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Rapid *in vitro* multiplication and biological potentialities of *Sericostoma pauciflorum* stocks ex Wight

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In the present study, an efficient *in vitro* plant regeneration protocol for *Sericostoma pauciflorum* stocks ex Wight, which is known for hypoglycemic efficacy, was achieved. Callus cultures from nodal explants were raised on Murashige and Skoog's (MS) medium with Indole acetic acid (IAA, 0.5 to 2 mg/l) and 2, 4-dichlorophenoxyacetic acid (2, 4-D, 0.5 to 2 mg/l). Shoot regeneration occurred from callus cultures inoculated on a medium supplemented with IAA (0.5 to 7 mg/l) and for root induction different concentrations of IAA (0.5 to 2.0 mg/l) and indole butyric acid (IBA, 0.5 to 2.0 mg/l) were used. Out of the various concentrations used, 2, 4-D (1.5 mg/l) proved to be better with 90% response for callus formation. Similarly, 3 mg/l IAA showed highest number of shoots (18.6±0.40). These shoots on MS medium supplemented with IAA (1.5 mg/l) gave maximum number of roots (5.40±1.98). For acclimatization, pure sand, garden soil and soil+compost (1:1) were used individually. The sand proved better by giving the survival rate 80%. The hardened plantlets were successfully transferred to the green house conditions and subsequently to the open field conditions. Different bioefficacies viz. antimicrobial using agar well diffusion method and antioxidant using 2,2 -diphenyl-2-picrylhydrazyl (DPPH) and Ferric ion reducing antioxidant potentials (FRAP) methods along with total phenolic content were studied and compared with those of *in vivo*.

Key words: *Sericostoma pauciflorum*, micropropagation, antimicrobial activity, antioxidant activity, DPPH, FRAP.

INTRODUCTION

Sericostoma is a small genus of family Boraginaceae. This family comprises of 8 species and is distributed through the tropical East and North East of Africa and North West India. These have close morphological resemblance to *Heliotropium* which are reputed to possess antitumor, carcinogenic, diuretic, laxative and emetic activities (Smith et al., 1962; Culvenor, 1968; Powis et al., 1979). *S. pauciflorum* is a short straggling under shrub growing widely throughout sea coast of Saurashtra and Maharashtra. This plant is used in making an important drug in Ayurveda named "Krishnavalli", which is used against cancer, diabetes and as health promoter (as described in "Nighantu Ratnakar"). Leaves are used in dehydration and acidity. Phytochemically, fernane, hopane and other type of triterpenoids were isolated (Afza et al., 1992; Ayatollahi et al., 1991, 1992 a, b). In view of introduction of this plant species, several protocols have been developed for the micropropagation of such medicinal herbs, but only a few have been demonstrated at the field level (Manjkhola et al., 2005; Lameira and Pinto, 2006). So far, not much work has been done about its micropropagation and biological activities on this species. Therefore, the present study was conducted to generate a protocol for micropropagation and acclimatization of *S. pauciflorum* and evaluation of biological potentialities, such as

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antimicrobial and antioxidant activity both *in vivo* and *in vitro.*

MATERIALS AND METHODS

Plant material

Whole plants of *S. pauciflorum* were collected from the fields locally during the months of July to October, 2008. The botanical identity was confirmed by Herbarium, Department of Botany, University of Rajasthan, Jaipur. (Voucher specimen no.110). The plant has been deposited at the Herbarium and Laboratory for further reference. Young nodal explants were washed under running tap water for 15 min to remove dust particles. Subsequently, nodal explants were cut into small pieces (2 cm) and treated with abs. alcohol for 30 sec followed by treatment with 0.1% (w/v) $HgCl_2$ for 4 min. After repetitive washings with sterilized distilled water (DW), the explants were transferred on sterile culture medium containing growth regulators of analytical grade (Merck, Germany).

