Phytochemical and Antimalarial Studies on *Swertia alata* Royle

Maninder Karan, S. Bhatnagar, P. Wangtak and K. Vasisht
Institute of Pharmaceutical Sciences, Panjab University
Chandigarh 160 014
India

Keywords: in vivo test, in vitro test, plant extracts, xanthones

Abstract

Different species of genus *Swertia* are used in different countries for a variety of ailments including malaria. More than 85 xanthones and 17 secoiridoid bitters have been isolated from different species of *Swertia*. In the present study, the whole plant of *Swertia alata* Royle was investigated and three xanthones; 1,8-dihydroxy-3,7-dimethoxyxanthone, 1,7,8-trihydroxy-3-methoxyxanthone and 1-hydroxy-3,7,8-trimethoxyxanthone were isolated from different extracts. The total methanolic extract, its hexane soluble portion, chloroform soluble portion of petroleum ether extract and three isolated xanthones were tested in vitro at different dose levels for antimalarial activity. The activity was expressed as percent invasion inhibition of the tested parasite. The results indicated that all extracts and xanthones possessed some protective effect, however only 1,8-dihydroxy-3,7-dimethoxyxanthone exhibited significant activity and was therefore, further tested in vivo in *Plasmodium berghei* test model. 1,8-dihydroxy-3,7-dimethoxyxanthone produced the most significant reduction in percent parasitaemia at 10 mg/kg dose level.

INTRODUCTION

The medicinal properties of the genus *Swertia* are well recognized in different parts of the world, especially in Asia. The genus is held in high esteem in the Indian system of medicine and is one of the top five drugs of Kampo medicine in Japan (Fuchino, 2002). In ancient medical texts of India, *S. chirata* is described as the principal species of *Swertia* (Chunekar, 2002). The plant is known for bitter, digestive and antimalarial properties and is one of the ingredients of a popular Ayurvedic formulation 'Ayush 64'. Unsustainable collection of this once abundantly available plant has nearly depleted its natural reserves in India resulting in diverted attention to other commonly available species like *S. alata*, *S. angustifolia*, *S. paniculata* and *S. purpurascens*. In view of this, comparative studies on different *Swertia* species were initiated in our laboratory to evaluate their industrial potential based on the presence of secoiridoid bitters and xanthones. It was observed in previous studies that chemically no other species is identical to *S. chirata* although most of them are akin in possessing derivatives of xanthones but different in bitter content of secoiridoids (Karan et al., 1997; Kumar, 2001).

There is a worldwide resurgence of interest in developing antimalarial agents from plants following the discovery of artemisinin. It has been realized that plants possess unique potential to provide novel structures for drug development. The traditional use of *S. alata* in malaria prompted our laboratory to investigate its antimalarial potential. The phytochemical investigations led to the isolation of three 1,3,7,8-tetraoxygenated xanthones namely 1,8-dihydroxy-3,7-dimethoxyxanthone, 1,7,8-trihydroxy-3-methoxy-xanthone and 1-hydroxy-3,7,8-trimethoxyxanthone. The structures of these compounds were elucidated on the basis of interpretation of their spectral details.

The different extracts and pure compounds were evaluated in vitro and in vivo for their antimalarial activity.

MATERIALS AND METHODS

Plant Material

The whole plant of *S. alata* was collected from Mussoorie (Uttaranchal State,

India). The plant was identified on the basis of taxonomic characters and by direct comparison with herbarium specimens available at the Forest Research Institute, Dehradun. The voucher specimen of the plant (No.1127) has been deposited at the Museum-cum-Herbarium of our Institute.

Preparation of Extracts for Preliminary Analysis, Fractionation and Isolation

Powdered plant material (1 kg), after defatting with petroleum ether was macerated with methanol at room temperature to yield 130 g of methanolic extract ('M_1'). This extract 'M_1' (100 g) was suspended in water and partitioned with hexane and chloroform to yield hexane soluble portion 'M_2' (11 g) and chloroform soluble portion 'M_3' (20 g). Both 'M_2' and 'M_3' were subjected to chromatographic separation by using eluants of increasing polarity (hexane, chloroform, methanol) which resulted in the isolation of 1,8-dihydroxy-3,7-dimethoxyxanthone (1) from 'M_2', and 1,7,8-trihydroxy-3-methoxyxanthone (2) and 1-hydroxy-3,7,8-trimethoxyxanthone (3) from 'M_3' (Fig. 1). The structures of these isolated compounds were elucidated on the basis of spectral studies including UV, IR, ^{1}H-NMR, and MS.

