58	00.00

Values represent the mean of 3 experiments. AS-AS (alcohol soluble), AS-MS (methanol soluble), AS-PS (pet.ether soluble).

Table 2. Nematicidal activity of different fractions isolated from *A. scholaris* on the larval mortality of *M. incognita* (root-knot nematode).

Fractions	Percent mortality/concentration after 48 h (%)				
	1.0	0.5	0.25	0.125	0.00 (control)
AS-AS	95	83	60	55	3.00
AS-MS	80	78	72	65	2.00
AS-PS	40	35	28	22	4.00
Azadirachta indica	90	87	80	60	2.00

Values represent the mean of 3 experiments.

Table 3. Nematicidal activity of compounds (1-7) on *M. incognita* larvae after 24 h.

Compound	Percent mortality/concentration after 24 h (%)				
	1.0	0.5	0.25	0.125	0.00 (control)
Sweroside (1)	80	68	40	20	0.0
α-Amyrin acetate (2)	36	31	22	21	2
β-sitosterol (3)	57	44	42	22	2
Ursolic acid (4)	71	64	50	30	4
β-sitosterol-*3-O-,β-D* glucopyranoside (5)	72	55	47	35	4
Lupeol (6)	89	41	36	36	2
Lupeol acetate (7)	91	61	55	55	4
Azadirachta indica	88	85	73	58	0.0

Values represent the mean of 3 experiments.

and 32% mortality, respectively, after 24 h, of *M. incognita* larvae. Seven pure compounds (1-7) were isolated from the methanol and hexane fractions and their nematicidal activity tested on *M. incognita* larvae. The results of the *in vitro* valuation are shown in Tables 3 to 4.

Compound 1 showed highest mortality (92%) at 1.0% concentration, while 4 and 5 showed 88 and 80% mortality, respectively at the same concentration after 48 h. Conventional nematicide *A. indica* showed 90% mortality at the concentrations used in the present studies. The pure compounds 2, 3, 6 and 7 showed 36, 57, 89, 91% mortality, respectively, after 24 h, while after 48 h, compounds showed 40, 69, 74, 76% mortality, respectively for 1.0% concentration. Nematicidal activity on 1, 0.5, 0.25 and 0.125% concentration and control is given in Tables 3 to 4.

It was noted that at all the concentrations, all the tested fractions and pure compounds exhibited significant larval mortality against the test nematode but the activity decreases with a decrease in concentration in all the cases (Tables 1 to 4).

Conclusion

The plant is of economic importance and has nematicidal value. Phytochemicals are used in many medicines, insecticides, pesticides, especially for plant diseases. Those plants which have these proportions can be used in the manufacture of nematicide (Javed et al., 2006). It is evident from the above discussion that there is a great likelihood of use of biocontrol agents for disease control by nematodes (Javed, et al., 2007; Jiskani et al., 2005).

Table 4. Nematicidal activity of compounds (1 -7) on *M. incognita* larvae after 48 h.

Compound	Percent mortality/concentration after 48 h (%)				
	1.0	0.5	0.25	0.125	0.00 (control)
Sweroside (1)	92	85	78	71	3
□-Amyrine acetate (2)	40	34	25	22	2
β-Sitosterol (3)	69	64	45	40	3
Ursolic acid (4)	88	70	68	55	5
β-sitosterol'*3-O-,β-D* glucopyranoside (5)	80	73	50	30	5
Lupeol (6)	74	70	57	45	3
Lupeol acetate (7)	76	71	53	40	2
Azadirachta indica	90	87	80	60	2

Values represent the mean of 3 experiments.

Although, several potential bio-control agents have been isolated and tested for their efficacy against soil born root pathogens, there is need to discover new potential antagonists or improve strains of already isolated antagonists for better crop production (Jiskani et al., 2005). Development of a sample, cheap and effective method for mass production of bio-control agents is a pre-requisite for the replacement of chemical fungicides by a bio-control agent which also needs investigation.

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