Academic Sciences Asian Journal of Pharmaceutical and Clinical Research
Vol 6, Issue 1, 2013 ISSN - 0974-2441

Research Article

IN VITRO PRODUCTION OF BIOACTIVE COMPOUNDS FROM STEM AND LEAF EXPLANTS OF *JUSTICIA GENDARUSSA* BURM. F.

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Received: 15 October 2012, Revised and Accepted:7 December 2012

ABSTRACT

In the present study, a preliminary phytochemical analysis for the production of bioactive compounds in the *in vitro* grown cultures of stem and leaf derived calli of *Justicia gendarussa* Burm. f. was carried out. The phytochemicals produced *in vitro* was compared with that of the stem and leaf samples. The study showed the presence of phenolics, flavonoids, alkaloids and terpenes in all the test samples. The spectrophotometric estimation for phenolics, flavonoids, alkaloids and phytosterols showed the presence of equal or slightly increased concentrations of all these compounds in the cultures compared to the plant samples. Hence, the study forms an alternative method for the extraction of bioactive compounds by reducing the pressure on the naturally grown plants.

Keywords: *Justicia gendarussa*, *in vitro* culturing, phenolics, flavonoids, alkaloids, phytosterols

INTRODUCTION

Justicia gendarussa Burm. f. (Acanthaceae) is an important medicinal plant used in the treatment of various health problems. The plant is known for its medicinal properties such as antioxidant and free radical scavenging, anti-arthritic, anti-inflammatory, analgesic, antifertility, anticancerous, hepatoprotective and larvicidal properties[1-3]. The plant was also reported to contain phenolics, alkaloids and sterols[3-8].

It has become a common practice to harvest the plants in large quantity for the industrially important compounds to be used for the welfare of human beings. The unscientific harvesting of the plants led to the destruction of natural habitat of the plants including their extinctions. At this juncture, biotechnological tools are important for the multiplication and genetic enhancement of the medicinal plants by adopting suitable techniques. In the present study, an attempt has been made to produce the bioactive compounds through callus cultures followed by their extraction and detection.

MATERIALS AND METHODS

Plant material

Justicia gendarussa was collected from natural forests of Dakshina Kannada District, Karnataka, India, and identified following the Flora of Udupi and Dakshin Kannada[9] and the voucher specimen (MU/AB/BN-02) were deposited at the herbarium of Department of Applied Botany.

The healthy stem and leaf samples were collected from the plant, cleaned, dried at 40°C and powdered using Cyclotech lab mill for the extraction of phytochemicals.

Callus induction and harvesting

The surface sterilization of the stem and leaf samples were carried out as reported by Bhagya and Chandrashekar[10]. The stem and leaf explants were washed thoroughly under running tap water for 20-30min to remove the surface debris and treated with a systemic fungicide Bavistine for 30-45min. The explants with ~0.5cm size were then sterilized with 70% alcohol (2min) and 0.1% $HgCl_2$ (8 min). After each step, the explants were washed using sterile distilled water. The explants were inoculated to MS medium with NAA + BAP (1+0.1mg/l) and incubated at 25±2°C with 16 hrs of photoperiod and 40.0±3.0µmol m^{-2} s^{-1} light intensity for the induction of callus. The cultures maintained under these conditions for 320-360d were inoculated to both solid and liquid MS medium and harvested at the stationary phase i.e. between 30-35th day, washed in distilled water and freeze dried.

Chemicals and reagents

All the chemicals used were of analytical grade purchased from Merck, Himedia and SRL.

Extraction of phytochemicals

The stem, leaf and stem derived callus and leaf derived callus samples were used for the extraction of phytochemicals as per Harbone[11]. The phenolics were extracted in methanol, ethanol and ether. Alkaloids were extracted using ammonium hydroxide precipitation method. The terpenes were extracted in methanol and methanol: water mixture. The extract was concentrated to dryness and the yield was noted. The dried extract was redissolved in minimum quantity of respective solvents and used for qualitative and quantitative detection.

Qualitative detection of phytochemicals

Phenolics, alkaloids and steroids were detected using color reactions and chromatographic methods as per Harborne[11] and Mallikharjuna *et al*[12].

Spectrophotometric estimation of phytochemicals

Total phenolic content in the extracts of stem/leaf and callus samples were determined as per Malic and Singh[13] and expressed as mg/g of gallic acid equivalents (GAE).