Experimental

MS medium (Murashige and Skoog, 1962) with 3% (w/v) sucrose and 0.8% (w/v) agar (HiMedia, India) were used in all the experiments. After adding the growth regulators, the pH of the medium was adjusted to 5.8±0.2 and autoclaved at 121°C and 15 psi for 20 min. These cultures were incubated in the culture room maintained at 25±2°C at fluorescent light of 300 to 400 lux for 16 h of photoperiod. The MS medium augmented with different concentrations of IAA (0.5 to 2 mg/l) and 2, 4-D (0.5 to 2 mg/l) were tested individually for callus induction. Developed callus was transferred on MS medium supplemented with different concentrations of IAA (0.5 to 7 mg/l) for shoot formation. Regenerated shoots were further sub-cultured to multiply the number. For root induction, individually *in vitro* raised micro-shoots (1 to 4 cm long), were excised and transferred to MS medium supplemented with different concentration of IBA and IAA (0.5 to 2 mg/l). 15 replicates were used in each experiment and the experiment was repeated twice. The percentage of rooting was recorded after 60 day. *In vitro* plantlets with well developed roots (4 to 5 cm in length) were washed carefully with sterilized DW to remove traces of agar and treated with abs. alcohol to prevent any microbial infection and then transferred to the plastic pots (8 cm diameter) containing sterilize potting mixtures viz. sand, soil and sand+compost mixture (1:1) individually. The pots were covered tightly with glass beakers to avoid rapid changes in environment. 1/2 strength MS salt solution in sterilized DW is used for irrigation. During hardening procedure, glass covers were gradually removed after 4 week in order to acclimatize the plants in green house condition. After two months, the plantlets were transferred to the greenhouse for the development into mature plants.

Biological activities

8 Weeks-old *in vitro* regenerated plantlets as well as *in vivo* plant were washed carefully, shade-dried, powdered and extracted in a Soxhelt apparatus with 100 ml of ethanol (8 x 2 h). Extract were filtered, concentrated *in vacuo,* weighed and stored at 4°C, till further studies. For antibacterial screening pure cultures of test bacteria*, Bacillus subtilis* (MTCC 441)*, Enterobacter aerogenes* (MTCC 111), *Escherichia coli* (MTCC 443)*, Pseudomonas aeruginosa* (MTCC 741), *Raoultella planticola* (MTCC 530) and *Staphylococcus aureus* (MTCC 740), were obtained from the IMTECH, Chandigarh, India. For preparation of inoculums, these cultures were grown and maintained on Nutrient Broth medium (NB) at 27°C for 48 h. For antifungal screening, *Aspergillus flavus* (ATCC 16870), *A. niger* (ATCC 322), *Candida albicans* (ATCC 4718*)*, *Penicillium crysogenum* (ATCC 5476*)* and *Tricophyton rubrum* (ATCC 2327) obtained from IARI, New Delhi, India. These were cultured on Sobouraud Dextrose Agar medium (SDA) at 37°C for 48 h. Antimicrobial assay was performed by Agar Well Diffusion method (Boyanova et al., 2005).

The inoculum was prepared by suspending bacteria in NB medium and fungus in SDA medium overnight at 37°C (10^6-10^7 CFU/ ml concentration). Bacterial and fungal suspensions were inoculated in NA and SDA plates, respectively. 4 mg extract concentration was used for each well. Plates were then incubated at 37°C for bacteria and 25°C in case of fungi for appropriate time periods under aerobic conditions. The diameter of the inhibition zone around each well was measured and recorded by Inhibition Zone Recorder (HiMedia, India). Three replicates were used and the average value was statistically analyzed (Mean ±S.E.). Gentamycin (10 mcg/disc) for bacteria and ketonocozole (10 mcg/disc) for fungi, were used as positive controls. The total phenolic content was determined with Folin-Ciocalteau reagent (Bray and Thorpe, 1954). Optical density (OD) was measured at 750 nm (Pharmaspec UV-Vis spectrophotometer, Shimadzu). A standard calibration curve of gallic acid (1 to 50 mg/ml) were prepared and total phenolics in extend were expressed in mg of gallic acid equivalents (mg GAE /g) of extract. All determinations were carried out in triplicate and statistically analyzed (Mean ± S.E.). Free radical scavenging activity is determined using DPPH method (Fogliano et al., 1999). OD was measured at 517 nm using a UV-Vis spectrophotometer. Quercetin was used as standard. The capability to scavenge the DPPH radical was calculated using following equation:

