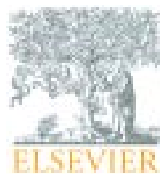

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Antibacterial activity of leaves and inter-nodal callus extracts of *Mentha arvensis* L

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ABSTRACT

Objective: To determine the anti-bacterial efficacy of chloroform, ethanol, ethyl acetate and water extracts of inter-nodal and leaves derived calli extracts from *Mentha arvensis (M. arvensis)* against *Salmonella typhi(S. typhi)*, *Streptococcus pyogenes(S. pyogenes)*, *Proteus vulgaris(P. vulgaris)* and *Bacillus subtilis(B. subtilis)*. **Methods:** The inter-nodal and leaves segments of *M. arvensis* were cut into 0.5-0.7 cm in length and cultured on Murashige and Skoog solid medium supplemented with 3% sucrose, gelled with 0.7% agar and different concentration of 2, 4-Dichlorophenoxyacetie acid (2,4-D) either alone or in combinations. The preliminary phytochemical screening was performed by Brindha *et al* method. Antibacterial efficacy was performed by disc diffusion method and incubated for 24 h at 37 ℃. **Results:** Maximum percentage of callus formation (inter-nodal segments 84.3 ± 0.78; leaves segments 93.8 ± 1.27) was obtained on Murashige and Skoog's basal medium supplemented with 3% sucrose and 1.5 mg/L of 2, 4-D. The ethanol extracts of leaves derived calli showed the maximum bio-efficacy than other solvents. The leaves and stem derived calli extracts on *Proteus* sp. showed that the plants can be used in the treatment of urinary tract infection associated with *Proteus* sp. Through the bacterial efficacy studies, it is confirmed that the *in vitro* raised calli tissue was more effective compared to *in vivo* tissue. **Conclusions:** The bio-efficacy study confirmed that the calli mediated tissues showed the maximum zone of inhibition. The present study paved a protocol to establish high potential cell lines by *in vitro* culture.

1. Introduction

Medicinal plants have become the focus of intense study in terms of validation of their traditional uses through the determination of their actual pharmacological effects. Medicinal plants as a group comprise approximately 8 000 species and account for around 50% of all the higher flowering plant species of India. Many efforts have been made to discover new antimicrobial compounds from various kinds of sources such as micro-organisms, animals, and plants. Systematic screening of them may result in the discovery of novel effective compounds[1]. In recent years, pharmaceutical companies have spent considerable time and money in developing therapeutics based upon natural products extracted from plants[2,3]. The rising incidence of multidrug resistance amongst pathogenic microbes has further necessitated the need to search for newer antibiotic sources[4]. Contrary to the synthetic drugs, antimicrobials of plant origin are not associated with many side effects and have an enormous therapeutic potential to heal many infectious diseases[5,6]. Although hundred of plant species have been tested for antimicrobial properties, the vast majority have not been adequately evaluated[7]. Global researches have shown that all different parts of the plants which include; stem, root, flower, barks leaves, etc. possess antimicrobial property[8-12].

Plants from the genus *Mentha* are used for antimicrobial, antiviral and insecticidal activity[13-20]. The plants are aromatic, stimulant and carminative. The infusion of leaves affords a remedy for rheumatism and indigestion[21-23].

