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In vitro Development of Callus from Node of *Mimusops elengi* - As Substitute of Natural Bark

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ABSTRACT
In this study, results of *in vitro* callus development from *Mimusops elengi* as substitute of bark were formulated. By different auxine/cytokinine ratio from young nodal explant compact, globular and fragile types of callus were developed. Methanolic extract of all types of callus and bark were studied for extractable matter, antibacterial activity, phytochemical profiling, biouautography and Thin Layer Chromatography (TLC) banding patterns. Results of all calluses were compared with results of natural bark of *M. elengi*. TLC pattern of three types of callus and bark revealed similar banding pattern, and same bioactivity was also observed in bioautography. Our results indicate that natural bark & *in vitro* developed callus of *M. elengi* posses similar chemical profiling and bioactivity regardless the type & colour of callus.

Keywords: Bioactivity, *Mimusops elengi*, antibacterial activity, bioautography.

INTRODUCTION

Mimusops elengi Linn. is belong to the family Sapotaceae. It is an evergreen tree, distributed in Deccan peninsular region and the Andamans region. [1] Plant is frequently cultivated in North India. [2] Apart from its medicinal value plants are cultivated for its ornamental appearance and fragrant flowers throughout India.

Medicinally all parts of *M. elengi* were reported for treatment of several human ailments. [3] Among all parts bark is extensively studies for its pharmacological activities [4-5], it is used as an astringent and applied externally too. Bark extract is also given orally to cure diseases of gums and teeth, in biliousness as an anthelmintic, stomachic and cardiotonic. [6] Currently bark extract of *M. elengi* reported for its moderate inhibitory activity against HIV type 1 protease. [7] Bark is also used as a gargle for odontopathy treatment. [8]

The natural vegetation of *M. elengi* is negligible, because seeds of most species within *sapotaceae* are reported to be short-lived in open storage at room temperature, and were thought to be recalcitrant [9], seeds if remained on plant then they lose their viability. [10] In such cases if overexploitation will continue, survival of *M. elengi* will be in danger. Same accident was happened with 15 years old *M. elengi* plant in our campus, and this episode encourage author to find out some alternative way as precaution for protect long survival of *M. elengi.*

In present investigation we developed *in vitro* technology of callus formation with same bioactivity and biochemical composition for *M. elengi*. Antibacterial activity and secondary metabolite profiling of all types of callus was compared with mature bark of *M. elengi*.

MATERIALS AND METHODS

In vitro Callus development and maintenance

Young internodes were used as explants. Plant material was thoroughly washed with 2-4 drops of surfactant in 1% Tween 20 for surface cleaning and subsequent washing for 20 min under running tap water. Than the treatment was given by the bleaching agent 4% sodium hypochlorite for 20 min and washed under running tap water for 30 min. The final sterilization was done with 0.01% $HgCl_2$ solution in sterile condition for 5 min. The explants were washed several times with sterilized distilled water and inoculated on the MS medium. [11] The MS basal medium used was, fortified with 3% sucrose. The pH of medium was adjusted to 5.6 to 5.8 with the help of 1 N NaOH and 0.1 N HCl and solidifies with 0.8% agar. The medium was autoclaved at 15 lbs/square inch pressure for 15 min. Plant growth regulators used were IBA (indole 3- butyric acid), Kn (kinetin), IAA (indole 3- acetic acid), BA (N^6-benzyladenine), 2, 4-D(2, 4-dichlrophenoxyacetic acid), NAA (1-naphthylacetic acid), alone and in combination.

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Comparative antimicrobial activity

Comparative antimicrobial activity of methanol extract of callus (green, fragile, globular) and bark was performed.

Preparation of Extract

Two month old callus powder (15 g) and bark (15 g) ware extracted at room temperature for 24 h in methanol. The extracts were filtered through filter paper and evaporate at temperature below 40°C in water bath and calculate the extractable matter percentage.