Total flavonoid contents in the crude extract was determined according to Jia *et al*[14] and expressed as mg/g of quercetin equivalents (QE).

The alkaloids in the samples were determined as per Sreevidya and Mehrotra[15] using $Bi(NO_3)_3.5H_2O$ (10-90µg/ml) as standard and expressed as mg/g of bismuth complex.

The phytosterols in the samples were determined as per Sackett[16] and calculated using cholesterol (40-400µg/ml) as standard and expressed as mg/g of cholesterol.

Statistical analysis

The data presented are the average of three replicates and expressed as Mean±SD. The statistical analysis of all the data were carried out using SAS 9.0 version and the treatment means were compared using DMRT at a level of 5% significance.

RESULTS AND DISCUSSION

Extract yield of phytochemicals

The extract yield of phenolics, alkaloids and terpenes in *J. gendarussa* is given in Table 1. The extract yield of phenolics in ether, methanol and ethanol showed a significant difference between the samples. The highest on par yield of 280.3 ± 20.8, 274.1 ± 53.1 and 264.0 ± 50.5mg/g of dried sample was observed in ethanol extract of stem derived callus cultured on solid medium, methanol extract of stem derived callus cultured in liquid medium and methanol extracts of leaf derived callus cultured in liquid medium respectively. The higher yield of phenolics and flavonoids in methanol observed in the present study may be due to the polarity and solubility of these phytochemicals in the respective solvents. According to Jayaprakasha *et al*[17] flavonoid aglycones are extracted in ethyl acetate and medium polar to polar compounds like phenolic acids, flavonoid glycosides, polysaccharides and sugars are extracted in ethanol and methanol depending on the polarity. A higher yield of phenoics was observed in semisolid cultured cells of *Maytenus aquifolium* Mart. compared to suspension cells[18].

A significantly higher yield of 12.76 ± 1.76mg/g of alkaloids in the leaf samples of *J. gendarussa* followed by 9.93 ± 6.08mg/g in the liquid grown callus of stem explants was observed. Similar to this result, the callus cells of *Camptotheca acuminata* showed the reduced yield for an indole alkaloid camptothecin and 10-hydroxycamptothecin[19]. However, the *in vitro* cultured cells of *Pinellia ternata*[20] and *Coscinium fenestratum*[21] showed higher yield of alkaloids in the *in vitro* grown cultures compared to the plant samples.

The extraction of terpenes from suspension callus of stem using mortar and pestle method recorded significantly high yield of 437.0 ± 78.4mg/g of dried callus. Our result is in agreement with the report of Takazawa *et al*[22], wherin, they reported an increased production of triterpenes in the stem derived callus of *Actinidia arguta*, *Actinidia chinensis* and *Actinidia polygama*.

Qualitative detection of phytochemicals

The color reactions for the qualitative detection of phenolics, flavonoids, alkaloids, saponins and steroids showed the presence of all the compounds in all the samples except saponins (Table 2). Similarly, the color reactions for the detection of phytochemicals in *in vitro* cultured cells of *Brassica nigra*[23], *Mimusops elengi*[24] and *Ricinus communis*[25] were reported.

The chromatographic detection of phytochemicals in various samples of *J. gendarussa* is shown in Figure 1. The MeOH and ether extracts of stem, leaf, and their respective callus samples separated in TLC for phenolics using 10% acetic acid solvent showed the presence of fluorescing spots under the UV light (Figure 1a). The increased intensity of the bands by spraying FC reagent and fuming the plates with ammonia vapor further confirmed the presence of plant phenolics. Paper chromatogram treated with ammonia vapor indicated the presence of flavonoids (Figure 1b). TLC for furanocoumarins failed to show the characteristic furanocoumarin colors under UV. However, a few fluorescing spots were observed under UV which may be of some other classes of phenolics. Although, ether and methanol extracts showed the similar spots in the chromatogram for phenolics, the better separation was observed in ether extracts. The NH_4OH precipitated extract separated using TLC showed the presence of one single orange yellow colored spot in all the samples which confirmed the presence of alkaloids in the test sample (Figure 1c).