$$\% \text{ Inhibition} = 1-(OD_{sample}/ OD_{control}) \times 100$$

Total reducing power of extracts was determined according to FRAP method (Yen and Chan, 1995). The OD was measured at 700 nm using a UV-Vis spectrophotometer. Higher absorbance of the reaction mixture indicated greater reducing power. Ascorbic acid used as positive control. Phytochemical studies are done using thin layer chromtography (TLC) profile on silica G plates (0.4 to 0.5 mm) along with reference markers (Harborne, 1973). Heptane-benzene-alcohol (100:100:1) and butanol: 27% aqueous acetic acid (1:1 v/v) were used as mobile phase. Plates were visualized by spraying with 10% $SbCl_3$ in chloroform and 10% methanolic $AlCl_3$. Several spots coinciding reference compounds as markers were scrapped from unsprayed plates, eluted with methanol, filtered, evaporated to dryness, reconstituted and crystallized in methanol. The melting point of the isolated compounds was determined in capillary tubes (Toshniwal melting point apparatus) and subjected to Infrared (IR) spectrum (Perkin Elmer 337, Grating Infra-red spectrophotometer).

RESULTS

In *S. pauciflorum* callusing was observed after 15 day of inoculation on IAA supplemented medium and after 7 day on 2, 4-D supplemented medium. Among all the tested concentrations, 2, 4-D at 1.5 mg/l proved to be best followed by IAA (1.5 mg/l, Table 2 and Figure 1A). Callus developed on 2, 4-D was dull in color and friable in nature, whereas callus developed on IAA brown and compact. Moisture content was recorded 96% in 4 week of the age. Shoot formation was achieved on IAA (0.5 to 7 mg/l) when 4 week callus was transferred to fresh MS

Table 1. Chromatographic and chemical characteristics of isolated compounds from *S. pauciflorum* (stem callus).

Isolated compound	R_F (× 100)		Color after spray		m.p. (°C)	IR (v_{max}) cm^{-1} (KBr)
	I	II	I	II		
Friedelin	81	-	Pink	-	198 to 200	1720, 1380, 1365, 1255, 1230, 1200, 920
α- Amyrin	26	-	Pink	-	183 to184	3350, 1640, 1480, 1360, 1130, 1050, 930
β- Amyrin	20	-	Pink	-	197 to198	3350, 1650, 1190, 1140, 1100, 1050
β- Sitosterol	06	-	Blue	-	136 to137	1730, 1640, 1240, 735, 725
Caffeic acid	-	76	-	Yellow	210 to121	812, 849, 899, 972, 1118, 1172, 1212, 1448, 1640, 3440

I: Heptane-benzene-alcohol (100:100:1), spray with 10% $SbCl_3$ in chloroform; II: Butanol: 27% aqueous acetic acid (1:1 v/v): spray with 10% methanolic $AlCl_3$.

