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The present study was aimed to develop an efficient protocol for callus induction and to investigate the antimicrobial activity of *Baliospermum montanum*. Callus induction was induced from leaf explants on Murashige & Skoog (MS) medium supplemented with 2, 4-dichlorophenoxyacetic acid (2, 4-D) and 6-furfuryl amino purine (KIN). Aqueous, methanol and chloroform extracts of root, stem, leaf and callus were screened for potential antibacterial activity against selected bacterial strains (*Bacillus subtilis, Escherichia coli, Klebsiella pneumoniae, Pseudomonas fluorescence* and *Staphylococcus aureus*) by agar well diffusion method. Antifungal activity was carried out by food poison technique against *Fusarium oxysporum*. Maximum callus induction was observed in 2,4-D (9.05 µM/l) and KIN(23.24 µM/l).Methanolic extract of leaf and callus showed maximum zone of inhibition against *Staphylococcus aureus*. Whereas, aqueous extract of the stem and root exhibited maximum zone of inhibition against *Bacillus subtilis*. Methanolic extracts showed higher antifungal activity compared to other extracts. In conclusion methanolic extract was proved to be a better solvent for extraction of antimicrobial metabolites from *in vivo* and *in vitro* leaf derived callus.

Key words: Alkaloids, *Baliospermum montanum,* , growth regulators, pathogens**,** secondary metabolites, sterilants

INTRODUCTION

Medicinal plants are the important natural resources which have been used to treat a variety of diseases all over the world. Plants acts as an anti-infectious agent due to the diversity of their secondary metabolites like alkaloids, flavonoids, phenols, saponins, tannins, glycosides etc. (Dzotam *et al*. 2016). These secondary metabolites are produced for self-defense and they are divided into different categories based on their mechanism of function i.e., chemotherapeutic, bacteriostatic, bactericidal and antimicrobial (Baskaran *et al*. 2016). Medicinal plants are used in various traditional system of medicine because of minimal side effects and less expensive (Poonam and Pratap, 2012). Recently, much attention has been directed towards plant extracts and biologically active compounds isolated from popular plant species. The global demand of plant origin bioactive compounds is very high, which could not fulfill by field grown plants. An alternative source for commercial exploitation was plant cell cultures which will produce high yield compared to field grown plants (Johnson *et al*.2010).

Baliospermum montanum locally known as Danti, is a vulnerabl emedicinal plant belonging to the family Euphorbiaceae. The species is distributed throughout sub-himalayan tracts and peninsular India. Roots are rich source of phorbol esters belonging to diterpene hydrocarbon viz., montanin, baliospermin, 12-deoxyphorbol-13-palmitate, 12-deoxy-5B-hydroxyphorbol-B-myristate and 12-deoxy-16-hydroxy phorbol-13-palmitate. Leaves are rich source of secondary metabolites such as 8-sitosterol, 8-D-glucoside and hexacosanol. Seeds, roots and leaves are used to treat jaundice, skin disease,rheumatism,snakebite, piles, asthma, bronchitis and abdominal tumors(Johnson *et al.* 2010). Due to indiscriminate collection and over exploitation for medicinal use, this plant species has been disappearing very fast. Hence, tis-

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sue culture technique helps in rapid multiplication of this important medicinalplant. Based on above facts, the present study was designed to develop an effective protocol for callus induction and to investigate the antimicrobial activity of root, stem, leaf and callus extracts of *B. montanum.*

MATERIALS AND METHODS

Collection of plant material and sterilization

Seeds and seedlings were collected from Sirsi, Western Ghats of Karnataka and it was maintained in green house conditions. The plant material was authenticated at the Department of Botany, Bangalore University, Bengaluru and a voucher specimen is deposited in the herbarium (BUB, No. 2264).Disease free young leaves were washed thoroughly under running tap water followed by teepol and bavastin for 30 min, rinsed with distilled water, and then in 70% ethanol for 2min. The explants were then treated with 0.1% $HgCl_2$ for 2min under aseptic condition and then washed with sterile water for 3-5 times to remove the traces of sterilants. Finally surface sterilized explants were cultured on MS medium.

Callus induction

Sterilized leaf segments were cultured on MS medium supplemented with 30 g/l sucrose, 8 g/ lagar with different concentrations of auxin and cytokinin. Cultures were incubated at 25±2ºC in a culture room with 70-80% relative humidity and 16hr of photoperiod.

Preparation of extracts

The extracts were prepared according to procedure of Samydurai and Saradha (2016) by soxhlet extraction method. Briefly, 10g of dried root, leaf, stem and callus was finely powdered using blender and extracted with 100ml of different solvents (aqueous, methanol and chloroform) for 24hr. After extraction, the extracts were concentrated by evaporation. The dried extract was stored at 4ºCuntil further analysis.

Inoculum preparation

Loopful of overnight grown bacterial culture was inoculated in 5ml of nutrient broth (NB) and incubated at 37ºC for 6-8hr. The actively growing culture suspension was adjusted with NB so as to obtain aturbidity that could be visually comparable with 0.5 Mac Farland standard. The turbidity is approximately equal to $1x10^8$ CFU/ml (Jayadevi *et al*. 2013).

