British Microbiology Research Journal
4(8): 869-884, 2014

SCIENCEDOMAIN *international*
www.sciencedomain.org

Phytochemical and Antimicrobial Analysis of Callus Extracts of *Biophytum sensitivum* (Linn) DC

Sirigiri Chandra Kala[1], Muvva Vijayalakshmi[1], Shaik Ibrahim Khalivulla[2] and Kokkanti Mallikarjuna[1*]

[1]Department of Botany and Microbiology, Acharya Nagarjuna University, Nagarjuna Nagar, Guntur 522510, India.
[2]Department of Pharmaceutical Chemistry, Faculty of Pharmaceutical Sciences, No.1, Jalan Menara Gading, 56000 Cheras, Kuala Lumpur, Malaysia.

Authors' contributions

This work was carried out in collaboration between all authors. Authors KM and MV designed the study, authors KM and SCK were wrote the protocol and the first draft of the manuscript. Author SCK performed the experimental part and managed the literature searches and author SIK performed statistical analysis. All authors read and approved the final manuscript.

Original Research Article

Received 30th September 2013
Accepted 13th March 2014
Published 6th May 2014

ABSTRACT

Aims: *In vitro* studies are highly instrumental in selecting a drug for a particular disease and also in getting the preliminary evidence to proceed for further *In vivo* pharmacological research. Hence, the study is designed to screen and identify the therapeutic suitability of this plant extract for the treatment of a particular disease. And to find out the presence of phytochemicals and antimicrobial activity of leaf callus cultures of *Biophytum sensitivum* Linn.
Place and Duration of Study: Department of Botany and Microbiology, Acharya Nagarjuna University, Nagarjuna Nagar, Guntur 522510, India during June 2010 to Dec 2010.
Methodology: Here we induced the callus from the leaf explants of this species on Murashige and Skoog basal medium supplemented with various concentrations of BA and NAA. BA 1.0 mg/l with NAA 1.0mg/l is the best concentration for optimal results. The callus was extracted sequentially with hexane, chloroform, ethyl acetate and methanol for

**Corresponding author: Email: mallikarjunaanu@gmail.com*;

24h by using Soxhlet apparatus. These extracts were used to investigate the presence of phytochemicals which was performed according to the Aiyelaagbe and Osamudiamen [29] and Egwaikhide et al. [30] methods. The mean values were statistically analyzed with the MINITAB 14 by the general one way (un stacked) analysis of variance (ANOVA) to find out the most effective extracts

Results: The qualitative phytochemical analysis of various solvent extracts showed the presence of phytochemicals viz., Terpenoids, phenols, flavonoids, saponins, quinones and phenols. All the extracts except hexane showed highest zone of inhibition against gram positive and gram negative bacteria (4.46-22.9mm) as well as fungi (7.64-144.4mm) by agar well diffusion method at 100ppm concentrations. The results of present study indicate that the callus of this plant is a potential source of antimicrobial agents and drugs and need to be investigated further.

Conclusion: From the present study, it is evident that, the antibacterial active constituent of *Biophytum sensitivum* is having a constant expression pattern over different pathogens. This plant leaf callus can be further subjected to enhancement and isolation of the therapeutic antimicrobials and carry out further pharmacological evaluation.

Keywords: Biophytum sensitivum; phytochemical study; antimicrobial activity; callus extracts; In vitro culture.

1. INTRODUCTION

Since their discovery during 20th century, antimicrobial agents have substantially reduced the threat posed by infectious diseases. The use of these “wonder drugs” has led to a dramatic drop in deaths from diseases that were previously wide spread, untreatable and frequently fatal. These drugs have also contributed to the major gains in life expectancy. The bacterial infections which contribute mostly to human diseases such as acquired infections are also developing resistance against drugs used to treat them [1]. The treatment of infectious diseases still remains as an unsolved global problem because of newly emerging diseases and increasing number of multi-drug resistant Gram-positive and Gram-negative bacteria. The usage of antimicrobials by human has been increased from 100 to 1000 times.

The most important bioactive constituents of plants are terpenoids, alkaloids, tannins and flavonoid compounds [2]. The presence of these secondary metabolites could be responsible for some of the observed antimicrobial activity. Plant based antimicrobial compounds serve without side effects than synthetic compounds and has enormous therapeutic potential. Although very few plant cell processes are operating commercially, the most successful commercial pharmaceuticals produced from undifferentiated cell cultures are antibiotic compounds [3].

There has been increasing interest in the production of secondary metabolites using cell and callus cultures for commercial use by many medicinal plants [4,5,6,7,8,9]. Cell and callus cultures represent the best source of cell mass for production of secondary metabolites due to unique environmental stress [2,3]. *In vitro* studies are highly instrumental in selecting a drug for a particular disease and also in getting the preliminary evidence to proceed for further *In vivo* pharmacological research. Hence, the study is designed to screen and identify the therapeutic suitability of this plant extract for the treatment of a particular disease.

Biophytum sensitivum Linn. DC. (Syn. *Oxalis sensitiva* Linn.) belongs to the family Oxalidaceae is a small, sensitive annual herb found throughout the tropical regions of South

Asia, Africa and Madagaskar. It is well known in Andhra Pradesh, India with its vernacular name as Jalapushpa, Attapatti and used as a folk medicine against diabetes. It grows in shady places in dry parts of India during the rainy season. It is extensively used in traditional oriental herbal medicines [10].

B. sensitivum is widely used in Ayurveda and Siddha systems of Indian traditional medicine to treat various ailments. The pharmacological studies of this plant have reported antioxidant, antibacterial, antitumor, antimetastatic, antiangiogenesis, antidiabetic, anticancer, antiinflammatory, immunomodulatory, chemoprotective, cardioprotective, radioprotective and wound healing properties. Phytochemical analysis of this species has reported important medicinal compounds such as amentoflavone, cupressuflavone and isoorientin. Recently this species has gained enormous pharmacological importance.