Table 1: Extract yield (mg/g of dried sample) of *J. gendarussa* phytochemicals in different solvents

Solvent	Stem callus and stem (Mean±SD)*			Leaf callus and leaf (Mean±SD)*			
	Suspension callus	Solid callus	stem	Suspension callus	Solid callus	Leaf	
Phenolics							
Ether	21.2±1.0 b-l	16.7±4.5 b-l	13.3±1.9 b-l	29.3±2.8 b-l	18.8±3.3 b-l	38.5±15.4 b-k	23.0±10.4 B
Methanol	274.1±53.1 a	46.6±5.4 b-k	174.7±5.4 b-d	264.0±50.5 a	103.0±3.6 b-f	136.2±37.4 b-f	166.4±89.1 A
Ethanol	141.3±3.7 b-e	280.3±20.8 a	107.7±2.8 b-f	139.3±42.2 b-e	73.3±8.6 b-i	196.6±17.8 b-d	156.4±70.9 A
	145.5±112.7 #	114.5±125.5 $	98.6±70.3 ¶	144.2±106.9 #	65.0±37.3 ¶	123.8±72.6 $	
S.E/plot = 23.27, C.V(%) = 20.18							
Alkaloids							
Ammonium hydroxide precipitation	9.93±6.08 ab	7.89±0.24 a-c	5.54±1.74 bc	3.050±1.55 c	2.95±0.15 c	12.76±1.76 a	
S.E/plot = 6.90, C.V(%) = 39.24							
Terpenes							
Methanol in mortar	437.0±78.4 a	4.9±0.3 b-h	82.1±7.7 b-h	343.6±42.1 b	186.9±32.7 b-f	183.8±29.1 b-f	206.3±154.6 A
Methanol in soxhlet	337.2±47.4 b	3.7±0.8 b-h	186.8±24.1 b-f	348.6±137.1 b	143.5±18.7 b-f	287.2±37.9 b	217.8±135.5 A
Methanol:water in soxhlet	127.1±6.3 b-h	1.1±0.2 b-h	200.3±0.9 b-f	213.5±67.7 b-e	164.0±43.4 b-f	220.7±6.2b c-e	154.4±82.6 B
	300.4±144.4 #	3.2±1.7 ♫	156.4±57.4 ¶	301.9±103.3 #	164.8±34.3 ¶	230.6±51.4 $	
S.E/plot = 47.00, C.V(%) = 24.38							

*** Values represent the Mean ± SD. Means with the different letters indicate significant differences at 5% level. Lowercase letters indicate the interaction means, uppercase and symbols indicate the marginal means.**

Table 2: Color reactions for phytochemicals in *J. gendarussa*

Class of compound	Name of the test	Stem	Stem solid callus	Stem liquid callus	Leaf	Leaf solid callus	Leaf liquid callus
Phenolics	Phenol test	+	+	+	+	+	+
	Ellagic acid test	+	+	+	+	+	-
Flavonoids	Lead acetate test	+	+	+	+	+	+
	Zn/HCl test	-	-	-	-	-	-
Tannins	$FeCl_3$ test	+	+	+	+	+	+
	Gelatin test	+	+	+	+	+	+
Alkaloids	Mayer's test	+	+	+	+	+	+
	Wagner's test	+	+	+	+	+	+
	Dragendorff's test	+	+	+	+	+	+
Steroids	Salkowski test	+	-	+	+	+	+
	Libermann-Burchard test	+	-	+	+	+	+
Saponins	Foam test	-	-	-	-	-	-
	Heamolysis test	-	-	-	-	-	-