Antimicrobial assay
Antibacterial screening

Antibacterial activity of methanol, aqueous and chloroform extracts were determined by agar well diffusion method (Johnson *et al*. 2010).Gram positive bacteria *Bacillus subtilis* (ATCC6051), *Staphylococcus aureus* (ATCC12600) and gram negative bacteria *Escherichia coli* (ATCC25922), *Klebsiella pneumoniae* (ATCC13883) and *Pseudomonas fluorescence* (ATCC 9027) were obtained from Department of Microbiology, Bangalore University, Bengaluru. For experiments, loopful colonies of active cultures were transferred to NB from stock and incubated at 37ºC for 6hr. Muller Hinton agar (MHA) plates were prepared by pouring 20 ml of media and allowed to solidify, then the plates were swabbed uniformly with microbial inoculums over the entire agar surface. The wells were bored with 8mm diameter sterilized cork borer. Different concentrations of extracts (50-150µg) were added into the wells for bacterial sensitivity test. Plates were incubated for 24hr at 37ºC and zone of inhibition were recorded in mm and compared with standard antibiotic streptomycin.

Antifungal activity

Antifungal activity was performed by food poison technique according to the procedure of Suresh *et al.* (2018) with slight modifications. The sterilized potato dextrose agar (PDA) media was supplemented with different concentration of extracts (200 to 800 µg). The medium without extract was decanted into petri plates with fungal mycelia of *Fusarium oxysporum* which was bored with the help of 5mm sterile cork borer. Such mycelial agar was inoculated to each petri dish containing different concentration of extracts and control (positive and negative control). All the Petri plates were incubated for 7days at 25±3ºC. The antifungal activity was determined by measuring the radial growth (mm) and is compared with traditional fungicide bavastin. The percentage of inhibition was calculated according to the formula.

$$\text{Percentage of inhibition} = \frac{dc-dt}{dt} \times 100$$

where dc, the average increase in mycelial growth of control and dt, the average increase in mycelial growth of tested sample.

Statistical analysis

The data are expressed as Mean ± SD. The results were analyzed by one way analysis of variance followed by Duncan's multiple range tests using SPSS software. Probability values P<0.05 were considered significant.

RESULTS AND DISCUSSION

Callus induction from leaf explants on MS medium

Growth regulators such as auxins and cytokinins play an important role in the growth and differentiation of cultured cells and tissues. The individual or combination of different hormones in the medium helps in the maintenance of balanced organic and inorganic contents in the growing tissue (Ngomuo *et al*. 2013).Different concentrations of growth hormones 2, 4-D (2.26-22.62 µM/l) and KIN (2.32-23.24 µM/l) were used to promote callus induction on MS medium (Table 1).In the present study, maximum induction of creamish friable callus was observed in 2,4-D (9.05 µM/l) and KIN (23.24 µM/l) after four weeks of inoculation (Fig.1). Callus induction was higher with increase in the concentration of KIN. Whereas, 2,4-D shows poor callus initiation in higher concentration.Previous studies by Johnson *et al.* (2010) and Saradha *et al.* (2014) reported maximum callus induction in 2,4-D (2 mg/l) from leaf explants of *B.montanum* and *Hildegardia populifolia*. Cheruvathur *et al.* (2012) and Umesh *et al.* (2014) observed that higher concentration of KIN showed maximum callus response from leaf explants of *Rhinacanthus nasutus* and *Asystasia gangetica*.

Table.1: Influence of 2, 4-D and KIN on callus induction from leaf explants of *B.montanum*

Growth hormones	Concentration in µM/l	% of Callus induction ± S.D
2, 4-D	2.26	85.78±2.27
	4.52	83.55±2.86
	9.05	90.33±1.52
	13.56	71.45±1.71
	18.09	69.88±1.82
	22.62	69.33±1.15
KIN	2.32	67.45±1.26
	4.65	65.21±1.33
	9.29	68.88±1.82
	13.94	67.11±1.17
	18.59	69.88±1.82
	23.24	89.66±1.52

Values represent the Mean ± SD in triplicates

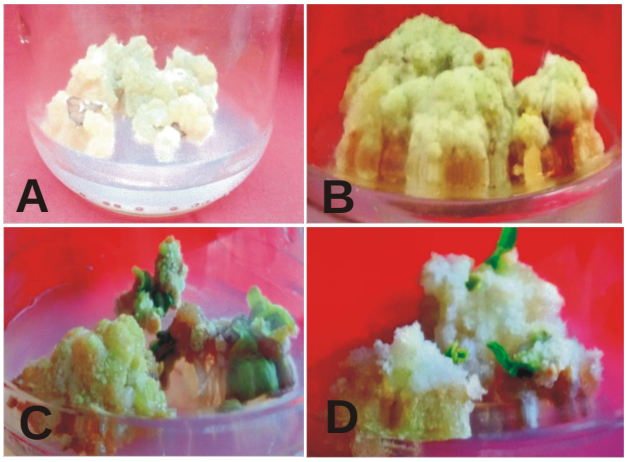

Fig. 1: Effect of auxin and cytokinin on callus induction from leaf explant:A) MS+2,4-D (2.26 µM/l), B) MS+2,4-D (9.05 µM / l), C) MS+KIN (18.59 µM /l) and D) MS+KIN (23.24 µM /l)