Microorganism used

Test organism were bacteria, *Enterobacter aerogens* (ATCC13048), *Pseudomonas aeruginosa* (ATCC25668), *Micrococcus luteus* (ATCC4698), *Klebsiella pneumoniae* (ATCC15380), *Bacillus subtilis* (ATCC6051) and *Bacillus cereus*, *Escherichia coli*, *Salmonella paratyphi-A*, collected from microbiology department, Sardar Patel University, V. V. Nagar, Gujarat. Pure cultures of bacteria were maintained by periodical transfer on a fresh nutrient agar slant from the previous stock at an interval of one month and stored at 4°C. [12]

Culture media

Culture inoculums of test organisms were prepared by transforming a loopful of bacterial culture from the maintained stocks in to 10 ml of sterile Mueller –Hinton Agar No. 2 (Hi-media), and kept on shaker at room temperature to incubate for 8 to 10 hrs (for *Micrococcus luteus* (ATCC4698) 17 hrs). Inoculum turbidity was maintained constant throughout the experiment to 0.8 OD at 660 nm. Level of turbidity is equivalent to approximately 1×10^8 CFU/ml.

Agar diffusion Assay

Antimicrobial screening was done using agar well diffusion methods. [13] For this 25 ml of sterile Mueller –Hinton Agar No. 2 (Hi-media) containing 100µl bacterial culture, was poured in sterile autoclaved petri plates, and then allowed to stand for solidify completely. The wells were prepared with the help of sterile 10 mm diameter cork-borer. Then 100µl of prepared plant extract solution were poured into the wells. Then the plates were sealed with plasticine and transferred to the refrigerator to diffuse out for 30 min. The plates were then incubated in the incubator at 37°C for 24 h. Triplicate plates were prepared for each treatment and the average zone of inhibition excluding well, were recorded. 40% DMSO was used as negative control, and 0.001mg/ml tetracycline was used as positive control.

Antibiotic susceptibility of selected bacterial strains

Susceptibility of selected bacterial strain was done against the standard antibiotics (Ampicillin (10µg), Tetracycline (25µg), Gentamicin (30µg), Streptomycin (10µg), Co-Trimoxazole (25µg), by ready-made disc (Hi-media, Mumbai). Twenty ml of sterilized nutrient agar seeded with activated bacterial culture broth was poured in petri dish, allowed to solidify and disc was placed gently on surface by pointed forceps. Seal the plates with plasticine and incubate in the incubators at 37°C for 48 h.

Phytochemical analysis & bioautography

Separation of Secondary metabolites on TLC plate

Chloroform fraction was extracted from dried methanolic extract powder of bark and callus. Extract (20µl) was loaded on pre-coated aluminium plates, 200 µm thick, Silica gel $60F_{254}$-Merck (7.5cm × 5.5 cm) with graduated capillary. TLC was developed with solvent toluene: ethyl acetate (9:1). Two plates were loaded at the same time and were developed in solvent system. One plate was used for bioautography and other was used for fingerprinting pattern documentation by without treatment (under UV light), and after treatment (by sprayed with anisaldehyde & heated at 105°C for 15 min).

Bioautography

Agar overlay method described by Slusarenko [14] was used for bioautography assay. Seeding medium was prepared with *E. coli* into 20 ml of Mueller –Hinton Agar No. 2 (Hi-media) medium. Developed TLC plate was placed in glass plate (150 mm diameter) and overlayed with seeded media. Agar was allowed to solidify and incubated at 37°C for 24h. On next day, the plate was flooded with 2, 3, 5 triphenyl tetrazolium chloride (0.1%) to visualize inhibition. The area of inhibition, remained colour less against red background.