Phytochemical studies of *B. sensitivum* showed the main bioactive constituents amentoflavone (in methanolic extract the amounts to be 0.26% in roots, 0.33% in stems, and 0.012% in leaves) with minute amount of cupressoflavone. From aerial parts, three flavonols (luteolin 7-methyl ether, isoorientin, 3-methoxyluteolin 7-O-glucoside) and, two acids (4-caffeoylquinic acid and 5-caffeoylquinic acid) [11] were reported. From methanolic extract of leaves, isoorientin, orientin, isovitexin, isoorientin 7-O-glucoside, isoorientin 2-O-rhamnoside and; from roots, (–)-epicatechin and epicatechin-(4β-8)-epicatechin (proanthocynidin B2) were isolated, indicating the presence of highest amount of C-glycosyl flavones in leaves [12]. The aerial parts of water extract contain a bioactive polysachcharide, BP100 III, galacturonic acid and rhamnose [13]. It has antioxidant [14] anti-inflammatory [15,16] antidiabetic [17,18], antiangiogenic [19], anticancer [20,21,22], chemo protective [23], immunomodulatory [24], radioprotective [25] and wound-healing properties. Natarajan et al., [26] and Mini et al., [27] in 2010 reported antimicrobial activity of wild plant leaf extracts of this species on human pathogenic bacterial strains and there is no work on antimicrobial screening of callus or pure compounds of it.

Flowering occurs during August to January and due to seed dormancy, the cultivation of this plant has become very difficult. It thrives only on slightly acidic and damp soil. Shivanna et al. [10] in 2009 established a protocol for *In vitro* regeneration of *B. sensitivum*. Owing to the great medicinal importance of this species and due to difficulty in propagation, in our current study we induced the calli of *B. sensitivum* and carried out solvent extraction of *In vitro* callus with increasing polarity viz., hexane, chloroform, ethyl acetate and methanol. These extracts were collected and used for phytochemical and antimicrobial study.

2. MATERIALS AND METHODS

2.1 Plant Material

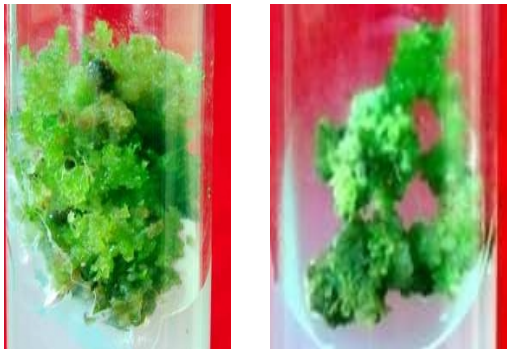
The fresh matured 100 plants of *B. sensitivum* were collected from Acharya Nagarjuna University Campus, Guntur District, Andhra Pradesh, India during June 2010 to Dec 2010 and used as a source of explants. The leaf explants were excised into 1 cm long segments and were washed with liquid detergent (Teepol, Qualigens, India), Bavistin (1% w/v) for 3 min and Mercuric Chloride (0.1% w/v) for 1min, followed by 70% ethanol. These are inoculated on Murashige and Skoogs medium [28] supplemented with various concentrations and combinations of phyto hormones for induction of callus. The combination of NAA+BA (BA 0.5-5.0mg/l, NAA 0.5-5.0mg/l) produced callus.

2.2 Callus Culture

The leaf explants were cultured on M.S. basal medium supplemented with various concentrations of BA+NAA for callus induction. BA 1.0mg/l + NAA 1.0mg/l is the best concentration for callus induction. After 30-60 days, old callus was collected and sub cultured on fresh medium with same growth regulator combinations twice in four week time interval. All the cultures were incubated at 24±2ºC under 16h photoperiod provided by cool white florescent lights.

2.3 Extraction from Callus Cultures

About 6-8 week-old calli (Fig. 1) derived from the leaf cuttings were collected and dried in an oven at 40±1ºC for 5h. Dried calli was homogenized to a fine powder and stored in airtight bottles. 25g of shoot calli powder was extracted with 150ml solvent of each of hexane, chloroform, ethyl acetate and methanol for 24h by using Soxhlet apparatus. All the extracts were concentrated separately on rotary evaporator at 40ºC. 100mg/ml were prepared by redissolving the extracted powder in the same solvent which was used for the extraction. This crude callus extract is used for qualitative phytochemical analysis and anti-microbial activity.



A B

Fig. 1. (A) *In vitro* regenerated callus of *Biophytum sensitivum* after six weeks of culture with BA 1.0mg/l + NAA 1.0mg/l. (B) Callus after six weeks of culture with BA 0.5mg/1+NAA 1.0mg/l

2.4 Phytochemical Screening

The preliminary phytochemical analysis was performed according to the Aiyelaagbe et al. [29] and Egwaikhide et al. [30] methods.

2.5 Test Bacteria

Gram positive bacteria - *Bacillus megaterium, Bacillus subtilis* (ATCC-6633)*, Staphylococcus aureus (*MTCC 3160)*, Streptococcus mutans* MTCC- 497, *Lactobacillus acidophilus* (MTCC 447), *Lactobacillus casei* (MTCC 1423) and *Pseudomonas aeruginosa* (ATCC-9027).

Gram negative bacteria - *Escherichia coli* (ATCC-35218), *Enterococcus faecalis* (MTCC-439), *Proteus vulgaris* (MTCC 7299), *Xanthomonas campestris* (MTCC 2286) and *Salmonella typhi* (ATCC 14028).

2.6 Test Fungi

Fungal - *Candida albicans* (ATCC-10231), *Fusarium solani* (MTCC- 6773), *Fusarium oxysporum* (MTCC-3075), *Aspergillus niger* (MTCC-872), *Helminthosporium solani* (MTCC–296), *Rhizoctonia solani* (MTCC-4634), *Trichoderma viridae* (MTCC–793) *and Botrytis cinerea* (MTCC–359).