The three extracts 'M_1', 'M_2', 'M_3' and three isolates were tested for antimalarial activity.

Experimental Animals, Chemicals and Reagents

Adult Swiss mice (BALBc strain of either sex), organism *Plasmodium berghei* (NK 65 strain) and RPMI 1640 culture medium (Gibco-USA) were used in the biological studies. All other chemicals and solvents used were of analytical grade (E. Merck).

In Vitro Antimalarial Studies

The in vitro antimalarial activity was studied using cultures of *P. berghei* maintained in the laboratory following standard reported procedure (O'Neill et al., 1985; Desjardins et al., 1979; Ekong et al., 1990). The test samples were dissolved in minimal volume of chloroform and diluted with RPMI to desired strength. A suspension (0.9 ml) of red blood cells infected with *P. berghei* (0.1% parasitaemia, 3-5% cell hematocrit) was added to each well of a standard 24-well tissue culture plate containing different doses of test substance or control. Each test compound was assayed in duplicate. Microtiter plates were incubated for 20 h at 37°C in a sealed candle jar and the assay was terminated by aspirating the culture medium. The smears were prepared, air dried, fixed in methanol (2 min), stained with Giemsa solution (30 min), washed under running water and air dried. The number of parasites per 2000 RBC's in each smear were counted under microscope. The results are expressed as per cent invasion inhibition.

$$\% \text{ Invasion inhibition} = 100 - \left[\frac{\text{No of rings in test}}{\text{No of rings in control}} \times 100 \right]$$

In Vivo Antimalarial Studies

The 4-day suppressive test model was used in *P. berghei* infected mice to determine the in vivo activity (Peters, 1987; Dutta, 1992). The doses were dispensed using 2% aqueous solution of gum acacia. Chloroquine at a dose of 4 mg/kg prepared in vehicle was used as a reference standard. The mice were randomly divided into different groups (control, test and reference) of six animals each. On day '0', animals in all the groups were inoculated with $1x10^7$ *P. berghei* infected RBC's (Table 1). The animals in test group were treated with test substance while the animals of control and reference group received vehicle and chloroquine respectively on first four days. On day '4', the tail blood smears of all the animals were prepared, stained with Giemsa solution and

examined under microscope to determine the level of parasitaemia. The per cent parasitaemia was calculated using the expression.

$$\% \text{ Parasitaemia} = \frac{\text{No of infected erythrocytes}}{\text{Total erythrocyte count}} \times 100$$

The results were analysed statistically using ANOVA and student's 't' test (Bolton, 1995).

RESULTS

Characterization of 1,8-Dihydroxy-3,7-dimethoxyxanthone (Swertiaperennine)

Yellow crystalline compound (59 mg); mp 184-187°C; UV λ_{max} (MeOH) nm 238, 262, 312 (sh), 329 and 383; IR ν_{max} (KBr) cm^{-1} 3590, 3010, 2960, 2900, 2660, 1890, 1670, 1640, 1610, 1580, 1500, 1465, 1380, 1290, 1200, 1150, 1090, 960, 820, 745, and 665; ^{1}H -NMR (300 MHz, $CDCl_3$) δ 12.09 (1H, *s*), 11.96 (1H, *s*), 7.26 (1H, *d*, *J* = 9 Hz), 6.84 (1H, *d*, *J* = 9 Hz), 6.37 (1H, *d*, *J* = 2.5 Hz), 6.32 (1H, *d*, *J* = 2.5 Hz), 3.94 (3H, *s*) and 3.89 (3H, *s*); MS m/z (%) 288 (M^+, 81), 273 (40), 270 (21), 259 (10), 254 (100), 242 (6), 202 (28) and 123 (7).

Characterization of 1,7,8-Trihydroxy-3-methoxyxanthone (Swertianin)

Yellow crystalline compound (12 mg); mp 224-226°C; UV λ_{max} (MeOH) nm 208, 238, 266, 314 and 325; IR ν_{max} (KBr) cm^{-1} 3860, 3730, 3460, 1660, 1610, 1590, 1460, 1280, 1160, 1050, 870, 820 and 630; ^{1}H -NMR (300 MHz, $CDCl_3$) δ 11.92 (1H, *s*), 11.85 (1H, *s*), 7.29 (1H, *d*, *J* = 9 Hz), 6.84 (1H, *d*, *J* = 9 Hz), 6.41 (1H, *d*, *J* = 2.2 Hz), 6.34 (1H, *d*, *J* = 2.2 Hz), 5.41 (br *s*), 3.9 (3H, *s*); MS m/z (%) 274(M^+, 100), 245 (31), 231 (15), 216 (6), 202 (7) and 123 (13).