Phytochemical analysis

Two-month-old green compact callus, globular callus, fragile callus and bark of *M. elengi* were used for the qualitative phytochemical investigation for tannin, alkaloids, terpenoids, saponin, and steroids, as described by Harbone. [15]

RESULTS AND DISCUSSION

***In vitro* callus development**

Tissues culture is a versatile tool for rapid clonal propagation of woody [16] as well as herbaceous plants. [17] Today's biotechnology has become more of a necessity than curiosity. It is being extensively used for afforestation programmes [18] and tapping useful compounds from plants. [19] Different types of callus with different characteristics were developed from the young node of *M. elengi* (Table 1). Phytohormones like 2, 4-D (0.1 mg/l), Kn (0.1 and 0.5 mg/l), BA (0.1, 0.5, 1.0 and 2.0 mg/l), NAA (0.1 mg/l) and IAA (0.1 mg/l) were not able to induce a callus from *M. elengi* node. But 2, 4-D at > 0.1 mg/l produced good callus biomass (97.7 mg to 145.3 mg on dry basis), and thus it was used in combination with Kn, NAA, and IAA. However, the production of in vitro callus is the result of the interaction of environmental conditions and the genotype of the cultured plant cells. Morphological and phytochemical differences in callus are attribute to culture conditions, composition of the medium [20], orientation of the explant [21], and growth and morphogenesis ability of plants itself. Type and concentration of hormone present in medium directly affect the nature, colour and biochemical composition of callus. [22] In present investigation, three type of callus were produced from same explants as shown in Fig. 1A (compact), Fig. 1B (globular) and Fig. 1C (fragile). Plant growth regulators are influence nature of callus like presence of IAA in growth medium will induced compact green colour callus from explant [23], similar result was observed in present study. BA alone was not able to initiate the callus from node, but with combination to other hormone BA showed positive results. And thus, BA is widely preferred with IAA and NAA for callus induction. [24] IAA, Kn, NAA and 2, 4-D alone were sufficient to induce callus from node of *M. elengi* and similar results were found in *Morus alba.* [25]

Antimicrobial activity of callus

Despite the wide availability of clinically useful antibiotics and semi synthetic analogues, a continuing search for new anti-infective agents remains indispensable because some of the major antibacterial agents have considerable drawbacks in terms of limited antimicrobial spectrum or serious side effects. [26] Extracts from higher plants can be serving as good source of antibiotics [27], several plants have been

studied for callus development and their antimicrobial activity comparison with adult mother plant like; *Baliospermum axillare* [28], *Alternanthera maritima* [29], *Catha edulis* [30], *Rauvolfia tetraphylla* and *Physalis minima.* [31] In present study the organic crude extract from callus (globular, fragile, compact) and mature bark of *Mimusops elengi* were found active against Gram-positive and Gram-negative bacterial strain, and results recorded in Table 2. All three type of callus showed antimicrobial activity, nevertheless, the values of inhibition zones were little smaller, as compared to bark extract. Similar results were reported for callus and adult plant of *Alternanthera maritime* by Marcos. [29] Callus extract had showed comparative lesser antimicrobial activity as compare to adult plant extract; these may be due to the presence of smaller concentration of the antimicrobial compounds in the callus. [29] Antibiotic susceptibility was studied using octadisc and zone of inhibition data were recorded in Table 3. Retrieved data from experiment were illustrated; chloramphonicol, tetracycline, gentamicin, streptomycin, kanamycin, and amikacin were active against all selected bacteria.