2.7 Antimicrobial Assay

The antimicrobial activity of the crude extracts of *B. sensitivum* was determined by agar well diffusion method of Cappuccino and Sherman [31] and Volk et al. [32]. Nutrient agar (NA) and Czapek-Dox (CD) agar media were used for culturing the test bacteria and fungi respectively. NA medium (100ml) was sterilized at 15 lbs pressure (12ºC) for 15min, cooled and inoculated with 0.2ml of test bacterial suspension. After thorough mixing, the seed medium was poured into Petri plates under aseptic conditions. After solidification of agar medium, wells of about 4 mm diameter were punched into it with sterilized cork borer. In case of antifungal assay, spore suspension of test fungus (10^5 Spores/ml) was mixed with the cooled, molten CD agar medium and poured into Petri dishes. Wells were made in the medium after solidification. The crude extract dissolved in hexane, chloroform, ethyl acetate and methanol at a concentration of 25, 50, 75 and 100ppm was added to each well. Streptomycin served as positive control while hexane, chloroform, ethyl acetate and methanol served as negative control for both bacterial and fungal strains. Fluconazole (0.1ppm/well) served as the positive experimental control for all fungi strains assayed. The plates were incubated at 37ºC for 24h for bacteria, 24-72h for yeast and filamentous fungi and the diameter of the inhibition zones was measured.

2.8 Statistical Analysis

The mean values were statistically analyzed with the MINITAB 14 by the general one way (un stacked) analysis of variance (ANOVA) to find out the most effective extracts and the most sensitive test organisms. Differences between antimicrobial assays by ANOVA followed by LSD ($P<0.05$).

3. RESULTS AND DISCUSSION

The calli of *B. sensitivum* extracted with hexane, chloroform, ethyl acetate and methanol were subjected to phytochemical screening to identify alkaloids, flavonoids, saponins, phlobatannins, terpenoids, tannins, quinones, anthraquinones, phenols, gum and oils, and the results are presented in Table 1. The results obtained are in consonance with published work [3,6,10,12-15,19-28,36] showing the evidence of the presence of flavonoids, terpenoids, saponins and quinone chemical constituents in this species.

Table 1. Qualitative phytochemical analysis of leaf callus extracts of *Biophytum sensitivum* Linn

Test for	Hexane extract	Chloroform extract	Ethyl acetate extract	Methanol extract
Alkaloids	--	--	--	--
Flavonoids	--	++	++	++
Saponins	++	++	++	++
Phlobatanins	--	--	--	- -
Terpenoids	++	--	++	++
Tannins	--	--	--	--
Anthraquinones	--	--	--	--
Quinones	++	--	--	--
Phenols	--	--	--	++
Gums	--	--	--	--
Oils	--	--	--	--

+ indicates presence of the phytochemical and – indicates absence of the phytochemical

3.1 Antimicrobial Activity

From Table 2, all the extracts except hexane of *B. sensitivum* have similar and great affinity to inhibit 12 bacterial human pathogens. The activity was positively correlated to the concentration of the extract, as there is an increase in the zone of inhibition with increased dose. Hexane extract failed to show any antimicrobial activity except in the case of *Bacillus megaterium,* where the zone of inhibition (4.26-6.20mm) (Table 2) was observed at higher concentrations (75 & 100ppm). The activity was positively correlated to the concentration of the extract, as there is an increase in the zone of inhibition with increased dose. Among all the bacterial species observed, *Lactobacillus casei* showed strong resistance to the effect of various extracts applied. The least values of zone of inhibition (2.42mm) in minimal concentration 25ppm and maximal concentration (12.48mm) at 100 ppm justifies above observation (Table 2). Strong inhibition of growth measured as zone of inhibition observed in all gram positive and negative bacteria which indicate that the various callus extracts of *B. sensitivum* contain valuable phytochemicals (Table 1) and the observed activity might be due to them.

The minimal and maximal values of zone of inhibition by various extracts are as follows, *Bacillus megaterium* (6.12–17.54mm) (Plate 1A), *Bacillus subtilis* (5.18-19.12 mm) (Plate 1B), *Pseudomonas aeruginosa* (6.76-22.90mm) (Plate 1J), *Staphylococcus aureus* (6.10-19.42 mm) (Plate 1G), *Lactobacillus acidophilus* (5.20-17.10mm) (Plate 1D), *Lactobacillus casei* (2.42-12.48mm) (Plate 1K), *Escherichia coli* (6.20-19.12mm) (Plate 1E), *Streptococcus mutans* (6.10-18.58 mm) (Plate 1C), *Enterococcus faecalis* (6.12-20.32mm), *Proteus vulgaris* (6.24-20.24mm) (Plate 1H), *Xanthomonas campestris* (5.12-20.66mm) (Plate 1F), *Salmonella typhi* (6.22-19.20mm) (Plate 1I), at 25 -100 ppm and showed gradual increase of zone of inhibition to 100ppm.

Table 2. Antibacterial activity of callus extracts of *Biophytum sensitivum*

Name of Bacteria	Zone of Inhibition (mm)										Positive Control (Streptomycin) Standard
	Hexane extract (ppm)				Hexane (ppm) N.C	Chloroform extract (ppm)				Chloroform (ppm) N.C	
	25	50	75	100	100	25	50	75	100	100	0.1 ppm
Gram positive											
B.m	-	-	4.26±0.20	6.20±0.05	-	6.12±0.02	7.03±0.13	10.02±0.02	14.09±0.07	0.40±0.02	21.50±0.20
B.s	-	-	-	-	-	6.24±0.14	7.12±0.20	12.08±0.11	15.06±0.18	-	10.10±0.50
S.a	-	-	-	-	-	-	6.10±0.18	8.76±0.43	13.33±0.52	-	12.22±0.16
L.a	-	-	-	-	-	5.20±0.12	9.20±0.16	10.20±0.24	14.10±0.42	-	7.36±0.08
L.c	-	-	-	-	-	-	-	2.42±0.15	4.46±0.26	-	10.30±0.30
Gram negative											
P.a	-	-	-	-	-	-	7.42±0.12	8.10±0.18	12.96±0.35	-	13.42±0.80
E.c	-	-	-	-	-	6.20±0.10	11.76±0.67	13.76±0.45	16.55±0.32	-	12.22±0.04
S.m	-	-	-	-	-	6.12±0.16	7.85±0.21	11.49±0.01	14.88±0.32	-	11.30±0.37
E.f	-	-	-	-	-	-	9.12±0.16	12.02±0.23	15.45±0.62	-	14.90±0.26
P.v	-	-	-	-	-	7.20±0.24	8.24±0.12	10.12±0.12	13.16±0.12	-	7.88±0.18
X.c	-	-	-	-	-	5.12±0.16	7.12±0.11	11.12±0.08	14.18±0.10	-	7.60±0.16
S.t	-	-	-	-	-	-	9.42±0.26	12.42±0.18	14.10±0.13	-	8.90±0.12

