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PJBS

ISSN 1028-8880

**Pakistan
Journal of Biological Sciences**

ANSI*net*

Asian Network for Scientific Information
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan

***In vivo* and *in vitro* Evaluation of Sterols from *Gymnema sylvestre* R. Br**

Sharad Vats and Raka Kamal

Laboratory of Medicinal Plant Biotechnology, University of Rajasthan, Jaipur-300004, Rajasthan, India

Abstract: *Gymnema sylvestre* R. Br. is an important medicinal plant known for its antidiabetic potential. In the present study, phytosterols from *G. sylvestre* was identified and quantified *in vivo* and *in vitro*. Maximum callus induction was observed in MS medium supplemented with 0.5 mg L⁻¹ of 2, 4-D. The protein content was significantly high both in aerial plant parts and callus tissue. Phytosterols were identified using chromatographic and spectral studies. β -sitosterol, campesterol and stigmasterol were identified both *in vivo* and *in vitro*. Lanosterol was identified only in callus culture. Phytosterols have reported for the first time in callus culture of *G. sylvestre*.

Key words: *Gymnema sylvestre*, MS medium, callus, protein, phytosterols

INTRODUCTION

In recent years there has been an upsurge in the use of plant based medicine all over the world. This is mainly because of the side effects and residual toxicity associated with synthetic drugs. The resistivity shown by various pathogenic organisms towards synthetic drugs due to their non restricted use has also favoured the inclination towards phytodrugs.

Sterols are important constituent of cell membranes in both animals and plants. The basic ring structure is common to various sterols and the difference lies in the side chain. More than 40 plant sterols have been identified (Law, 2000). Natural dietary intake varies from about 167-437 mg day⁻¹. It has been reported that phytosterols are absorbed in the human intestine at a very low amount. Dietary consumption of plant sterols helps in lowering the absorption of cholesterol (Ostlund, 2002). β -sitosterol has been reported as an antitumor and hypoglycaemic agent (Kaufman *et al.*, 1999). β -sitosterol in corn oil and other vegetables oil affect the cholesterol level in the plasma and also help in enhancing the functional activity of liver (Ye *et al.*, 2010). There are other reports trying to validate the anticancer activity of plant sterols (Awad *et al.*, 2000; Awad and Fink, 2000; Bradford and Awad, 2007).

Gymnema sylvestre R. Br. (Asclepiadaceae) is commonly called as *gurmar* which means 'sugar destroyer'. It is an antidiabetic plant which has an important place in traditional and Ayurvedic medicine. The plant is also used as a digestive, antiviral, diuretic, antiallergic, antidiabetic and hypolipidemic agent

(Bone, 1997). The plant contains gymnemic acids, conduritol A, gymnemasaponins, gymnemasins, gymnemosides, lupeol and quercitol (Kanetkar *et al.*, 2007). Excessive use of natural products of this plant for commercial purpose has led to its marked depletion from its natural habitat. Thus the need of the hour is to have effective *in vitro* system to harness its metabolites for therapeutic purpose and save the plant from being nearly extinct.

The present investigation was carried out to isolate and identify sterols from aerial plant parts and callus culture of *G. sylvestre*, which probably is the first ever report to the authors' best knowledge.

MATERIALS AND METHODS

Nodal explants (1-2 cm) were first washed in running tap water for 10 min and then given a liquid soap treatment with 1% (v/v) Teepol for 3 min with constant agitation. The explants were then treated with HgCl₂ (0.1%)+Ciprofloxacin(0.25%) for 6 and 4 min, respectively. Sterilants were washed off with four washes of sterile distilled water in a laminar airflow cabinet. Nodal segments were inoculated in the flasks containing MS medium supplemented with 2, 4-D (0.5 and 1.5 mg L⁻¹, each) and 2, 4-D:KN (5:0.02 mg L⁻¹). Cultured flasks were incubated in the growth chamber maintained at 26±1°C. The light intensity (1200 lux) was provided from fluorescent tubes (40 watt) and incandescent bulbs (40 watt). A photoperiod of 16 h light was used. The cultures were observed and examined every week and final data were recorded.

Primary metabolites: Total soluble sugars and starch was estimated using method of Dubois *et al.* (1951). Proteins were estimated according to the method of Lowry *et al.* (1951). Total lipid was estimated using method of Jayaraman (1981).