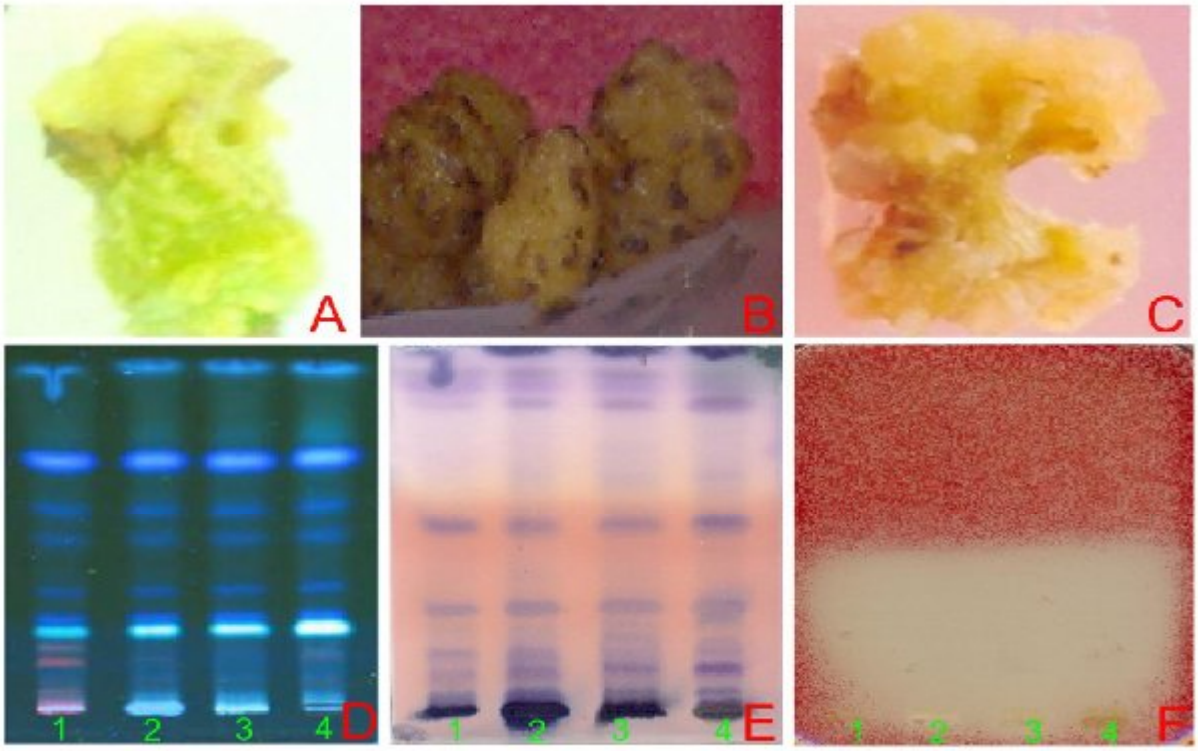

Fig. 1: Different types of callus, TLC fingerprint and bioautography of *Mimusops elengi.*
A. Green compact callus, B. Brown globular callus, C. Yellowish fragile callus, D. TLC fingerprint under UV at366nm, E. TLC fingerprint after spray with Anish-aldehyde, F. Bioautography against *E.coli.*

1. Separation of secondary metabolites of green compact callus,
2. Separation of secondary metabolites of globular callus,
3. Separation of secondary metabolites of fragile callus,
4. Separation of secondary metabolites of mature plant (bark)