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The genus *Mentha* includes 25 to 30 species that grow in the temperate regions of Eurasia, Australia and South Africa[24]. Mint is valued for its multipurpose uses in the field of pharmaceuticals, cosmetics as well as for flavoring foods, beverages and tobacco[25,26]. *Mentha arvensis* L. is an important medicinal plant belongs to the family Lamiaceae. It is commonly called Mint in English and Pudina in Tamil. The plant is aromatic, stimulant and carminative[23]. The essential oil of *M. arvensis* contains d-carvone, d-sylvesterene and citronellol. Clark[27] estimated the world total production of menthol is 11.8 M tons. Most of the production (9 400 M tons) is from the crude oil of *M. arvensis*. India is the leading producer and exporter of corn mint oil and its products include menthol crystals, dementholised oil, mint terpenes, *etc*[28]. The cultivation of corn mint or menthol mint (*M. arvensis f. piperascens*) is largely confined to northern and northwestern India[29]. To fulfill the commercial requirements a lot of researches conducted on large scale production of this commercially important *Mentha* using shoot tip, nodes, leaves, axillary buds, inter-nodal, leaf segments as explants through tissue culture[23,30-33]. Chakraborty *et al*[34] produced a protocol for menthol production via cell suspension culture from the leaves segments of *Mentha piperita*. Dhawan et al[35] produced "Saksham" the menthol tolerance plants for the superior production of menthol. Islam *et al*[36] optimized the cryo-storage for four mints (*Mentha* spp.) accessions. In recently, a lot of studies related to antimicrobial activities of plant extracts[37-40] and callus extracts[41-43]. But there is no report on the antibacterial efficacy of the callus extracts of *M. arvensis*. The present investigation was carried out on inter-nodal and leaves derived calli extracts of *M. arvensis* in order to determine the anti-bacterial efficacy of chloroform, ethanol, ethyl acetate and water extracts against *Salmonella typhi (S. typhi), Streptococcus pyogenes (S. pyogenes), Proteus vulgaris (P. vulgaris)* and *Bacillus subtilis (B. subtilis)*.

2. Materials and methods

Inter-nodal and leaves segments of *M. arvensis* L were collected from the young top shoot cuttings of mature plants. The explants were washed thoroughly under running tap water for 5 min. and then washed with a commercial detergent Tween-20 for 4 min followed by thorough washing with sterile distilled water. Surface sterilization was done with mercuric chloride solution (0.5 % w/v) for 2 min. Then washed thrice with sterile distilled water, the explants were cut into 0.5 cm in length and cultured on Murashige and Skoog[44] solid medium supplemented with 3% sucrose, gelled with 0.7% agar and different concentration of 2,4-Dichlorophenoxyacetic acid (2, 4-D) and Kin either alone or in combination. The pH of medium was adjusted to 5.8 before autoclaving at a pressure of 1.06 kg/cm^2 (121 ℃ for 15 min). The cultures were incubated at 25±2 ℃ with 12/8 h photoperiod under white fluorescent tubes (1 500 lux). Each and every experiment was performed with ten replicates and repeated thrice. The callus cultures were maintained for a period of over 10 months by periodic sub-culturing with 2 to 4 weeks intervals on to fresh multiplication medium. Consequently, the callus were harvested at the transfer age of 3 to 4 weeks, kept at above 90 ℃ for 3 to 5 min at hot air oven to inactivate the enzyme activity followed by continuous drying at 50 to 60 ℃, till a constant weight was obtained and these callus were further exploited for extraction and bacterial efficacy analysis. Dried whole plants as also the *in vitro* derived callus were powdered using the electric homogenizer exhaustively extracted by cool extraction with ethanol, Ethyl acetate, chloroform and water for 72 h[45]. The solvent-containing extracts were decanted and filtered. All the extracts and fractions were stored at 4 ℃ for antibacterial activity. Stock cultures were made fresh every seven days on agar slants during this scheme of work. Pure bacterial cultures viz., *B. subtilis, P. vulgaris*, *S. pyogenes* and *S. typhi* were maintained on nutrient broth at 37 ℃ for 24 h. Bacterial strains were cultivated at 37 ℃ and maintained on nutrient agar (Himedia, Mumbai) slant at 4 ℃. Antimicrobial activity was determined against four bacterial pathogens by the agar disc diffusion assay[46]. The crude and fractionated extracts were dissolved in Dimethyl Sulfoxide (DMSO) with the exception of the water fraction and then antimicrobial effects of crude fractionated extracts were tested using four different concentrations viz., 100, 250, 500 and 1 000 μg/mL. Petri dishes (measuring 90 mm each side) containing 20 mL of Mueller Hinton agar (Himedia, Mumbai). At the same time, 6 mm diameter sterile Whatman Antibiotic disc were placed on the surface of the inoculated agar plates, and then appropriate concentration of the extracts in DMSO and water were applied onto the discs, 100, 250, 500 and 1 000 μg/mL final concentrations were obtained for each discs. The plates were incubated at 37 ℃ for 16-18 h. The antibacterial activity was evaluated by measuring the zone of growth inhibition surrounding the discs. Standard discs of the antibiotic gentamycin (10 mg) and ampicillin (10 mg) served as the positive antibacterial controls. Negative controls were done using paper discs loaded with 20 mL of DMSO and water. The experiments were repeated in triplicate and the inhibition zone and antibacterial activities were documented.