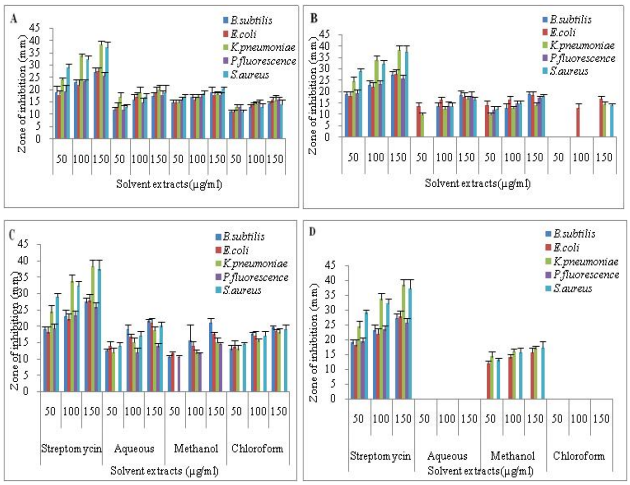

Fig. 2: Zone of inhibition on different extracts of *B. montanum*A) Leaf B) Stem C) Root and D) Callus extarcts against bacterial strains by agar well diffusion method

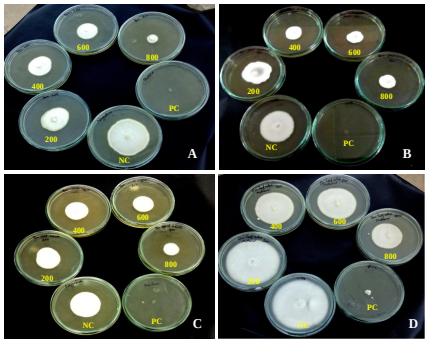

Fig. 3: Antifungal activity on methanolic extractsof *B.montanum*A) Leaf B) Stem C) Root and D) Callus extarcts

Table. 2: Antifungal activity on different extracts of *B. montanum* against *F. oxysporum* by food poison method

	Concentration in µg	Methanol	Aqueous	Chloroform
Leaf	200	21.66±2.08	18.33±1.52	13.66±1.15
	400	28.00±1.73	22.00±2.00	22.00±1.73
	600	49.33±1.15	36.00±2.00	35.00±3.00
	800	63.33±2.88	54.00±2.00	41.60±3.51
Stem	200	07.60±2.51	22.00±2.64	08.70±2.82
	400	35.16±3.40	33.00±3.60	23.80±1.04
	600	42.10±1.04	42.00±1.73	36.80±2.56
	800	58.43±0.81	46.60±1.52	41.10±1.25
Root	200	09.33±4.04	06.35±2.26	06.00±0.28
	400	20.00±3.00	19.88±4.17	16.20±2.01
	600	34.18±1.73	28.00±0.21	26.75±2.65
	800	61.19±3.05	50.33±0.70	36.00±3.86
Callus	200	12.13±0.52	4.63±2.18	05.34±1.01
	400	28.99±0.59	25.24±1.37	16.27±0.34
	600	32.73±0.84	32.73±0.86	28.19±0.84
	800	41.20±2.72	37.46±1.12	35.40±2.47
Bavistin	500	100	100	100
DMSO	500	0	0	0

Values represent the Mean ± SD in triplicates

Agar-well diffusion method

The screening of antibacterial activity against selected five pathogens were carried out by agar well diffusion method. The root, stem, leaf and callus extracts of *B.montanum* were showed varying degree of antibacterial activities. The results were evaluated by measuring the diameter zone of inhibition in mm. Methanolic extracts of leaf and leaf derived calli showed maximum antibacterial activity against *S.aureus* (20.33±2.08 and 17.33±2.08). Whereas, aqueous extracts of stem and root showed highest zone of inhibition against *P.fluoresence* (20.00±2.00) and *B. subtilis* (21.50±0.70) respectively (Fig.2). Arumugam *et al.* (2011) reported that methanolic extract of leaf and callus showed higher zone of inhibition against *E. coli* from *Centella asiatica*. Studies by Auwal *et al*. (2013) and Malar (2016) showed that aqueous extract of root bark and stem showed maximum inhibition activity against *B. subtilis* of *Jatropha caucus* and *Salacia oblonga* respectively.

Food poison method

Food poison method is used to evaluate the ef-

fect of antifungal activity against different solvent extracts. In the present study, methanolic extract proved to be a best solvent compared to other solvents against *Fusarium oxysporum*. Leaf and root extracts exhibited maximum percentage of inhibition (63.33±2.88 and 61.19±3.05) compared to the stem and leaf callus (Table 2; Fig.3). Sharma *et al.* (1997) reported that ethanolic extract of root and callus of *Cassia italica* showed maximum inhibition against *F. moniliforme*. Studies by Mahesh (2008) showed higher zone of inhibition in methanolic extract of leaf compared to the root extract against *F. verticillioides.*

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