Characterization of 1-Hydroxy-3,7,8-trimethoxyxanthone (Decussatin)

Light yellow crystalline compound (16 mg); mp 154-156°C; UV λ_{max} (MeOH) nm 212, 238, 258, 311 and 373; IR ν_{max} (KBr) cm^{-1} 1670, 1610, 1590, 1485, 1430, 1380, 1290, 1160, 1060, 970 and 800; ^{1}H -NMR (300 MHz, $CDCl_3$) δ 13.25 (1H, *s*), 7.33 (1H, *d*, *J* = 9.2 Hz), 7.16 (1H, *d*, *J* = 9.2 Hz), 6.33 (1H, *d*, *J* = 2.2 Hz), 6.31 (1H, *d*, *J* = 2.2 Hz), 4.00 (3H, *s*), 3.93 (3H, *s*), 3.88 (3H, *s*); MS m/z (%) 302 (M^+, 89), 287 (100), 284 (19), 273 (23), 259 (41), 244 (9), 216 (14), 201 (16), 149 (10), 144 (12) and 123 (7).

Antimalarial Results

The results of in vitro and in vivo studies are given in Table 2, Fig. 2 and Table 3 and Fig. 3 respectively.

DISCUSSION

Identification of Isolates

The isolates 1, 2, 3 (Fig. 1) each produced a single spot on TLC that brightened on spraying with 5% aqueous KOH solution and fluoresced under UV light indicating benzo-γ-pyrone moiety. The presence of a typical three peak pattern in the range of 1670-1590 cm^{-1} in IR spectrum and UV absorption maxima at 223, 262, 312 and 383 nm is characteristic of xanthones having 1,3,7,8 oxygenation pattern (Chaudhuri and Ghosal, 1971). The tetraoxygenated substitution was also confirmed from the observed signals for only four aromatic protons in ^{1}H-NMR spectrum. The mono-, di- and trihydroxy nature of these isolates was interpreted from the signals for one, two and three hydroxy protons respectively in their proton NMR.

The appearance of three sharp 3H singlets of three methoxyls and another sharp singlet of strongly chelated hydroxyl in a far downfield region confirmed the isolate 3 as

1-hydroxy-3,7,8-trimethoxyxanthone (decussatin) which was in accordance with the observed molecular ion peak at 302 in its mass spectrum. Presence of one pair of meta coupled protons (δ 6.33 and 6.31, J = 2.2 Hz) and one pair of ortho coupled protons (δ 7.33 and 7.16, J = 9.2 Hz) further confirmed its structure.

The isolate 1 showed presence of two singlets in the far downfield region corresponding to two hydroxyls placed in peri position to carbonyl at C-1 and C-8. It showed presence of two sharp 3H singlets at δ 3.94 and 3.89 corresponding to two methoxyls. The signals for one pair of meta coupled aromatic protons (δ 6.37 and 6.32, d, J = 2.5 Hz) confirmed presence of one of the methoxyl at C-3. The second methoxyl could be placed at C-7 or C-5 to account for another pair of ortho coupled aromatic protons (δ 7.26 and 6.84, d, J = 9 Hz). The lack of abundance of M^+-15 peak, which is usually 100% for xanthones with methoxyl at C-5 (Chaudhuri and Ghosal, 1971) confirmed that the second methoxyl is substituted at C-7. These observations led to identification of the isolate 1 as 1,8-dihydroxy-3,7-dimethoxyxanthone (Swertiaperennine) which was fully supported by its mass spectrum.

The preliminary indication of 1,3,7,8-tetraoxygenated pattern of isolate 2 was further supported by the presence of four substituents as only four aromatic protons out of eight could be accounted for in the ^{1}H-NMR spectrum. One of these substituents was confirmed as methoxyl (δ 3.9, 3H, s) and the remaining three were identified as hydroxyls, two of which were placed peri to carbonyl at C-1 and C-8 (δ 11.92 and 11.85, typical of chelated hydroxyls). The compound was insoluble in 5% aqueous Na_2CO_3 which explained that the C-3 position was occupied by methoxyl (C-3 hydroxyl derivatives are soluble; Chaudhuri and Ghosal, 1971). These observations together with signals for a pair of two ortho and two meta coupled protons and a molecular ion peak at 274 confirmed the isolate 2 as 1,7,8-trihydroxy-3-methoxyxanthone (Swertianin).