Table 2 Continued……

Name of Bacteria	Zone of Inhibition (mm)										Positive Control (Streptomycin) Standard
	Ethyl acetate (ppm)				Ethyl acetate (ppm) N.C	Methanol (ppm)				Methanol (ppm) N.C	
	25	50	75	100	100	25	50	75	100	100	0.1 ppm
Gram positive											
B.m	7.08±0.12	9.04±0.13	12.14±0.16	15.08±0.10	0.6±0.1	7.10 0.11	8.08 0.14	12.5±0.21	17.54±0.13	0.8±0.15	21.50±0.20
B.s	5.18±0.19	6.34±0.10	16.20 0.08	19.12±0.22	0.5±0.1	7.10 0.26	8.50±0.12	13.22±0.24	16.20±0.16	0.6±0.11	10.10±0.50
S.a	6.19±0.62	8.20±0.24	14.50±0.15	18.82±0.61	-	11.24±0.34	15.57±0.10	16.01±0.11	19.44±0.23	-	12.22±0.16
L.a	10.08±0.34	11.40±0.10	12.20±0.52	14.24 ±0. 8	0.6±0.8	9.62 ±0.16	10.14±0.13	14.12±0.88	17.10±0.08	-	7.36±0.08
L.c	4.62 ±0.12	5.40±0.06	7.80±0.11	8.40±0.18	0.6±0.5	6.55 ±0.66	8.20 ±0.32	11.42±0.24	12.48±0.88	-	10.30±0.30
Gram egative											
P.a	6.76±0.43	7.10±0.18	57.15±0.21	19.19±0.97	0.8±0.5	10.45±0.67	10.11±46	19.42±0.23	22.90±0.52	-	13.42±0.80
E.c	7.12±0.15	9.14±0.46	12.98±0.13	16.11±0.62	-	6.85 ±0.65	9.76 ±0.43	16.55±0.32	19.12±0.97	-	12.22±0.04
S.m	11.58±0.39	12.03±0.13	14.06±0.45	17.62±0.28	0.6±0.2	6.10 ±0.43	10.45±0.67	14.20±0.15	18.58±0.16	-	11.30±0.37
E.f	6.12±0.10	8.24±0.18	11.12±0.21	13.24±0.34	-	7.62 ±0.14	11.55±0.16	18.72±0.15	20.32±0.14	-	14.90±0.26
P.v	12.24±0.26	13.46±0.34	14.24±0.82	16.28±0.12	-	6.24 ±0.12	9.26 ±0.12	17.88±0.12	20.24±0.12	-	7.88±0.18
X.c	6.12 ±0.16	7.16 ±0.22	11.05±0.24	14.12±0.44	0.6±0.8	6.60±0.16	9.04 ±0.55	12.74±0.55	20.66±0.18	0.4±0.5	7.60±0.16
S.t	6.22±0.44	10.42±0.22	13.20±0.66	16.40±0.80		8.22 ±0.05	13.40±0.42	15.10±0.22	19.20±0.14	-	8.90±0.12

Each value represents the mean of triplicate analysis. B.m-Bacillus megaterium, B.s-Bacillus subtilis, *S.a-Staphylococcus aureus, L.a-Lactobacillus acidophilus and L.c-Lactobacillus casei.* Gram (-) strains were*, P.a-Pseudomonas aeruginosa E.c-Escherichia coli, S.m- Streptococcus mutans*, *E.f-Enterococcus faecalis*, *P.v-Proteus vulgaris*, *X.c-Xanthomonas campestris and S.t-Salmonella typhi*

Table 3. Antifungal activity of callus extracts of *Biophytum sensitivum*

Name of Fungi	Zone of Inhibition (mm)											Positive Control (Fluconazole) Standard 0.1 ppm
	Hexane extract (ppm)				Hexane (ppm) N.C		Chloroform extract (ppm)				Chloroform (ppm) N.C	
	25	50	75	100	100		25	50	75	100	100	
F.s	-	-	-	-	-		-	-	-	-	-	10.42±0.18
F.o	-	-	-	-	-		-	-	-	-	-	12.82±0.06
R.s	-	-	-	-	-		-	-	5.48±0.12	7.24±0.22	-	12.30±0.39
T.v	10.14±0.08	12.8±0.16	14.40±0.88	17.66±0.33	-		-	-	10.40±0.36	12.82±0.04	-	16.60±0.68
H.s	-	7.45±0.84	11.40±0.11	14.80±0.92	-		-	10.40±0.44	21.40±0.14	23.46±0.46	0.52±0.12	12.32±0.06
B.c	-	-	-	-	-		-	-	-	-	-	12.88±0.42
C.a	-	-	-		-		9.40±0.62	11.60±0.51	14.40±0.21	17.64±0.02	-	14.4±0.08
A.n	-	-	-	-	-		-	-	-	-	-	10.50±0.62

Name of Fungi	Zone of Inhibition (mm)										Positive Control (Fluconazole) Standard 0.1 ppm
	Ethyl acetate extract (ppm)				Ethyl acetate (ppm) N.C	Methanol extract (ppm)				Methanol (ppm) N.C	
	25	50	75	100	100	25	50	75	100	100	
F.s	-	-	-	-	-	-	-	-	-	-	10.42±0.18
F.o	-	-	-	-	-	-	-	-	-	-	12.82±0.06
R.s	-	-	-	7.12±0.24	4.18±0.12	8.24±0.26	10.40±0.42	12.30±0.06	14.40±0.14	0.52±0.02	12.30±0.39
T.v	-	-	-	-	-	-	-	-	-		16.60±0.68
H.s	5.82±0.28	9.40±0.26	10.45±0.18	13.40±0.84	0.46±0.11	4.34±0.42	6.50±0.82	8.32±0.04	10.40±0.12	0.54±0.11	12.32±0.06
B.c	7.22±0.52	8 .40±0.07	11.42±0.34	15.40±0.46	0.52±0.33	-	-	-	4.32±0.24		12.88±0.42
C.a	8.30±0.27	8.40±0.66	11.40±0.26	14.40±0.26	0.42±0.14	-	-	-	-	-	14.4±0.08
A.n	-	-	-	7.22±0.44	-	-	-	-	4.24±0.11	-	10.50±0.62

