# *In vitro* Antimicrobial Activity of Callus Extracts of *Micrococca mercurialis* (L.) Benth. on Western Ghats

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## Introduction

*Micrococca mercurialis* is a perennial uncommon herb and the plant has flowering and fruiting throughout the year. The plant is used to treat children with fever and the plant-sap is instilled into the nose, eyes or ears to treat headache, filariasis of the eye or otitis, respectively (1). It has 14 species, native to tropical Africa. Most are herbs, but some, especially in the tropics, are also shrubs or trees. Some are succulent and resemble cacti (Jeyachandran *et al.*, 2013).

*Micrococca mercurialis* wild food plants contribute to local household food and livelihood security especially for the economically disadvantaged, the young or the elderly. They are important to local food security because they are free and are easy to access by the local communities. (FAO, 1988; Banana and Turiho-Habwe, 1997; Shackleton *et al*., 1998; Somnasang and Moreno-Black, 2000). There are more than 30 000 plant species known to man as food (FAO, 1996). The majority of these are harvested locally and are not widely used at the global level. Raju and Rao (1977) reported paracytic, anisocytic, anomocytic and diacytic type in 50 species of the family. They also recorded tetracytic and cyclocytic types. Rao and Raju (1975) observed paracytic, anomocytic, anisocytic and diacytic type in case of *Micrococca mercurialis*, however paracytic type is predominant. Wild food plants also provide nutritional security by adding essential nutrients as well as variety to diets, making staples more appealing to the taste. In addition, they also contribute to household economies (Ladio, 2001). Wild relatives of crop plants are important to plant breeding because they are a source of genes which can be used to improve existing crop varieties (Iltis, 1988; Frisvold and Condon, 1998; Smith, El Obeid and Jensen, 2000).

Medicinal plants are resources of new drugs. It is estimated there are more than 250,000 flower plant species. Studying medicinal plants helps to understand plant toxicity and protect human and animals from natural poisons (13). Wild food plants also provide nutritional security by adding essential nutrients as well as variety to diets, making staples more appealing to the taste. In addition, they also contribute to house hold economies (Ladio, 2001). Wild relatives of crop plants are important to plant breeding because they are a source of genes which can be used to improve existing crop varieties (Iltis, 1988; Frisvold and Condon, 1998; Smith, El Obeid and Jensen, 2000).

## Materials and Methods

The aim of this study was planned to evaluate fresh leaves of *Micrococca mercurialis* were cultured on tissue culture MS media and fortified with different concentration of plant growth regulators using standard tissue culture techniques.

### Procurements of plant material

The fresh explants materials were collected in the month of September 2012 from the plants growing in Kolli Hills in Namakkal district in Tamil Nadu, India. They were identified and authenticated from the Rapinat Herbarium, at St. Joseph's College (Autonomous), Tiruchirappalli, Tamil Nadu. The voucher plant material was deposited in the same herbarium (No: RHT-10480). The collected fresh plant material were further maintained in department green house harden.

### Explants sterilization

The explants were washed with running tape water to remove the traces of soil particles. They have been further washed with Teepol liquid detergent under running tape water and may treated with 0.1% (w/v) Mercuric chloride (Hi Media) for 40 sec. After these, explants were then thoroughly washed 2-3 times sterilized with double distilled water to remove the traces of mercuric chloride and an again washed with fungicide 0.2% (Bavistin) for 1-2 minutes under aseptic conditions. After surface sterilization, the explants were then thoroughly washed 3-4 times sterilized with double distilled water to remove the traces of Bavistin. They already developed in the green house fresh plant was cut into shoot tip only for induction of callus culture. The leaf explants were washed with Teepol liquid detergent under running tap water to departure of dust particles. Further surface-sterilization treatment was conducted in the inner side of the laminar air flow chamber.

### Preparation of MS medium

The explants of leaf were inoculated in the MS medium (Murashige and Skoog, 1962) fortified with different concentrations of plant growth regulators 2,4-D (0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1.0 mg/l) and BAP (1.0, 0.9,.0.8, 0.7,.0.6, 0.5, 0.4, 0.3, 0.2, 0.1 mg/l) in combinations (Table 1) and sucrose (3%) was added in the MS medium. Before adding the agar (8%) to set the pH was adjusted to 5.6-5.7 with help of acidic (0.1 N HCL) and basic (0.1 N NaOH) solution. MS medium was separated by aliquot in ten different beakers (100 ml) and was added with different concentra-tions of plant growth regulators. The preparation of MS salt with agar solution was boiled and poured into culture tube around 30- 40 ml of the medium which was dispensed into 25×55 mm (Borosil, India) culture vessels before being media and apparatus are rendered sterile by autoclaving at 15 lbs/inch$^2$ (121°C) for 15 minutes.