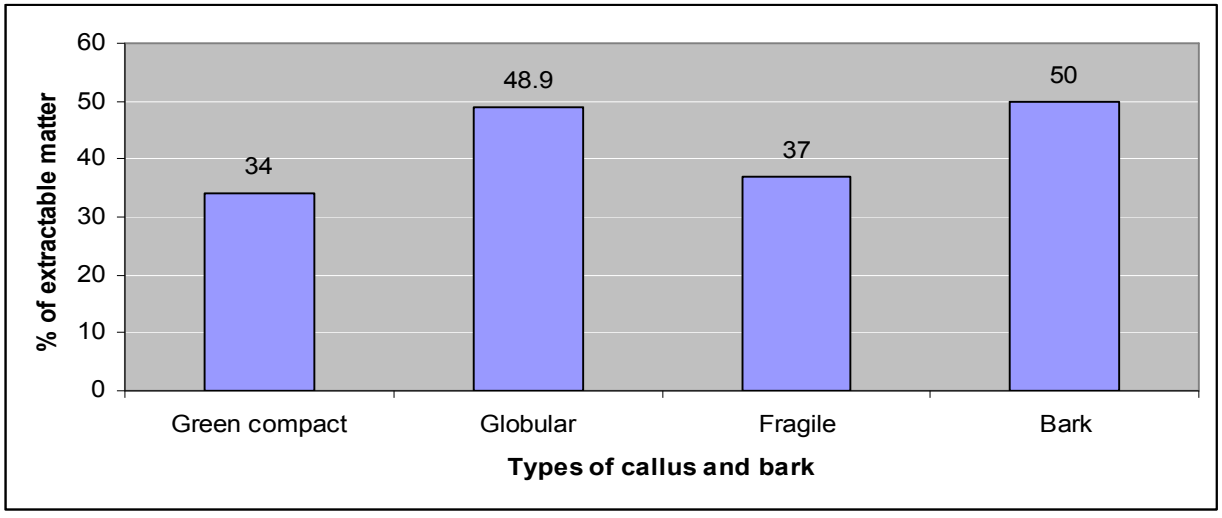

Fig. 2: Comparison of extractable matter (in methanol) of all types of callus and natural bark.

Table: 1 Effect of plant growth regulators on induction of callus from node of *Mimusops elengi* L. (Data are expressed as Mean± S.D of twelve replicate).

No.	Medium	Texture of callus	Colour	Fresh Weight (mg)	Dried Weight (mg)
1	MS	-	-	0	0
2	MS+2,4-D 0.1	-		0	0
3	MS+2,4-D 0.5	GL	Y	349±4.79	74.8± 0.632
4	MS+2,4-D 1.0	GL	Y	496±3.705	97.7±0.948
5	MS+2,4-D 2.0	GL	Y	753±3.496	124.7±0.823
6	MS+Kn 0.1	-	-	0	0
7	MS+ Kn 0.5	-	-	0	0
8	MS+ Kn 1.0	C	LG	255±2.0	47.5±0.707
9	MS+ Kn 2.0	C	LG	264±5.391	57±0.471
10	MS+ BA 0.1	-	-	0	0
11	MS+ BA 0.5	-	-	0	0
12	MS+ BA 1.0	-	-	0	0
13	MS+ BA 2.0	-	-	0	0
14	MS+NAA 0.1	-	-	0	0
15	MS+ NAA 0.5	F	Y	251±6.095	47.5±1.08
16	MS+ NAA 1.0	F	Y	385±3.777	74.9±0.567
17	MS+ NAA 2.0	F	Y	475±5.57	96.7±1.411
18	MS+ IAA 0.1	-	-	0	0
19	MS+IAA 0.5	C	G	195±3.50	32±1.49
20	MS+ IAA 1.0	C	G	266±1.81	47.1±0.99
21	MS+ IAA 2.0	C	G	391±7.196	74.3±0.67
22	MS+ 2,4-D 0.5 +Kn 1.0	C	YG	368±3.195	63.6±1.505
23	MS+2,4-D 1.0 +Kn 1.0	C	YG	654±6.915	123.5±1.711
24	MS+2,4-D 0.5 +NAA 1.0	F	Y	325±1.429	65±0.471
25	MS+2,4-D 1.0 +NAA 1.0	F	Y	747±5.27	133.6±1.429
26	MS+2,4-D 0.5 +IAA 1.0	GL	YLB	363±3.314	72±0.471
27	MS+2,4-D 1.0 +IAA 1.0	GL	YLB	757±1.885	145.3±1.059

Legends: GL –globular, C- compact, F-fragile, Y-yellow, G-green, LG-light green, YG-yellowish green, YLB – yellowish light brown.

Table 2: Antimicrobial activity of methanolic extract (15 mg/ml) (callus and bark) of *Mimusops elengi* L. (Data are expressed as mean ± SD of four replicate)

Bacterial strain	Green callus	Globular callus	Fragile callus	Bark
	Inhibition zone (mm)			
E. coli	8.32±0.23	10.17±0.12	8.17±0.12	10.37±0.47
S. paratyphi A	8.27±0.15	10.12±0.12	6.17±0.12	10.25±0.50
E. aerogens	8.05±0.12	8.05±0.12	6.15±0.12	9.5±0.57
P. aeruginosa	2.12±0.15	2.1±0.14	1.0±0.0	4.12±0.25
M. luteus	8.0±0.0	10.02±0.05	6.10±0.08	10.2±0.21
K. pneumoniae	8.35±0.12	10.02±0.05	6.10±0.08	14.22±0.28
B. subtilis	8.15±0.12	8.15±0.12	8.05±0.05	8.27±0.20
B. cereus	8.22±0.12	8.05±0.05	7.12±0.12	10.3±0.081