Each value represents the mean of triplicate analysis. Standard deviation was 0.5 for the values. F.s-Fusarium solani, F.o-Fusarium oxysporum, R.h-Rhizoctonia solani, T.c- Trichoderma viride, H.s- Helminthosporium solani, B.c-Botrytis cinerea, C.a-Candida albicans, A.n- Aspergillus niger

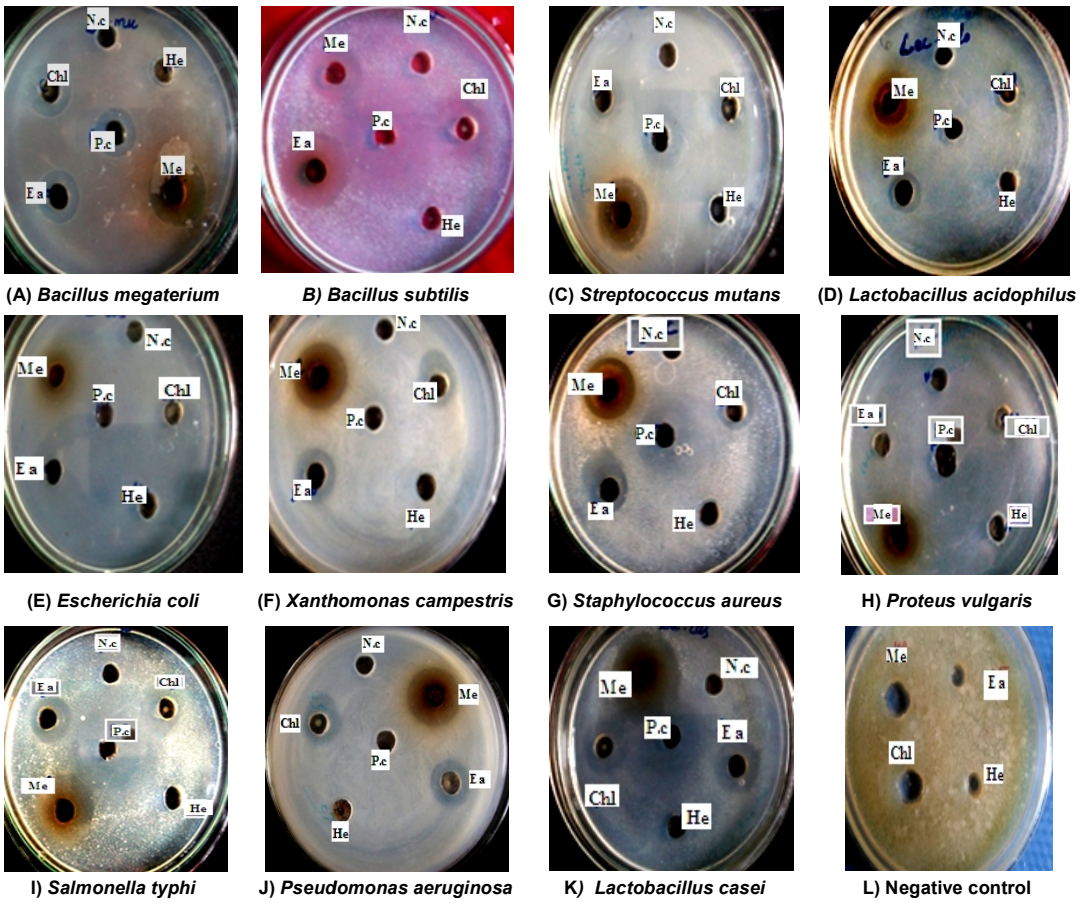


Plate 1. Antibacterial activity of leaf callus extracts of *Biophytum sensitivum*

He: Hexane extract (100 ppm), Chl: Chloroform extract (100 ppm), E.a: Ethylacetate extract (100 ppm), Me: Methanol extract (100 ppm). N.C: Negative control (He,Cl, Ea, Me 100 ppm), P.C: Positive Control (Streptomycin 0.1 ppm)

ANOVA data on the antimicrobial activity of chloroform, ethyl acetate and methanol extracts had significant effect ($P<0.05$) on the levels of concentrations of extracts. The results showed that the studied plant callus extracts were potentially a rich source of antimicrobial agents. However the plants differ significantly in their activity against the test microorganisms. According to one way ANOVA results, antimicrobial activity has also shown differences among the taxa ($P=0.0042$, $F=4.62$, $R=0.1$). Hexane, chloroform, ethyl acetate and methanol were used as negative control for which a number of few inhibitory effects could be observed.

In all these cases, the increased zone of inhibition is observed in the methanolic extracts. But ethyl acetate extract also showed high zone of inhibition in *B. subtilis* (19.12mm) (Plate 1B), indicating that ethyl acetate extracts contains specific phytochemicals needed for inhibition of growth of these bacteria. When the zone of inhibition of gram positive bacteria are compared with gram negative bacteria, increased zone of inhibition was observed with later indicating that the callus extracts of *B. sensitivum* contain highly important antibiotics. The qualitative phytochemicals analysis confirms the above observation (Table 1).

Methanolic extracts showed potent activity against all tested bacteria which could be due to various secondary metabolites like flavonoids, saponins, terpenoids and phenols as noticed in qualitative analysis of extracts (Table 1). Hence the methanol was noticed to be the best solvent among the three.