**Callus induction**

Callus initiation is the primary stage in many tissue culture processes for the establishment of cell suspension cultures (Kumar and Kanwar, 2007; Ngara *et al*., 2008), indirect somatic embryogenesis (Kulkarni *et al*., 2002; Rahman *et al*., 2006) and other application. In the present study, MS medium was supplemented with different concentra-tions of 2,4-D, and BAP hormones were tested for initiation of green friable, white friable and vigorous growing green callus from leaf explants to be used as inoculation. The leaf explants of *M. mercurialis* were brought from our department green house harden for inoculation of callus induction. Aseptically excised leaf explants of various sizes (3-5cm) were placed on the MS medium. The MS medium supplemented with different concentra-tions of plant growth regulators (2,4-D: 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1.0 mg/l) and (BAP: 0, 0.9,.0.8, 0.7,.0.6, 0.5, 0.4, 0.3, 0.2, 0.1 mg/l). After the seventh week of inoculation they were well developed green callus was appeared in the culture medium. All the callus cultures were incubated at a suitable controlled temperature at 25± 2ºC with cool white fluorescent tubes (Philips, India) 25 µmol $m^{-2}$ $s^{-1}$ under the photoperiod (16/8 hrs) daily. The growth pattern of callus induction was noted regarding quantity of callus colour, type and number of days to formation of callus was observed and results were recorded.

**Table 1**
**Effect of different concentrations of 2,4-D (mg/l) and BAP (mg/l) for induction of callus on leaf explants of *Micrococca mercurialis***

| S. No. | Plant growth regulators (mg/l) | | Productivity of callus in leaf explants of *Micrococca mercurialis* | | |
|---|---|---|---|---|---|
| | 2,4-D | BAP | Yellow friable callus | Green friable callus | % of explants producing callus (Mean±SE) |
| 1. | 0.1 | 1.0 | 0.44 ± 0.10 | 0.71 ± 0.08 | 0.67 ± 0.09 |
| 2. | 0.2 | 0.9 | 0.84 ± 0.10 | 0.74 ± 0.09 | 0.64 ± 0.17 |
| 3. | 0.3 | 0.8 | 0.72 ± 0.08 | 0.75 ± 0.10 | 0.72 ± 0.10 |
| 4. | 0.4 | 0.7 | 0.52 ± 0.10 | 0.79 ± 0.99 | 0.75 ± 0.09 |
| 5. | 0.5 | 0.6 | 0.51 ± 0.11 | 0.76 ± 0.96 | 0.76 ± 0.10 |
| 6. | 0.6 | 0.5 | 0.76 ± 0.11 | 0.73 ± 0.13 | 0.72 ± 0.11 |
| 7. | 0.7 | 0.4 | 0.73 ± 0.10 | 0.62 ± 0.16 | 0.80 ± 0.10 |
| 8. | 0.8 | 0.3 | 0.55 ± 0.13 | 0.72 ± 0.11 | 0.57 ± 0.09 |
| 9. | 0.9 | 0.2 | 0.56 ± 0.12 | 0.73 ± 0.10 | 0.80 ± 0.07 |
| 10. | 1.0 | 0.1 | 0.58 ± 0.14 | 0.71 ± 0.12 | 0.57 ± 0.09 |

**Statistical analysis**

All the data were presented in the average of three replicates and expressed as Mean ± SD. The statistical analysis of all the data were carried out using SPSS 16.0 version. Callus formation was recorded at percentage level on each explant after $7^{th}$ weeks of cultivation. Data present the average of triplicate experiments (means $\pm$ S.D) and significant different at $p < 0.05$) using t-Test.

**Antimicrobial activity**

The antimicrobial activity was determined by callus extracts of *Micrococca mercurialis* against four different solvents by ethyl acetate, ethanol, hexane, and chloroform extracts were tested by the disc diffusion method (NCCLS).

**Preparation of callus extracts**

The callus was washed under running tap water for remove their unwanted debris medium and their air dried under shade to fine powder with help of mixer grinder which was particle sieve size 40 to 70 mm. The fine powder of callus was extracted under soxhlet apparatus with using various solvents i.e., ethyl acetate, ethanol, hexane, and chloroform.

**Preparation of Inoculum**

The appropriate microorganisms were inoculated aseptically with the Muller-Hinton (Hi media, Mumbai) broth for testing of antibacterial studies. The suspension of inoculum was perfectly suited for culture of bacterial strains. The bacterial strains were incubated at 37°C and reached to the stationary phase of growth during 18-24 hr. The colony forming units (CFU/ml) of bacteria are approximately corresponding to 105 (CFU/ml) in the inoculum suspension.