Table 2. Callus regeneration from nodal explant in *S. pauciflorum.*

Hormone supplemented	Concentration (mg/l)	% Response	Callus growth	Texture	Color	Response
IAA	0.5	40	+	CM	GY	Embryogenic
	1.0	40	+	"	GY- BN	"
	1.5	80	++	"	GY	"
	2.0	60	++	"	GY- BN	"
2,4-D	0.5	20	+	FR	WT	Non-embryogenic
	1.0	40	++	"	WT	"
	1.5	90	++++	"	WT	"
	2.0	60	++	"	WT-BN	"

Evaluation after 8 week after of culture initiation. +, Low, ++, Moderate, +++, High, ++++, intense. FR, friable; CM, compact; GY, grey; BN, brown; WT, white.

medium (Table 2). Maximum number of shoots were observed in 3 mg/l (18.6 ± 0.40) followed by 2 mg/l (15.2±0.46) concentrations of IAA (Figure 1B) within 60 day. Maximum rooting (5.40±1.98) was recorded on medium supplemented with 1.5 mg/l IAA followed by IBA 0.5 mg/l (5.0± 0.31). Lower concentration of IAA did not show any response (Table 3 and Figure 1D and E). From *in vitro* plants, several triterpenoid compounds viz. friedelin, α-amyrin, β- amyrin, β- sitosterol and a phenolic compound name caffeic acid, were isolated and identified on the basis of their chromatographic behavior, melting points and spectral analysis (Table 1 and Figure 2). Fully grown plantlets (5 to 6 cm long shoot along with roots) were transferred to the pots (Figure 1G).

These plants are supplied with autoclaved half strength MS media. Plant has the arid zone adaptation so it survives best in sand as it show 80% survival on day 15 (Table 4). New leaf generated within 15 days after transferring the plantlet into pots (Table 5). Soil+compost mixture gave least survival rate (40%). The results of anti-microbial screening of test extracts of *S. pauciflorum* are summarized in Table 6. *In vitro* plant extract showed appreciable antibacterial activity (mm) against *P. aeruginosa* (IZ 14.66±0.34 mm) and notable antifungal activity against *P. chrysogenum* and *T. rubrum* (IZ 13.33±0.34 and 12.66±0.67 mm, respectively). In *in vivo* extracts appreciable activity against *P. aeruginosa, P. chrysogenum* and *T. rubrum* were recorded (IZ 15.33±0.34, 12.66±0.67 and 13.33±0.67 mm, respectively). The results of antioxidant activity are given in Table 7. Total phenolic contents were recorded as 73.75±0.72 mg GAE/g in *in vitro* extract and 99.75±1.60 mg GAE/g in *in vivo* extract. Highest antioxidant activities by mean of percentage inhibition of DPPH were 94.81% in *in vitro* plant as compare to *in vivo* plant extract (94.07%) at 80 µg/ml concentration. IC_{50} values were 6.5 and 7.5 µg/ml in *in vitro* and *in vivo* extract, respectively. Same results were obtained in FRAP method, that is, minor difference in the absorbance in *in vitro* and *in vivo* extracts.

DISCUSSION

To date any reports are not available on *in vitro* regeneration protocol of *S. pauciflorum* along with its biological efficacies. From the literature it is evident that 2, 4-D was most effective for the induction of somatic embryos and callus generation in family Boraginaceae (Raquel and Romanato 2000; Chithra et al., 2005; Xu et al., 2008). 2, 4-D involved in induction of gene expression,