- **Absent**

+ **Present**

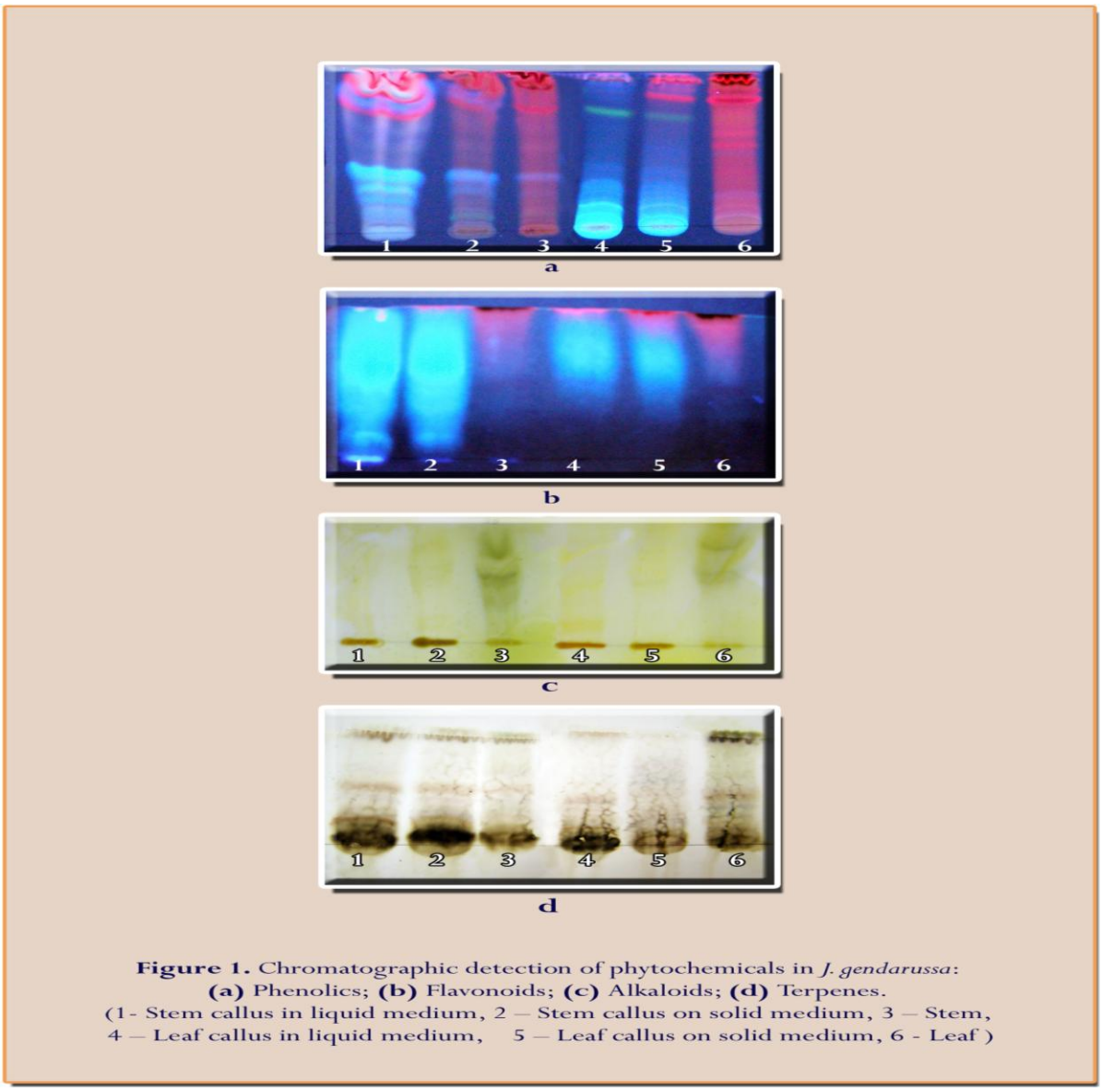


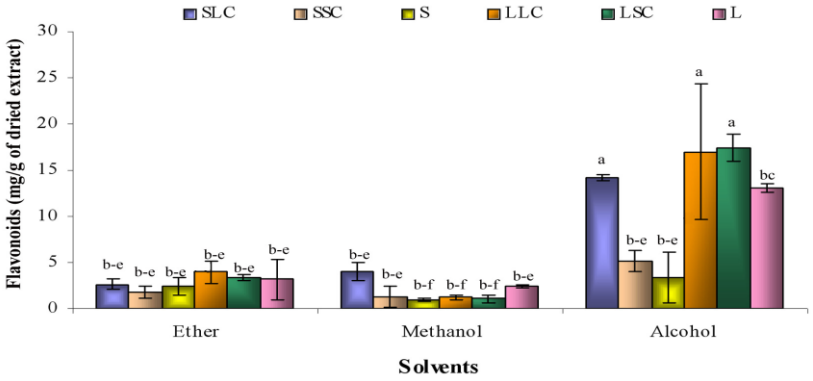
Figure 1. Chromatographic detection of phytochemicals in *J. gendarussa*:
(a) Phenolics; **(b)** Flavonoids; **(c)** Alkaloids; **(d)** Terpenes.
(1- Stem callus in liquid medium, 2 — Stem callus on solid medium, 3 — Stem,
4 — Leaf callus in liquid medium, 5 — Leaf callus on solid medium, 6 - Leaf)

The TLC for terpenes and phytosterols showed better separation in n-butanol:2M NH_4OH (1:1) solvent system compared to others and showed the presence of three major bands when sprayed with detecting agents such as Libermann-Burchard reagent or H_2SO_4 :H_2O (Figure 1d). The $CHCl_3$ extract of sapogenin showed negative result. Chromatographic techniques have been used for the detection of phenolics, flavonoids, alkaloids and terpenes in cultured cells and respective plant samples of *Brassica nigra*[23], *Mimusops elengi*[24] and *Ricinus communis*[25].