Antimalarial Activity

1. In Vitro. The in vitro antimalarial activity of crude extracts 'M_1', 'M_2' & 'M_3' and three pure isolates was tested at dose levels of 50 and 100 µg/ml (Table 2, Fig. 2). All three extracts showed some activity with per cent inhibition ranging from 34 to 50. Methanol extract exhibited maximum per cent inhibition (50%) at 100 µg/ml dose level. The activity of three xanthones ranged from 38 to 52 per cent inhibition. The xanthones were more active at higher dose of 100 µg/ml and 1,8-dihydroxy-3,7-dimethoxyxanthone was the most active showing 42 and 52% inhibition at a dose of 50 and 100 µg/ml respectively.

2. In Vivo. The isolated compound 1,8-dihydroxy-3,7-dimethoxyxanthone, which showed maximum in vitro activity, was subjected to in vivo assay at three dose levels (1, 5, 10 mg/kg). The compound produced significant reduction in parasitaemia and a noticeable increase in activity was observed when the dose was increased from 1 to 5 mg/kg. The most significant reduction (17.60%, $p < 0.05$) in parasitaemia was noted with a dose of 10 mg/kg. The in vivo results were complementary to in vitro findings where also the higher dose showed better activity.

Literature Cited

Bolton, S. 1995. Statistics. In: Remington's, The Science and Practise of Pharmacy. 19th ed., Mack Publishing Company Eston, Pennysylvania. 109p.

Chaudhuri, R.K. and Ghosal, S. 1971. Xanthones of *Canscora decussata* Schult. Phytochemistry 10:2425-2432.

Chunekar, K.C. 2002. Bhavaprakasa Nighantu (Indian Materia Medica). 28th ed., Chaukhambha Bharati Academy, Varanasi, India. p.72-75.

Desjardins, R.E., Canfield, C.J., Haynes, J.D. and Chulay, J.D. 1979. Quantitative assessment of antimalarial activity in vitro by semiautomated microdilution technique. Antimicrob. Agents Chemother. 16:710-718.

Dutta, G.P. 1992. Screening techniques for antimalarial activity: UNESCO-CDRI workshop on the use of pharmacological techniques for the study of natural products.

Central Drug Research Institute, Lucknow, 30 March-4 April. 85 p.
Ekong, R.M., Kirby, G.C., Patel, G., Warhurst, D.C. and Phillipson, J.D. 1990. Comparison of the in vitro activities of quassinoids with activity against *Plasmodium falciparum*, anisomysin and some other inhibitors of eukaryotic protein synthesis. Biochem. Pharmacol. 40:297-301.
Fuchino, H. 2002. Regulations for production and marketing of Traditional medicines in Japan. Workshop on 'Regulations for production and marketing of medicinal plant products'. ICS-UNIDO, Trieste- Italy, 18-20 November, unpublished.
Karan, M., Vasisht, K. and Handa, S.S. 1997. Morphological and chromatographic comparison of certain Indian species of *Swertia*. JMAPS. 19:955-963.
Kumar, A. 2001. Morphological and phytochemical comparison of certain Indian species of *Swertia*. M. Pharm. Thesis, University Institute of Pharmaceutical Sciences, Panjab University, Chandigarh.
O'Neill, M.J., Bray, D.H., Boardman, P., Phillipson, J.D. and Warhurst, D.C. 1985. Plants as sources of antimalarial drugs. Part I. In vitro test method for the evaluation of crude extracts from plants. Planta Med. 50:394-397.
Peters, W. 1987. Chemotherapy and drug resistance in malaria (revised 2nd ed.), Academic Press, London.

Tables

Table 1. Treatment schedule for in vivo antimalarial test model.

Group	Day 0	Day 1	Day 2	Day 3	Day 4
Control	IM[1], V[2]	V	V	V	Preparation of blood
Test	IM, D[3]	D	D	D	smears to determine
Reference	IM, CQ[4]	CQ	CQ	CQ	per cent parasitaemia

[1] inoculum containing *P. berghei* infected RBC's, [2] vehicle, [3] test substance, [4] chloroquine

Table 2. In vitro antimalarial activity of crude extracts and xanthones of *S. alata.*