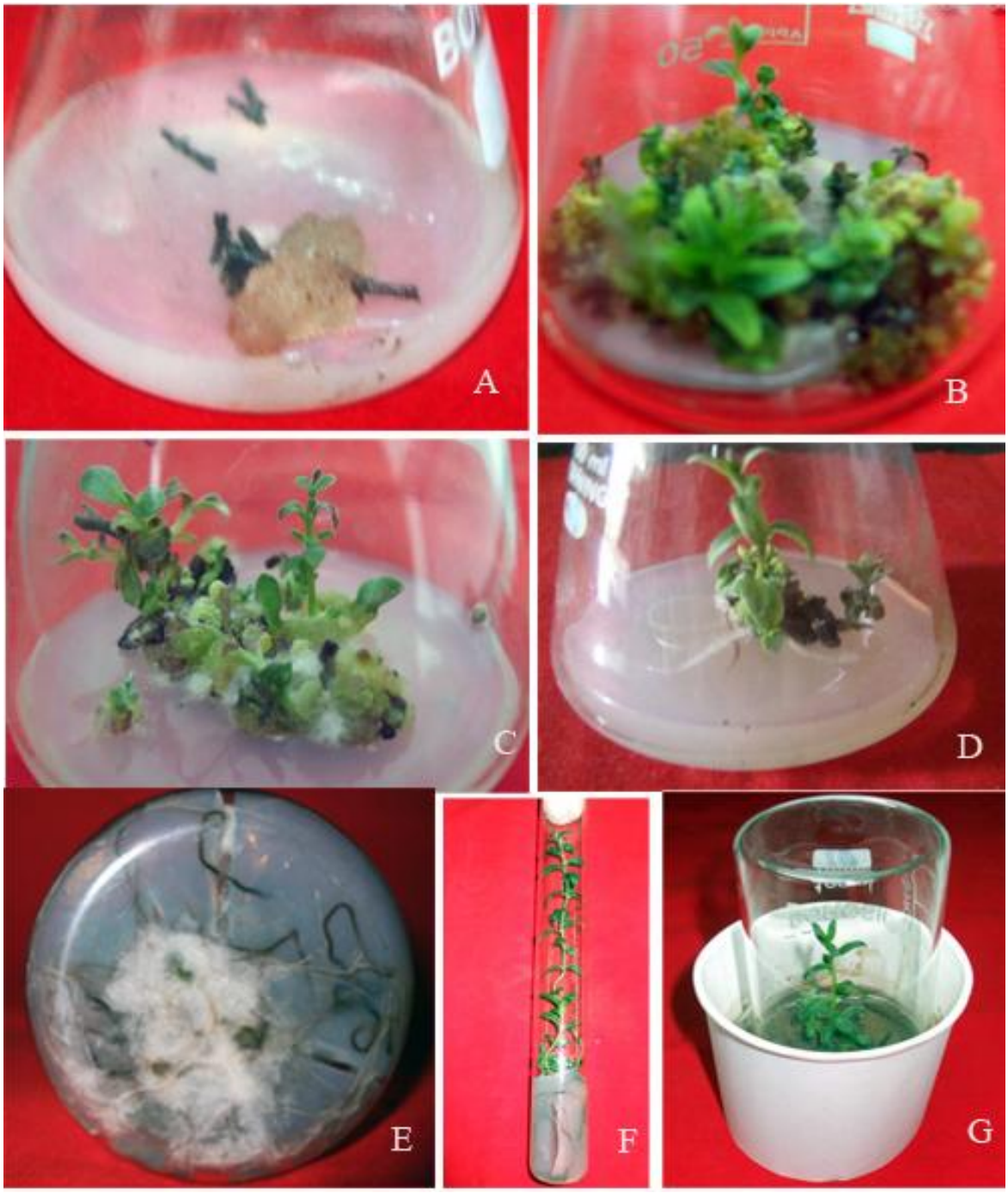

Figure 1. Multiple shoot formation in *S. pauciflorum.* **A** Callus initiation in shoot node explants cultured on MS medium; **B** Shoot generation from callus supplemented on different concentrations of IAA; **C** Multiplication of shoots; **D** root initiation; **E** rooting after 60 days; **F** *In vitro* regenerated plantlets; **H** 8 weeks-old plant acclimatized in culture room condition.

characteristic changes in ribonucleic acid (RNA), protein syntheses and deoxyribonucleic acid (DNA) synthesis (Yasuda et al., 1974; Caliskan, 2001). Similar results were obtained in *S. pauciflorum* where maximum callus induction was observed in 2,4-D supplemented medium. IAA regulates receptory complexes on plasmalemma by which transduction of environmental signals to cell compartments realized (Venis, 1985; Merkys et al., 1998). Auxins in its higher concentration than of optimal level reduce the number of shooting (Kukreja et al., 1990; Sen and Sharma, 1991). Similar results were obtained as maximum no. of shoots was observed in 3 mg/l IAA