3. Results

Callus induction was observed on the inter-nodal and leaves segments on MS medium supplemented with 2, 4-D and Kin alone or in combinations. Based on the concentration of plant growth hormone and age of the explants the callus induction frequency was varied (Table 1). Young explants cultured on the medium with below 1.5 mg/L of 2, 4-D and Kin showed high percentage of callus formation and multiplication. Above 1.5 mg/L 2, 4-D and Kin augmented medium produced compact and hard callus, they showed very low percentage of multiplication

and regeneration potential. Maximum percentage of callus formation (inter-nodal segments 84.3 ± 0.78; leaves segments 93.8 ± 1.27) was obtained on Murashige and Skoog's basal medium supplemented with 3% sucrose and 1.5 mg/L of 2, 4-D. MS medium augmented with 1.5 mg/L showed 76.5 ± 0.27 percentage of callus proliferation on leaves segments and 67.5 ± 0.54 from inter-nodal segments respectively. Different types of calli were obtained of which, the friable, semi friable and creamy white coloured showed high proliferation rate. The semi friable callus was showed highest rate of shoot proliferation. Friable calli were showed highest percentage of cell division and cell multiplication. Leaves derived calli more friable than the inter-nodal segments derived calli and they showed high frequency of proliferation rate. Leaves derived calli showed high percentage of multiplication in the cell suspension medium than inter- nodal segment derived calli, inter-nodal segment derived calli were more aggregative than the leaves derived calli. The results of antibacterial screening tests of leaves and inter-nodal segments, leaves and inter-nodal segment derived calli extracts of *M. arvensis* in different solvents viz., ethanol, chloroform, Ethyl acetate and water against pathogenic bacteria using disc diffusion techniques are depicted in Table 2. The ethanol extracts of leaves derived calli showed the maximum bio-efficacy than other solvents (Table 2).

Table 1

Influence of 2, 4-D and Kin on callus induction from the leaves and inter-nodal segments of *Mentha arvensis*.

MS medium + Plant growth regulator (mg/L)		Mean percentage of callus induction ± S.E.	
2, 4-D	Kin	Leaves	Inter-nodal
0.5	0.0	64.9 ± 0.56	57.8 ± 0.34
1.0	0.0	78.6 ± 0.63	79.6 ± 1.16
1.5	0.0	93.8 ± 1.27	84.3 ± 0.78
2.0	0.0	78.3 ± 0.78	68.7 ± 0.51
2.5	0.0	61.9 ± 0.91	54.8 ± 0.78
3.0	0.0	51.8 ± 0.35	43.4 ± 0.81
0.0	0.5	52.8 ± 0.48	36.6 ± 0.41
0.0	1.0	65.8 ± 0.74	51.3 ± 0.36
0.0	1.5	76.5 ± 0.27	67.5 ± 0.54
0.0	2.0	54.7 ± 0.63	53.3 ± 0.44
0.0	2.5	50.4 ± 0.57	46.9 ± 0.57
0.0	3.0	43.2 ± 0.54	39.7 ± 0.62
0.5	0.5	73.5 ± 0.82	52.6 ± 0.76
1.0	1.0	69.4 ± 0.81	59.7 ± 0.78
1.5	1.5	61.7 ± 0.67	63.4 ± 0.46
0.5	1.0	63.4 ± 0.93	69.2 ± 0.86
1.0	1.5	69.4 ± 0.46	58.7 ± 0.63
1.5	0.5	73.4 ± 0.63	51.9 ± 0.58

Table 2

In vivo and *in vitro* antibacterial efficacy leaves and inter-nodal derived calli extracts of *M. arvensis (*inhibition zone in mm).