Spectrophotometric estimation of phytochemicals

The spectrophotometric estimation of phenolics in the test samples showed a significantly highest on par concentrations of 183.33 ± 57.73mg/g in the solid grown stem callus extracted in methanol and solid grown leaf callus extracted in ethanol followed by 133.33 ± 14.43mg/g and 100.00 ± 0.00mg/g in solid grown stem callus in ethanol and liquid grown stem callus extracted in methanol respectively (Figure 2).

The concentration of total phenolics accumulated in the cultured tissue was higher in the callus of *Tecoma stans* (L.) Juss. ex Kunth.[26] and in the hypocotyl explants derived callus of *Brassica nigra*[23]. Even in the present study, the spectrophotometric estimations of phenolics in *J. gendarussa* showed the highest concentration in solid grown callus of stem explant extracted in methanol and solid grown callus of leaf extracted in ethanol. Amid *et al.*[27] estimated the phenolic content in the suspension culture from the leaf derived callus of *J. gendarussa* grown in MS medium supplemented with NAA + BAP (1 + 0.5mg/l). The phenolic content of 88mg/g observed by them was very low compared to the total phenolic content of 183.33 ± 57.73mg in the solid grown callus of stem and leaf explants extracted in methanol and ethanol (alcohol) respectively in the present study.

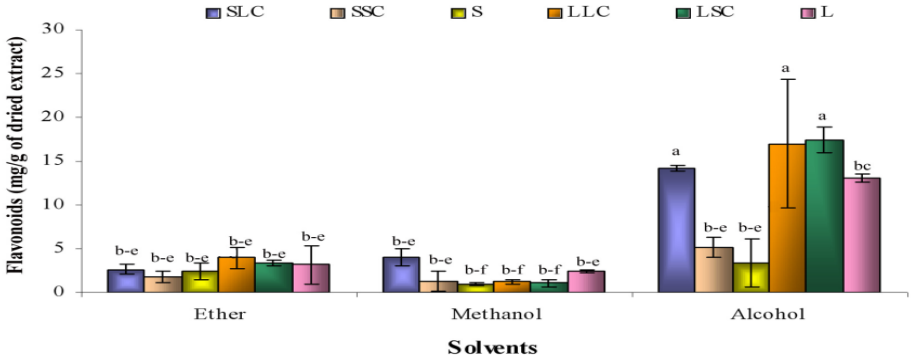


S.E/plot = 21.21, C.V. (%) = 36.26
Values represent the Mean±SD. Means with different letters indicate significant differences at 5% level.
SLC = stem callus in liquid medium, SSC = stem callus on solid medium, S = stem, LLC = leaf callus in liquid medium, LSC = leaf callus on solid medium, L = leaf

Fig. 2: Concentration of phenolics in different extracts of *J. gendarussa*

Ethanol (alcohol) was found to a better solvent for the extraction of flavonoids with a highest on par concentrations of 17.00 ± 7.31mg/g, 17.39 ± 1.50mg/g and 14.23 ± 0.28mg/g in liquid grown leaf callus, solid grown leaf callus and liquid grown stem callus respectively (Figure 3).

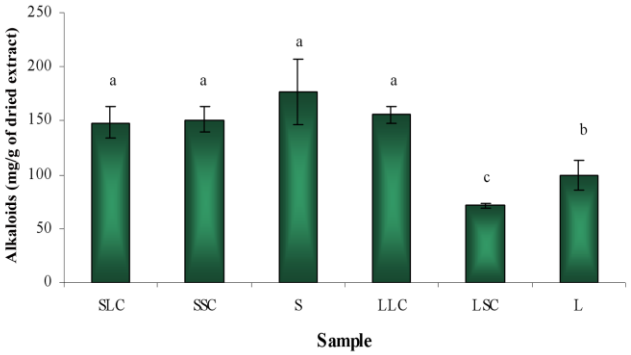
Establishment of cell suspension culture for the production of flavonoids was reported from the leaf derived callus of *Saussurea medusa* Maxim.[28] and callus cultures of *Saussurea involucrata*[29]. The cell suspension cultures from leaf, fruit and root explants of Indian Mulberry (*Morinda citrifolia*) in MS medium supplemented with NAA, BAP and Kn led to the increased production of flavonoids[31]. Similarly, in the present study, the suspension grown leaf callus, solid grown leaf callus and suspension grown stem callus showed the highest on par concentrations of flavonoids in *J. gendarussa*.