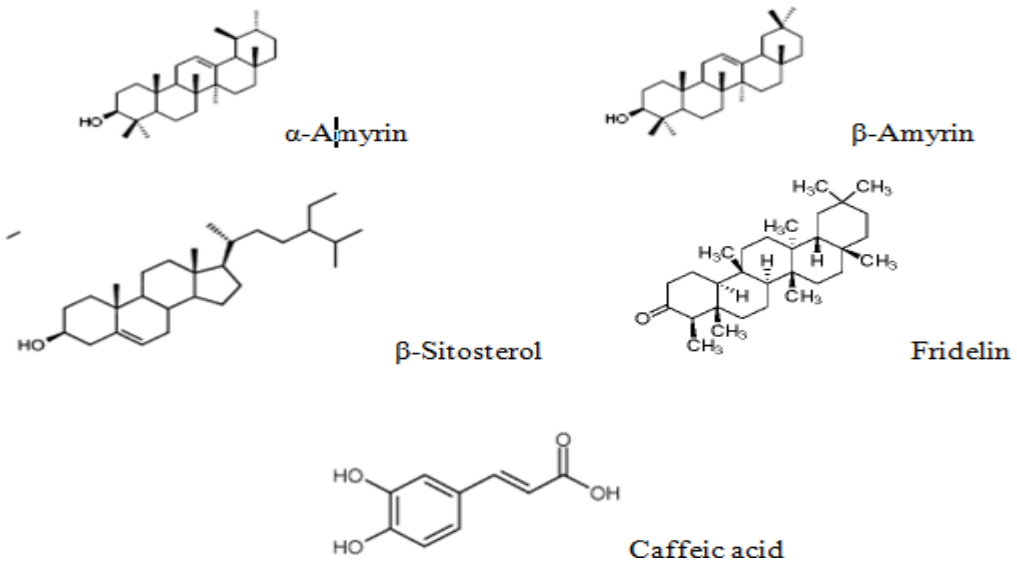

Figure 2. Isolated compounds from *S. pauciflorum in vitro* regenerated plants.

Table 3. Effect of IAA on shoot formation.

IAA(mg/l) supplemented	% Response	Mean no of shoots	Mean length of shoots (cm)
0.5	20	2.2 ± 0.372	2.0 ± 0.31
1.0	40	5.2 ± 0.45	2.8 ± 0.42
2.0	60	15.2 ± 0.46	3.0 ± 0.34
3.0	80	18.6 ± 0.40	3.2 ± 0.33
4.0	80	13.0 ± 0.59	4.2 ± 1.34
5.0	40	8.8 ± 0.56	0.91 ± 0.64
6.0	40	6.0 ± 0.31	0.50 ± 1.34
7.0	20	2.4 ± 0.24	0.74 ± 0.94

Evaluation after 8 week transfer of callus to shooting medium.

Table 4. Effect of IAA and IBA on root formation.

Hormone supplemented	Concentration (mg/l)	% Response	No of roots / shoots	Length of roots (cm)
IBA	0.5	60	5.0 ± 0.31	2.5 ± 0.35
	1.0	60	3.8 ± 0.28	3.62 ± 0.24
	1.5	80	3.4 ±1.03	4.76 ± 0.28
	2.0	40	2.2 ± 0.20	2.6 ± 0.23
IAA	0.5	-	-	-
	1.0	60	4.00 ± 0.67	3.45 ± 1.04
	1.5	80	5.40 ± 1.98	6.66 ± 2.01
	2.0	40	4.70 ± 0.87	5.01 ± 1.55

Evaluation after 8 week transfer of shoots to rooting medium.

concentrations and it decreased afterwards. Auxins especially IBA is involved in root formation in several species (Müller et al., 2005). In *Arabidopsis thaliana*, IBA hormone encodes by PEX5 gene responsible for peroxisomal function such as transport, signaling and response (Ludwig-Müller, 2000; Zolman et al., 2004).