Solvents	Concentration (μg/mL)	Leaves segments derived calli extracts				Inter-nodal segments derived calli extracts			
		S. t	*S. p*	*P. v*	*B. s*	*S. t*	*S. p*	*P. v*	*B. s*
Chloroform	100	12	00	00	14	28	00	00	11
	250	24	00	00	13	30	00	00	13
	500	25	00	00	20	32	00	00	16
	1 000	29	00	00	25	36	00	00	20
Ethyl acetate	100	14	11	13	13	11	12	14	11
	250	20	17	19	16	18	16	18	17
	500	22	19	21	19	22	19	20	20
	1 000	29	21	23	23	24	22	23	25
Ethanol	100	13	12	12	11	08	13	03	13
	250	14	17	16	15	12	17	08	15
	500	21	19	20	19	16	20	14	17
	1 000	23	20	22	24	19	22	19	23
Water	100	08	13	08	09	03	04	10	04
	250	10	18	11	14	06	11	15	09
	500	12	19	13	17	12	14	18	16
	1 000	15	22	16	22	20	16	23	20

S. t-Salmonella typhi; S. p-Streptococcus pyogenes; P. v-Proteus vulgaris; B. s-Bacillus subtilis.

4. Discussion

Influence of 2, 4-D and kin on callus induction was observed by number of tissue culturist[42,47-49]. In the present study we observed maximum percentage of callus on 2, 4-D and kin supplemented medium with varied percentage. Our result was directly consonance with the Manickam *et al*[48] and Rout *et al*[49] observations on *Withania somnifera* and *Plumbago zeylanica* respectively. In the present study we obtained maximum percentage of callus on MS medium supplemented with 2, 4-D. Several workers have reported that many plants possess antimicrobial properties including the parts which include; flower, bark, stem, leaf, and *in vitro* derived calli of leaves and stems etc. It has been shown that when solvents like ethanol, petroleum ether, chloroform, isopropanol, hexane and methanol are used to extract plants, most of them are able to exhibit inhibitory effect on both gram positive and gram negative bacteria[50]. In the present study also we observed the anti-bacterial activity of leaves and inter-nodal segments derived calli of *M. arvensis*. Phytochemical constituents such as tannins, saponins, flavonoids, alkaloids and several other aromatic

compounds are secondary metabolites of plants that serve as defense mechanisms against predation by many microorganisms, insects and other herbivores[51-54]. This can partially explain the demonstration of antimicrobial activity by the stem, leaves and stem and leaves derived calli extracts of *Rauvolfia tetraphylla, Physalis minima, Plumbago zeylanica, Passiflora mollussima, Phyllanthus amarus, Passiflora edulis*[47,49,50-53]. The demonstration of antimicrobial activity against both gram-positive and gram-negative bacteria may be indicative of the presence of broad spectrum antibiotic compounds[54].

Observation of the present study was supported by the previous observation on *Phyllanthus amarus, Rauvolfia tetraphylla, Physalis minima, Passiflora mollussima, Passiflora edulis* leaf and callus extracts[47,50-53]. Earlier observations on *Baliospermum axillare, Rauvolfia tetraphylla, Physalis minima* and *Mimosa hamata* leaf and callus extracts showed considerable antibacterial and antimicrobial activity[42,50,55]. The present observation augments the previous bio-efficacy studies on cell cultures. The present study observation is strengthening the bio-efficacy studies on cell cultures. The leaves and stem derived calli extracts on *Proteus* sp. showed that the plants can be used in the treatment of urinary tract infection associated with *Proteus* sp[56]. Through the bacterial efficacy studies, it is confirmed that the *in vitro* raised calli tissue was more effective compared to *in vivo* tissue. The ethanolic extracts of leaves and leaves derived calli were more effective against the selected bacteria than the inter-nodal segments. Potentially viable and reproducible callus cultures to biosynthesize similar compounds are established. Further work is required to find out the active principle from the plant extracts and to carry out pharmaceutical studies.

Conflict of interest statement

We declare that we have no conflict of interest.

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