3.2 Antifungal Activity

Like in the case of antibacterial activity, anti fungal activity is also exhibited by various extracts of *B.sensitivum* (Table 3). Extracts inhibited the growth of six fungal species and is ineffective against two species namely, *Fusarium solani* and *Fusarium oxysporum* (Table 3). *Aspergillus niger* (Plate 2F), and *Rhizoctonia solani* also showed minimal response with chloroform (5.48-7.24mm) and methanol extracts (8.24-14.40mm) (Plate 2A). In other extracts they are irresponsive. *Botrytis cinerea* (Plate 2C) failed to respond to hexane, chloroform and methanol extracts and responded only in ethyl acetate extracts (7.22-15.40mm). *Trichoderma viridae* (Plate 2D), *Helminthosporium solani* (Plate 2B) and *Candida albicans* (Plate 2E) growth is potentially inhibited by extracts of *Biophytum*. Hexane extract showed high inhibition (10.14-17.66mm) (Plate 2D) in the case of *Trichoderma viridae.*

Chloroform extracts produced high zone of inhibition in *Helminthosporium solani* (23.46mm) (Plate 2B) and *Candida albicans* (17.64mm) (Plate 2E). Among all these extracts, chloroform extract showed potent antifungal activity as against to methanol extract in the case of bacteria. Though the callus extracts of *B. sensitivum* showed antifungal activity in few species but the activities are very potent (Table 2), (Plate 2A-F). Though hexane extract failed to show good activity in the case of bacteria, but inhibited the growth of two fungi *Trichoderma viridae* (10.14-17.66mm) (Plate 2D) and *Helminthosporium solani* (7.45 mm - 14.80mm) (Plate 2B).

B. sensitivum exhibited strong to moderate activity against test microorganism. The effects of anti fungal activity were exhibited by hexane, ethyl acetate and chloroform extracts was significantly higher than that produced by methanol extract. However, the produced quantity of extracts have significant effect at $P>0.05$. The antimicrobial activity potential is only due to the callus extracts of the selected plant but not due to the negative controls such as hexane, chloroform, ethyl acetate and methanol solvents(Tables 2 & 3) (Plate 1L), (Plate 2G).

Medicinal plants are playing important role in producing the therapeutic drugs with minimal or without side effects compared to synthetic drugs. Discovery of natural substances has been showing the path to do the extensive work on medicinal plants. To minimize the maximum usage of the medicinal plants, it became an urgent need to propagate the plant in less time and space. *In vitro* culture is the only option that is left behind and so in current study, we propagated *B. sensitivum* by *in vitro* culture, carried out phytochemical, antibacterial and antifungal activities of callus extracts and reported for the first time. The callus was extracted sequentially with hexane, chloroform, ethyl acetate and methanol. Each extract was tested for active bio-constituents (Table 1) and tested for antibacterial (Table 2) and antifungal (Table 3) activities.

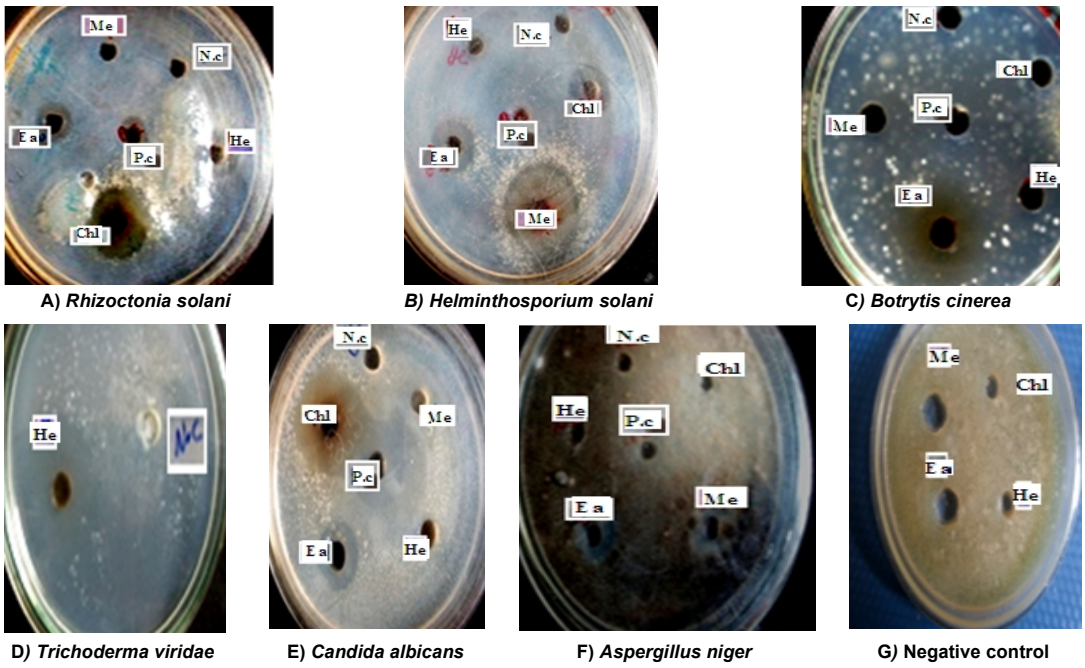


Plate 2. Antifungal activity of leaf callus extracts of Biophytum sensitivum

He: Hexane extract (100 ppm),, Chl: Chloroform extract (100 ppm), E.a: Ethylacetate extract (100 ppm), Methanol extract (100 ppm), N.C: Negative control (He, Cl, Ea, Me (100 ppm), P.C: Positive Control (Flucanazole 0.1 ppm)

In 2010, Natarajan et al. [26] reported the antibacterial activity of wild plant leaf extracts of *B.sensitivum* with petroleum ether, chloroform, acetone and methanol. It showed potent antimicrobial activity against *Bacillus subtilis, Staphylococcus aureus, S. pneumoniae, Klebsiella pneumoniae, Salmonella typhi, Proteus vulgaris* and *Escherichia coli* by agar well diffusion method. All the extracts inhibited the growth of almost all the selected bacteria in the range of 7-25 mm. Among these, acetone extract showed great antibacterial activity, chloroform and methanol extracts showed better activity than petroleum ether extract.