Table 5. Acclimatization of *in vitro* regenerated plants.

Potting mixture	No of plants transfer	Survival rate (%)		Regeneration of new leaf (in days)
		After 30 days	After 60 days	
Sand	5	80	60	15
Soil	5	60	40	20
Soil + Compost	5	40	40	20

4 weeks after transfer of plants in the respective potting mixtures.

Table 6. Antimicrobial activity of *S. pauciflorum in vitro* and *in vivo* plant extracts.

Microorganism		*In vitro* plants[a]	*In vivo* plants[a]
Bacteria			
B. subtilis	IZ[b]	12.33 ± 0.67	15.00 ± 0.34
	AI[c]	0.56	0.68
E. aerogenes	IZ	13.00 ± 0.57	15.33 ±0.34
	AI	0.92	1.09
E. coli	IZ	11.66 ± 0.67	13.00 ± 0.57
	AI	0.61	0.68
P. aeruginosa	IZ	14.66 ± 0.34	15.33 ± 0.34
	AI	0.73	0.76
R. planticola	IZ	11.33 ± 0.34	14.00 ± 0.57
	AI	0.53	0.63
S. aureus	IZ	8.00 ± 0.00	11.00 ± 0.00
	AI	0.38	0.5
Fungi			
A. flavus	IZ	14.00 ± 0.57	11.66 ± 0.33
	AI	0.51	0.43
A. niger	IZ	12.00 ± 0.00	10.66 ± 0.37
	AI	0.44	0.39
C. albicans	IZ	11.00 ± 0.00	11.66 ± 0.37
	AI	0.55	0.53
P. crysogenum	IZ	13.33 ± 0.34	12.66 ± 0.67
	AI	0.63	0.53
T. rubrum	IZ	12.66 ± 0.67	13.33 ± 0.67
	AI	0.60	0.45

[a]Test samples 4 mg/well. Standard test drugs: Gentamycin for bacteria, Ketonocozole for fungi (10 mcg/disc). [b] IZ=Inhibition zone (in mm) including the diameter of well (6 mm). AI[c] = Activity index = inhibition zone of the sample/Inhibition zone of the standard.

Likewise, IBA and IAA proved helpful in the induction of rooting in *S. pauciflorum.* Regarding the biological activities not much work on antimicrobial screening was performed but production of antioxidant compounds in callus cultures has been reported in family Borginaceae viz., rabdosiin, rosmarinicacid, eritrichin, lithospermic acid, caffeic acid–rosmarinic acid conjugate (Yamamoto et al., 2000; Mehrabani et al., 2005; Bryukhanov et al., 2008). The results presented herein suggest a practical and feasible method for multiplication and restoration of

Table 7. % Inhibition of DPPH in *S. pauciflorum* in both *in vivo* and *in vitro* extracts.

Extract	Total phenolics (mg GAE/ g)	IC_{50} (µg/ml)	% Inhibition (µg/ml)				
			10	20	40	60	80
In vitro	73.75 ± 0.72	6.5	75.18	83.00	91.48	92.23	94.81
In vivo	99.76 ± 1.60	7.5	67.85	75.05	88.93	91.56	94.07
Quercetin	-	6	64.42	80.58	93.38	93.82	94.71

% inhibition = 1-(Absorbance of the sample/Absorbance of the control) × 100.

S. pauciflorum, which so far has been achieved in only few borage family plants only. Incidentally, this plant is rich in phenolic compounds and good antibiotic and has good antimicrobial and antioxidant potentials. However, i*n vitro* plants have better antioxidant potentials than the *in vivo* plants but in antimicrobial activity *in vivo* extract was more effective than *in vitro*. The work on the solation and identification of bioactive compounds is in progress in our laboratory and will be published later.

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