Group (conc μg/ml)	Culture smear (hr)	No of parasites per 2000 RBC's			% Inhibition
		R[1]	T[2]	S[3]	
Control	0	21	41	94	-
	20	102	4	4	-
Methanol extract (50)		56	2	4	45
Methanol extract (100)		1	9	50	50
Hexane extract (50)		57	5	4	44
Hexane extract (100)		67	5	4	34
Chloroform extract (50)		2	4	42	60
Chloroform extract (100)		2	8	44	57
1,8-dihydroxy-3,7-dimethoxyxanthone (50)		59	5	14	42
1,8-dihydroxy-3,7-dimethoxyxanthone (100)		49	6	16	52
1-hydroxy-3,7,8-trimethoxyxanthone (50)		63	2	8	38
1-hydroxy-3,7,8-trimethoxyxanthone (100)		56	5	8	45
1,7,8-trihydroxy-3-methoxyxanthone (50)		54	4	5	42
1,7,8-trihydroxy-3-methoxyxanthone (100)		55	1	5	46

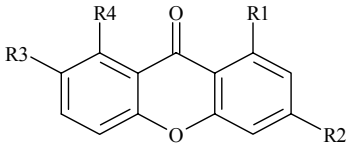
[1] ring, [2] trophozoite, [3] schizont

Table 3. In vivo antimalarial activity of 1,8-dihydroxy-3,7-dimethoxyxanthone.

Group/dose (mg/kg)	Percent parasitaemia (mean ± S.D.)
Control	36.50 ± 2.27
Test (1)	26.65 ± 2.4
Test (5)	19.91 ± 1.1
Test (10)	17.60 ± 0.92
Chloroquine	3.80 ± 3.08

(significant at $P < 0.05$)

Figures



	R1	R2	R3	R4
(1) 1,8-Dihydroxy-3,7-dimethoxyxanthone	OH	OCH3	OCH3	OH
(2) 1-Hydroxy-3,7,8-trimethoxyxanthone	OCH3	OH	OCH3	OCH3
(3) 1,7,8-Trihydroxy-3-methoxyxanthone	OH	OCH3	OCH3	OCH3

Fig. 1. Xanthons of *S. alata*.

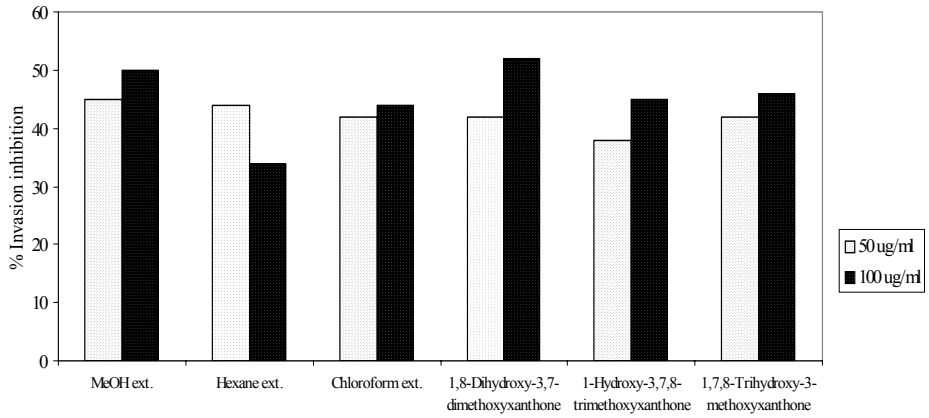


Fig. 2. In vitro antimalarial activity of extracts and isolates of *S. alata.*

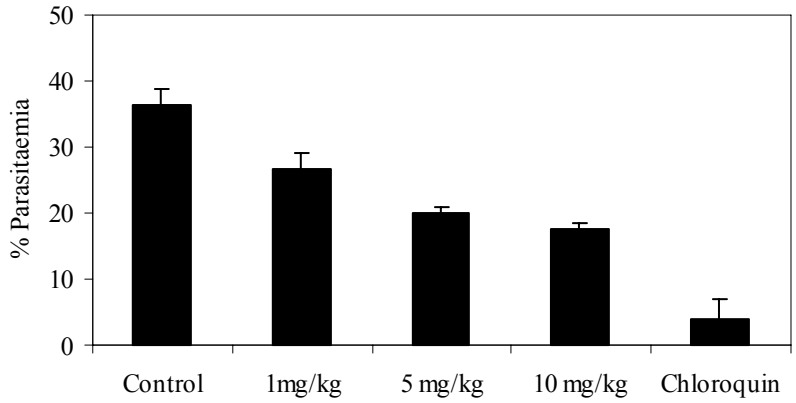


Fig. 3. In vitro antimalarial activity of 1,8-dihydroxy-3,7-dimethoxyxanthone.