S.E/plot = 2.04, C.V. (%) = 37.29
Values represent the Mean±SD. Means with different letters indicate significant differences at 5% level.
SLC = stem callus in liquid medium, SSC = stem callus on solid medium, S = stem, LLC = leaf callus in liquid medium, LSC = leaf callus on solid medium, L = leaf

Fig. 3: Concentration of flavonoids in different extracts of *J. gendarussa*

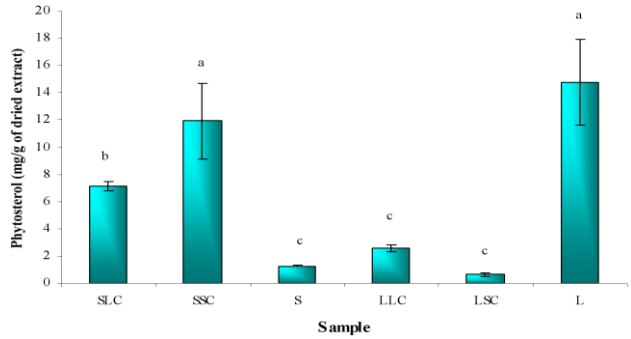
On par alkaloid concentration was observed in stem, liquid grown leaf callus, solid grown stem callus and liquid grown stem callus, the highest being in stem sample (176.99 ± 30.66mg/g) (Figure 4). Similar to our results, an enhanced accumulation of total alkaloids was observed in the *in vitro* grown cultures of *Stephania cepharantha*, a member of Menispermaceae[30] and in the *in vitro* grown cultures of *Ricinus communis* a member of Euphorbiaceae when compared to the root, hypocotyl and cotyledonary explants[25].



S.E/plot = 68.99, C.V. (%) = 11.98
Values represent the Mean±SD. Means with different letters indicate significant differences at 5% level.
SLC = stem callus in liquid medium, SSC = stem callus on solid medium, S = stem, LLC = leaf callus in liquid medium, LSC = leaf callus on solid medium, L = leaf

Fig. 4: Concentration of alkaloids in different extracts of *J. gendarussa*

Highest on par concentrations of 11.91 ± 2.76mg/g and 14.79 ± 3.15mg/g of phytosterol were observed in 90% methanol extract of solid grown stem callus and leaf sample respectively followed by 7.17 ± 0.34mg/g in liquid grown stem callus (Figure 5). The presence of fucosterols in seaweed[31], sterol in edible fats and oils of ten vanaspati ghee samples[32] and phytosterols in the succulent shoots of *Bambusa balcooa*[33] were detected and estimated using Liberman – Burchard reagent. Similar to the results obtained in the present study, a higher concentration of terpenes was observed in the cultured stem calli of Actinidiaceous species when compared to the stem samples[22].



S.E/plot = 1.72, C.V. (%) = 26.94
Values represent the Mean±SD. Means with different letters indicate significant differences at 5% level.
SLC = stem callus in liquid medium, SSC = stem callus on solid medium, S = stem, LLC = leaf callus in liquid medium, LSC = leaf callus on solid medium, L = leaf

Fig. 5: Concentration of phytosterols in different extracts of *J. gendarussa*

The present study illustrates the production of equal/near equal or slightly increased concentrations of total phenolics, flavonoids, alkaloids and phytosterols in the *in vitro* cultured cells when compared to the plant samples. Methanol was found to be a better solvent for the extraction of most of the phytochemicals. The crude extracts of the samples showed the antioxidant activity with a maximum activity being observed in solid grown stem derived callus extracted in ethanol and methanol (unpublished observation by Bhagya and Chandrashekar, 2012, under preparation and will be communicated soon). Therefore, *in vitro* culturing may be considered as a better method to harvest higher concentrations of bioactive phytochemicals than the stem/leaf samples.

ACKNOWLEDGMENTS

Senior author (BN) is grateful to UGC, New Delhi for financial assistance under RFSMS scheme. Authors are thankful to VGST, Department of IT, BT and S & T, Govt. of Karnataka, India for financial assistance.

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