Table 3: Antibiotic susceptibility of bacterial strains (data are expressed as mean of four replicate)

Bacterial Strain	A 10 mcg	T 25 mcg	G 30 mcg	S 10 mcg	CO 25 mcg
	Inhibition zone (mm)				
E.coli	2	12	10	12	10
E.aerogens	2	10	10	10	0
S.paratyphi A	22	16	16	14	20
P.aeruginosa	2	14	12	12	0
M.luteus	18	20	20	20	20
K.pneumoniae	20	22	16	16	18
B.subtilis	22	20	16	16	22
B.cereus	0	12	14	12	0

C: Chloramphonicol, A: Ampicillin, T: Tetracycline, G: Gentamicin, S: Streptomycin, K: Kanamycin, Co: Co-Trimoxazole, A: Amikacin.

Table: 4 Phytochemical analysis of callus & bark of *Mimusops elengi* L.

Chemical group	Bark	Green callus	Fragile callus	Globular callus
Alkaloids	-	-	-	-
Terpenoids	+	+	+	+
Tannin	+	+	+	+
Saponin	+	+	+	+
Flavanoids	+	+	+	+
Steroids	+	+	+	+

+ = present, - = absent

While *Bacillus cereus* and *Enterobacter aeruginosa* were completely resistant for ampicillin and co-trimoxazole respectively. *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Bacillus cereus* showed biostatic behavior for co-trimoxazole. While over all study of antimicroabial activity of callus regardless nature and colour were showed broad-spectrum inhibition zone, and will be act as good antimicrobial agent.

Phytochemical analysis & bioautography

Qualitative phytochemical comparison of methanolic extract and comparison of extractable matter of callus and fresh bark was evaluated and record results in Table-4 and Fig. 2 respectively. Extractable matter of globular callus (48.9%) is quite nearer to value of bark (50%). Alkaloids were absent in callus and bark, but terpenoids, tannin, saponin, flavanoids, and steroids were detected in all; similar results for secondary metabolite presence in *M. elengi* was reported by Misra & Mitra. [32] However, the production of *in vitro* bioactive metabolites is the result of the interaction of environmental conditions and the genotype of the cultured plant cells, in present study not much variation found in major secondary metabolite though the different color and nature were observed in callus.

It is essential that the medicinal properties are conserve in the plants produced through tissue culture. [33] Finger printing of secondary metabolite of tree callus and bark was resulted in same banding pattern when observed under UV light at 366nm (Fig. 1D), and after staining with anisaldehyde-sulphuric acid (Fig. 1E). Banding pattern of green callus extract appeared with one dark red colour band under UV light (Fig. $1D_1$), while these band in others (callus and bark) banding patterns, showed very light intensity (Fig. $1D_{2, 3, 4}$), these may be due to presence of chlorophyll in the callus. Bioautography was performed after developing chromatogram [34], which helps in making the position of the biologically active detected substances visible on chromatograms. [35] In present investigation bioautography was performed against *Escherichia coli* and strong inhibition zone was clearly observed in all three-callus extracts and

bark (Fig. $1F_{1, 2, 3, 4}$). Antibacterial activity revealed uniformity in callus and bark, and these results were correlated with TLC profiling for secondary metabolite pattern and their bioautography. Only lower substance near the loading point showed antibacterial activity, but these results were same for all callus extracts and bark too.

The study showed callus from node of *Mimusops elengi* has great potential as new antimicrobial agent. Callus developed from node showed similar phytochemical and bioactivity with mature plant bark, so callus can be used as the substitute for the bark. And in vitro callus development strategy can be used as tool to protect the biodiversity/natural vegetation, of *M. elengi*. Further callus can be used for somatic embryogenesis /differentiations for regeneration of *M. elengi* plant.

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