Sterol

Extraction: Aerial Plant parts and callus were dried, powdered and defatted in petroleum ether (60-80°C) for 24 h on a water bath. Defatted material was air-dried and hydrolyzed in 30% HCl (v/v) for 4 h. Each hydrolyzed sample was washed with water till pH 7 and dried. The dried preparation was again extracted with benzene for 24 h. The extract was filtered and dried *in vacuo*. The crude extract was dissolved in chloroform before chromatographic examination (Kaul and Staba, 1968).

Thin layer chromatography (TLC): Extracted samples were dissolved in chloroform and applied on activated TLC plates along with authentic standard. These plates were developed in airtight presaturated chromatographic chamber containing solvent mixture hexane: Acetone-8:2 (Fazli and Hardman, 1968). Other solvent systems such as benzene: Ethyl acetate-85:15 (Heble *et al.*, 1968) and benzene: ethyl acetate-3:1 (Kaul and Staba, 1968) were also used but hexane: Acetone (8:2) gave better separation. These plates were air-dried and visualized under UV light and fluorescent spots corresponding to that of standard markers were marked. These developed plates were sprayed with 50% H₂SO₄ and anisaldehyde reagent, separately and heated at 110°C for 10 min.

Preparative thin layer chromatography (PTLC): PTLC was performed using silica gel G coated plates (500 µm thick) along with the reference markers. These plates were developed in hexane: acetone (8:2), air-dried and examined under UV light. Each spot coinciding with that of standard markers was marked, scraped and eluted with chloroform. The eluted fractions were dried, weighed and subjected to crystallization for determination of melting point and mixed melting point. The isolated compounds were also subjected to UV and IR spectral studies.

Statistical analysis: Data were subjected to one-way Analysis of Variance (ANOVA) and means were separated using Duncan's Multiple Range Test (DMRT) at significance level p = 0.05.

RESULTS

The callus initiated from the cut ends of the nodal explant. Lower dose of 2, 4-D was found to be conducive for callus growth. The maximum percentage response and callusing was observed in 2, 4-D at concentration of 0.5 mg L⁻¹ (78±0.5%) with fragile yellowish white callus. Higher dose of 2, 4-D was not effective to induce callusing. The percentage response in MS medium supplemented with 2, 4-D (1.5 mg L⁻¹) was 72±0.8. Moreover, using 2, 4-D in combination with Kinetin (KN) at concentration of 5 and 0.02 mg L⁻¹, respectively the callus induction was minimum with least response (37±1.4%). The callus was light brown and compact.

Level of all the different primary metabolites tested was found to be more in aerial parts as compared to callus tissue. Maximum content was found to be of proteins both aerial plant parts (140 mg gdw⁻¹) and callus (102 mg gdw⁻¹). Minimum content was that of starch (16 and 10 mg gdw⁻¹ in aerial plant parts and callus, respectively). The lipid content was observed to be twice in aerial parts as compared to callus (Fig. 1).

Sterols identified in the present study were β-sitosterol, stigmasterol, campesterol and lanosterol (Table 1, Fig. 2-4). Total sterol was more in callus tissue (2.94 mg gdw⁻¹) as compared to aerial part (2.10 mg gdw⁻¹). Individually β-sitosterol was more in callus (1.20 mg gdw⁻¹), stigmasterol in aerial part (0.82 mg gdw⁻¹) and campesterol in aerial part (0.56 mg gdw⁻¹). The callus tissue of *G. sylvestre* showed the presence of lanosterol, which was not identified in aerial part. *In vitro* studies showed the least content of campesterol (0.50 mg gdw⁻¹) as compared to other sterols (Table 2).

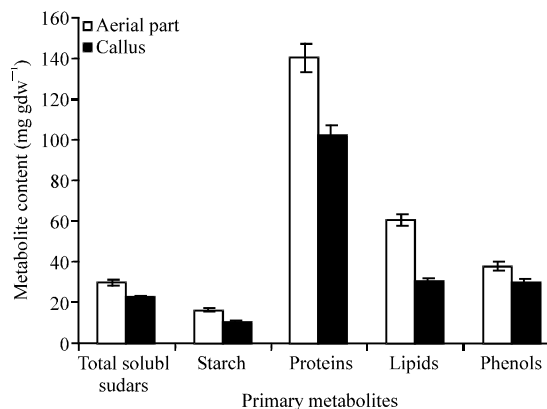


Fig. 1: Primary metabolites content in *G. sylvestre* *in vivo* and *in vitro*

Table 1: Chromatographic behavior and chemical characteristics of isolated phytosterols