Ribona et al. [33] in 2011 reported the antimicrobial activity of aqueous, ethanolic and acetone extracts of *B.sensitivum* on pathogens like *E. coli, Klebsiella and Proteus* by agar well diffusion method. In case of *Klebsiella, Proteus* and *E. coli* the inhibition zone was 9 mm for aqueous extracts in maximum concentrations. However in case of *Klebsiella* and *Proteus* inhibition zone of 11mm for ethanolic extracts was observed. Whereas *E. coli* and *Proteus* showed 9 mm zone of inhibition which was more effective when compared to acetone and aqueous extracts. Inhibition of growth of both gram positive and gram negative bacteria by these extracts indicates the potentiality for broad spectrum activity. The antibacterial potential activity of methanolic and chloroform extracts in other plant systems has been reported [34]. Other species from Oxalidaceae family were also showed good antimicrobial activities. *Oxalis corniculata* leaf extract showed antibacterial activity against several human pathogenic bacteria [7,35]. It was reported that stem bark extracts of *Averrhoa carambola* showed potent antibacterial and antifungal activities [36], antibacterial and cytotoxic activities using this plant fruit extract [37] and antioxidant activities on fruit at various stages of ripening [38].

The data pertaining to the antibacterial and antifungal potential of the callus culture explants of *B. sensitivum* were presented in Tables 2 and 3. The extracts from callus culture were obtained from two different hormonal combinations. Previously reported that BA 1mg/l and NAA 1mg/l is best concentration for leaf callus induction of *B. sensitivum* [39,40]. The results of the present study revealed the positive relation between production of active metabolites in the callus and growth regulator (NAA) in the media. The activity can be positively correlated to the dose, as there is an increase in the zone of inhibition with increased dose. All the extracts except hexane had good and similar activities against bacteria and ethyl acetate and methanol extracts showed good activity for fungal human pathogens. As chloroform, ethyl acetate and methanol are polar solvents than non polar hexane, extracts prepared by methanol provided more consistent antimicrobial activity. This might have resulted from the best of solubility of the active constituents in methanol extract.

Such screening of various natural organic compounds and identifying active agents is the need of the hour, because successful prediction of a lead molecule and drug like properties at the onset of drug discovery will pay off later in drug development. From the present study it is evident that, the antibacterial active constituent of *B. sensitivum* is having a constant expression pattern over different pathogens. This plant leaf callus can be further subjected to enhancement and isolation of the therapeutic antimicrobial and carry out further pharmacological evaluation. It is highly valuable species, with impressive range of medicinal uses. Further work is mandatory to isolate the active principle from the callus extracts and to carry out pharmacological studies.

4. CONCLUSION

It is a highly valuable species with wide range of antimicrobial activities. Further work is mandatory for isolation and identification of pure active antibacterial and antifungal constituents from the callus extracts and to carry out pharmacological studies.

ACKNOWLEDGEMENTS

The first author S. Chandra Kala is thankful to University Grants Commission, New Delhi for providing financial support with Rajiv Gandhi National Fellowship.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Priscila Ikeda Ushimaru, Mariama Tomaz Nogueira da Silva, Luiz Claudio Di Stasi, Luciano Barbosa, Ary Fernandes Junior. Antibacterial activity of medicinal plant extracts. Brazilian Journal of Microbiology. 2007;38:717-719.
2. Edeoga HO, Okwu DW, Mbaebie BO. Phytochemical constituents of some Nigerian medicinal plants. African J. Biotechnol. 2005;7:685-688.
3. Khafagi I,Dewedar A, Amein M. Oppertunities of finding novel anti-infective agents from plant cell cultures. *Curr. Med.* Chem.–Anti-Infect. Agent. 2003;2:191-211.
4. Rahman MA, Bari MA. Antibacterial activity of cell suspension cultures of castor (*Ricinuscommunis* L. cv. Roktima). Eur. J. Med. Plant. 2013;3:65-77.

5. Chintalwar GJ, Gupta S, Roja G, Bapat VA. Protoberberine Alkaloids from Callus and Cell Suspension Cultures of *Tinospora cordifolia*. Pharm. Biol., (Formerly Int. J. Pharmcog. 2003;41:81-86.
6. Furmanowa M, Nosov AM, Oreshnikov AV, Klushin AG, Kotin M, Starosciak B, Sliwinska A, Guzewska J, Bloch R. Antimicrobial activity of *Polyscias filicifolia* cell biomass extracts. Pharmazie. 2002;57:424-426.
7. Valsaraj R, Pushpangadan P, Smitt UW, Adsersen A, Nyman U. Antimicrobial screening of selected medicinal plants. Indian J. Ethnopharmacol. 1997;58:75-83.
8. Sokmen A, Jones BM, Eturk M. Antimicrobial activity of extracts from the cell cultures of some Turkish medicinal plants. Phytother. Res. 1999;13:355-357.
9. Wolters B, Eilert U. Antimicrobial substances in callus cultures of *Rutagraveolens.* Planta Med. 1981;43:166-174.
10. Shivanna MB, VasanthaKumari MM, Mangala MC. Regeneration of *Biophytum sensitivum* through organogenesis and somatic embryogenesis. Indian J. Biotech., 2009;8:127-131.
11. Yun-Lian L, Wan-Yi W. Chemical constituents of *Biophytum sensitivum*. Chin. Pharm. J., (Taipei, Taiwan). 2003;55:71-75.
12. Bucar F, Jachak SM, Kartnig T, Schubert-Zsilavecz M. Phenolic compounds from *Biophytum sensitivum*. Pharmazie. 1998;53:651-653.
13. Inngjerdingen KT, Coulibaly A, Diallo D, Michaelsen TE, Paulsen BS. A Complement Fixing Polysaccharide from *Biophytum petersianum* Klotzsch, a Medicinal Plant from Mali, West Africa. Biomacromolecules. 2006;7:48-53.
14. Guruvayoorappan C, Afira AH. Kuttan G. Antioxidant potential of *Biophytum sensitivum* extract *In vitro* and *In vivo*. J. Basic Clin. Physiol. Pharmacol. 2006;17:255-267.
15. Banerjee T, Vander Vliet A, Ziboh VA. Down regulation of COX-2 and iNOS by amentoflavone and quercetin in A549 human lung adenocarcinoma cell line. *Prostaglandins Leukot.* Essent. Fatty Acids. 2002;66:485-92.
16. Jachak SM, Bucar F, Kartnig T. Anti-inflammatory activity of extracts *Biophytum sensitivum* in carrageen in-induced rat paw oedema. Phytother Res. 1999;13: 73-74.
17. Puri D. Screening of mildly hypoglycaemic compounds: Obese British Angora rabbits with borderline glucose intolerance as animal model. Indian J. Pharm. Sci. 2006;68:579-583.
18. Puri D. The insulintropic activity of a Nepalese medicinal plant *Biophytum sensitivum*: Preliminary experimental study. J. Ethnopharmacol. 2001;78:89-93.
19. Guruvayoorappan C, Kuttan G. Inhibition of tumour specific angiogenesis by amentoflavone. Biochemistry (Mosc). 2008;73:209-18.
20. Guruvayoorappan C, Kuttan G. Amentoflavone Inhibits Experimental Tumor Metastasis Through a Regulatory Mechanism Involving MMP-2, MMP-9, Prolyl Hydroxylase, Lysyl Oxidase, VEGF, ERK-1, ERK-2, STAT-1, nm23 and Cytokines in Lung Tissues of C57BL/6 mice. Immunopharmacol. Immunotoxicol. 2008;30:711-727.

21. Guruvayoorappan C, Kuttan G. Amentoflavone stimulates apoptosis in B16F-10 melanoma cells by regulating bcl-2, p53 as well as caspase-3 genes and regulates the nitric oxide as well as proinflammatory cytokine production in B16F-10 melanoma cells, tumour associated macrophages and peritoneal macrophages. J. Exp. Ther. Oncol. 2008;7:207-218.
22. Guruvayoorappan C, Kuttan G. Anti-metastatic effect of *Biophytum sensitivum* is exerted through its cytokine and Immunomodulatory activity and its regulatory effect on the activation and nuclear translocation of transcription factors in B16F-10 melanoma cells. J. Exp. Ther. Oncol. 2008;7:49-63.
23. Guruvayoorappan C, Kuttan G. Evaluation of the chemoprotective effect of *Biophytum sensitivum* (L.) DC extract against cyclophosphamide induced toxicity in Swiss albino mice. Drug Metabol. Drug Interact. 2006;22:131-150.
24. Chandrasekharan C, Kuttan G. Methanol Extract of *Biophytum sensitivum* Alters the Cytokine Profile and Inhibits iNOS and COX-2 Expression in LPS/Con A Stimulated Macrophages. Drug Chem. Toxicol. 2008;31:175-188.
25. Guruvayoorappan C, Kuttan G. Protective effect of *Biophytum sensitivum* (L.) DC on radiationinduced damage in mice. Immunopharmacol. Immunotoxico. 2008;30:815-835.
26. Natarajan D, Shivakumar MS, Srinivasan R. Antibacterial activity of leaf extracts of *Biophytum sensitivum* (L.) DC. J. Pharm. Sci. & Res. 2010;2:717-720.
27. Mini NV, Ida B, Seema D, Shital D, Riva DS, Astrida R. Antimicrobial activity of ten common herbs, commonly known as 'Dashapushpam' from Kerala, India. Afr. J. Microbiol. Res. 2010;4:2357-2362.
28. Murashige T. and Skoog F.; A revised medium for rapid growth and bioassays with tobacco tissue cultures. Plant Physiol. 1962;150:473-497.
29. Aiyelaagbe OO, Osamudiamen PM. Phytochemical screening for active compounds in *Mangifera indica* leaves from Ibadan, Oyo State. Plant Sci. R. 2009;2:11-13.
30. Egwaikhide PA, Okeniyi SO, Gimba CE. Screening for antimicrobial activity and phytochemical constituents of some Nigerian medicinal plants. Adv. Biological Res. 2007;1:155-158.
31. Cappuccino JG and Sherman N. Microbiology, a laboratory manual. Pearson Education, Inc., New Delhi. 2004;282-283.
32. Volk RB. A newly developed assay for the quantitative determination of antimicrobial activity of both hydrophilic and lipophillic test compounds without any restriction. Microbiology Res. 2008;163:161-167.
33. Ribona J, Raj LS, Nethaji S, Ushadevi T. Antimicrobial Activity of *Biophytum sensitivum* Linn on Urinary Pathogens. Herbal Tech Industry. 2011;8:18-19.
34. Adegoke SA, Opata OM and Olajide JE. Antimicrobial activity of the aqueous, methanol and chloroform leaf extracts of *Cissus multistriata.* African J. Biotechnol. 2010;9(8):1168-1172.
35. Raghavendra MP, Satish S, Raveesha KA. Phytochemical analysis and antibacterial activity of *Oxalis corniculata,* a known medicinal plant. My SCIENCE. 2006;1:72-78.
36. Mia Md.M, Rahman MS, Begum K, Begum B, Rashid MA. Phytochemical and Biological Studies of *Averrhoa carambola. Dhaka Univ.* J. Pharm. Sc. 2007;6:125-128.

37. Das BN, Ahmed M. Antibacterial and cytotoxic activities of the fruit extract of *Averrhoa carambola*, Int. Res. J. Pharmaceticals. 2012;2:58-61.
38. Wakte SR, Patil DA. Antimicrobial and antioxidant activity of *Averrhoa carambola* L. fruit at various stages of ripening. J. Herbal Medicine and Toxicology. 2011;5:121-129.
39. Sirigiri Chandra Kala, Kokkanti Mallikarjuna, Patchala Aruna. An efficient protocol devised for rapid callus induction from leaf explants of *Biophytum sensitivum* (Linn) DC. International Journal of Phytopharmacy. 2014;4(1):20-24.
40. Sirigiri Chandra Kala, Kokkanti Mallikarjuna. *In vitro* analysis of cytotoxicity and 5-lipoxygenase inhibition activity by using callus extract of *Biophytum sensitivum* (Linn) DC. Int. J. Pharm. Sci. Rev. Res. 2014;34:215-218.

Peer-review history:
The peer review history for this paper can be accessed here:
http://www.sciencedomain.org/review-history.php?iid=489&id=8&aid=4476