Isolated compound	R value			Color after spray		M.P. (°C)	IR spectral peaks (rept.) v (Kbr) cm ⁻¹
	S ₁	S ₂	S ₃	R ₁	R ₂		
β-sitosterol	0.89	0.90	0.71	PU-BN	PK	136.137	33.50 (O-H), 2830, 1665 (C = C), 1470, 1300, 1052 (C-O) and 880
Stigmasterol	0.83	0.64	0.65	GY	PU	067.690	34.50 (O-H), 2950, 1750, 1640 (C = O), 1305 (C-O) 991, 957, 935, 810 and 715
Campesterol	0.29	0.23	0.21	GY	GY	157.158	3400 (O-H), 2950, 2850, 1640 (C = O), 1470, 1380, 1035, 880 and 820
Lanosterol	0.23	0.21	0.19	BL	GY	143.144	3450 (O = H), 2890, 1640, (C = O), 1385, 1360, 1310, 1290, 1270, 1110 and 880

S₁: Hexane:acetone (8:2), S₂: Benzene:acetone (2:1), S₃: Benzene:ethyl acetate (3:2), R₁: 50% H₂SO₄, R₂: Anisaldehyde reagent, BN: Brown, PK: Pink, PU: Purple, BL: Blue, GY: Gray

Table 2: Sterol content in *G. sylvestre* in vivo and in vitro

Samples	Sterols Content (mg gdw ⁻¹)				
	β-sitosterol	Stigmasterol	Campesterol	Lanosterol	Total
Aerial Part	0.72±0.10 ^b	0.82±0.08 ^a	0.56±0.20 ^a	ND	2.10±0.5 ^b
Callus	1.20±0.40 ^a	0.72±0.15 ^b	0.50±0.24 ^a	0.52±0.20	2.94±0.4 ^a

^{a, b}Mean±SE represented by the same letters within the same column are not significantly different at p>0.05, ND: Not detected

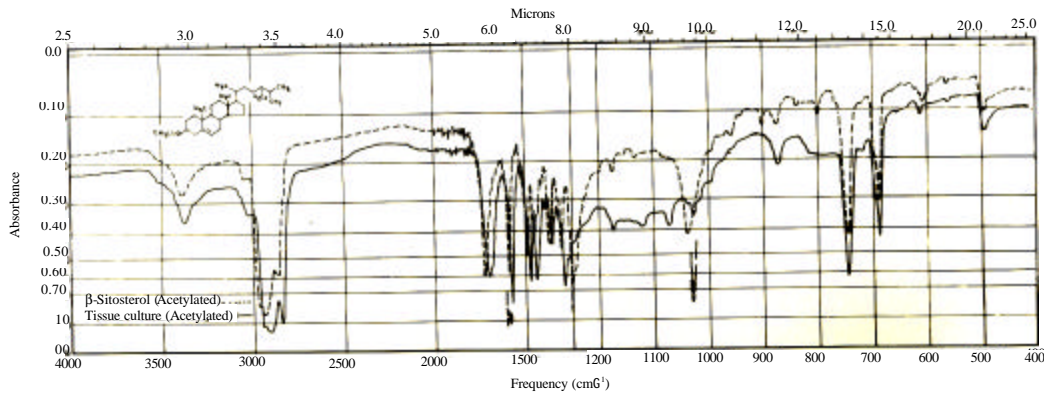


Fig. 2: IR spectra of isolated and standard β-sitosterol

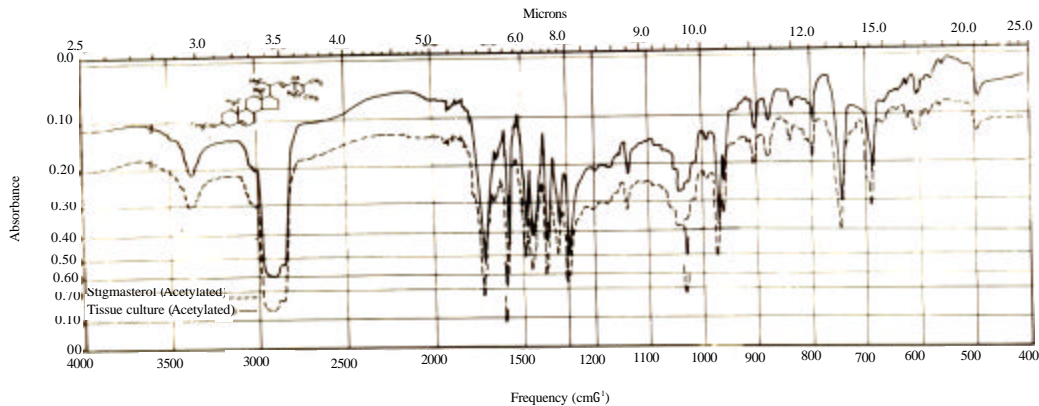


Fig. 3: IR spectra of isolated and standard stigmasterol

DISCUSSION

Various physical and chemical factors affect the growth of the plant tissue cultures and in order to achieve rapidly proliferating undifferentiated callus mass from

highly organized multicellular systems, use of optimum combination and concentration of plant growth hormones (auxins and cytokinins) is very essential.

In the present study *G. sylvestre* showed maximum callusing in lower doses of 2, 4-D. This was in

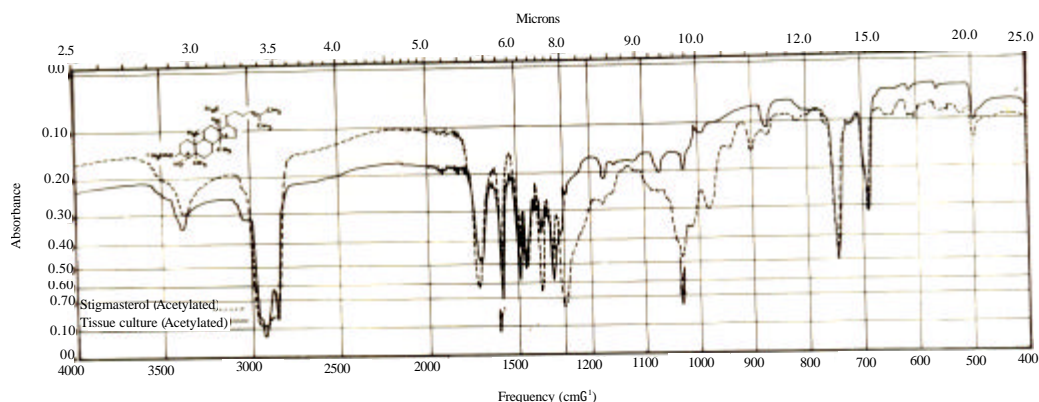


Fig. 4: IR spectra of isolated and standard lanosterol

confirmation with the study of various workers that different concentrations of auxins especially 2, 4-D inhibits differentiation and promotes callus formation. The callusing response of most of the plant growth regulators has been studied while multiplication of *G. sylvestre* by Reddy *et al.* (1998) and Komalavalli and Rao (2000). Ashok *et al.* (2002) reported the formation of callus from hypocotyl, cotyledon and leaf explants excised from seedlings of *G. sylvestre* using 2, 4-D and BAP. Roy *et al.* (2008) obtained maximum callus induction from nodal explants on 2, 4-D supplemented media from nodal explants.

Primary metabolites are essentially needed for the growth and development of the plants. They are the building blocks of various components of the cell like membranes, cytoskeletons, nucleic acids etc. The content of proteins was observed to be highest in aerial parts of *G. sylvestre* and callus tissue. This suggests that the experimental plant is a good source of protein *in vivo* and *in vitro*. The plant has been traditionally used as antidiabetic agent. Thus consumption of the same can be of useful medicinally as well as nutritionally.

β -sitosterol and campesterol has been reported in many plant sps (Galliard, 1968; Habib *et al.*, 2007). Secondary metabolites are usually not the end products of metabolism but show high degree of turnover (Wink, 1988). In plants production of bioactive compounds seems to be equilibrium between synthesis, storage and degradation and it depends on the developmental stage as to which component dominates. The presence of certain metabolites *in vitro* and absence *in vivo* may be due to the channeling of the same metabolite for degradative pathway with the help of enzymes. This can be the reason for the absence of lanosterol *in vivo* in *G. sylvestre* and subsequent presence in the callus culture.

Due to intake of diets rich in cholesterols and rather sedentary life style the risk of cardiac disorder and cholesterol associated risks has increased manifold. The isolated sterols in the present study have been reported to have immense therapeutic potential and can be useful in reducing above mentioned problem. Phytosterols/ phytostanols might have regulatory effect on proteins implicated in cholesterol metabolism thereby reducing serum Low-density Lipoprotein (LDL)-cholesterol level. It has been suggested that food products containing phytosterols taken as therapeutic dietary option reduce atherosclerotic risk (Calpe-Berdiel and Blanco-Vaca, 2009).

The presence of isolated sterols in the present investigation has been reported for the first time in the tissue culture of *G. sylvestre*. The study gives a reproducible system to harness phytosterols *in vitro* which can be of immense therapeutic potential and simultaneously help in conserving the endangered plant from near extinction.

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