**Disc diffusion method**

Disc diffusion experiment was performed by the method of NCCLS (1993). The plant extracts of *M. Mercurialis* were tested by human pathogenic bacteria with the commercially available antibiotics. The commercially purchased Muller Hinton Agar (Hi-Media, Mumbai) was prepared by sterile conditions and medium was poured (30 ml/plates) on to the autoclaved Petri dishes ($150 \times 20$ mm) and allowed to solidify. For the purpose of inhibiting the fungal pathogens, 2 ml of antifungal agent (fluconazole) per 100 ml of medium was added. After solidification, the test strains were seeded on the surface of agar plates with help of sterile cotton swab. The sterile impregnated (plant extract 100 mg/ml) paper disc was placed on the agar petridishes. The plates were incubated under anaerobic condition at 37°C for 48 h. After the completion of incubation period, the diameter of the zone of inhibition was measured in mm and also was recorded.

**Antifungal Activity**

The four types of callus extracts of *Micrococca mercurialis* were tested for antifungal studies using three types of fungal species by disc diffusion method.

The Sabouraud Dextrose Agar (SDA) (Hi-Media, Mumbai) plates were prepared and inoculated in each fungal inoculum with help of sterile cotton swab over the surface of the Sabouraud Dextrose Agar (SDA) plates. The sterilized filter paper discs (5 mm in diameter) impregnated with plant extracts (100 mg/ml) were placed on swabbed test organism-seeded on the plates. The sterilized paper disc was impregnated with DMSO solvents were used as negative controls. The impregnated disc was completely evaporated the solvents before introduced into the test plates. The commercially purchased Nystatin (10 µg/ disc) compound was used as positive control. All the plates were incubated at 28°C for 72 h under incubator. After the incubation period the activity was determined and the diameter of inhibition zone was measured in mm.

**Results and Discussion**

The induction of callus was initiated from leaf explants of *Micrococca mercurialis* on MS medium fortified with 1.0 mg/l 2,4-D and 0.1 mg/l BAP. The yellowish and green nodular callus culture was showed a good number of result and optimized at the concentration of 2,4-D (1.0 - 0.1 mg/l), and BAP (1.0-0.1 mg/l) in the 3$^{rd}$ weeks of culture. The high content of callus was initiated from the concentrations of 2,4-D (0.79 $\pm$ 0.99) on yellow friable callus and green friable callus was initiated from the concentra-tions of BAP (0.80 $\pm$ 0.07). Three differences have been observed during the initiation of callus 1. a single organ was successively developed into leaf; 2. a complete leaf primordial was formed; 3. shoot was successively developed from the single organ. Shoot buds developed and elongated from callus culture on the same medium. The high morphogenic efficiency of nodal segments derived callus may be due to the presence of some internal components from the pre-existing auxillary buds for induction of caulogenesis (Martin, 2002).

The well developed callus culture was subculture onto fresh MS medium and supplemented with 2,4-D and Kinetin plant growth regulators which was carried out after an interval third weeks. The shoot bud was regenerated on the subcultured callus after the first week of inoculation. Best shoots appeared in the concentration of plant growth regulators was achieved in 2,4-D (1.4 mg/l) and Kinetin (0.8 mg/l). As the potentiality of shoot multiplication from callus continued for a long time, regenerates may be characterized by somaclonal variation. Several species of *Amaranthus* (Bennici *et al.*, 1997), *Ananas comosus* (Akbar *et al*., 2003), *Cuphea* (Millam *et al.*, 1997), *Dubosia* (Lin and Griffin, 1992) and *Salvia* (Liu *et al.*, 2000) produced regenerates through callus-mediated adventitious shoot differentia-tion. Such regenerates may prove to be a potential source of somaclonal variants, giving birth to traits agronomic importance. The regenerated plants of *Paedaria foetada* L. are currently being screened for agronomically useful genetic variants.

Among the four extracts the chloroform extract as it was clearly exhibited in (Table 2) *Bacillus subtilis*, *Escherichia coli*, *Serratia marcescens*, and *Staphylococcus aureus* are exhibited no activity against among the tested bacteria. The maximum inhibition zone exhibited against *Bacillus subtilis*, *Vibrio cholera* and *Proteus vulgaris*. The maximum zone of inhibition was observed using positive control, chloramphenicol against *E.coli* (2.00+0.33), *Bacillus subtilis* (1.99+0.33) and *Pseudomonas aeruginosa* (1.98+ 0.32). The negative control of DMSO solvent showed clearly a significant activity against certain types of bacteria *Salmonella typhi* (0.39+0.15), *Vibrio cholera* (0.42+0.13) and *Serratia marcescens* (0.40+0.14). Callus extract of hexane was showed good for inhibition zone against much number of tested strains. On other hand Ethyl acetate, Ethanol, and Chloroform extracts were showed better zone of inhibition activity against the certain types of bacterial species. They did not show any zone of inhibition activity against the following bacterial species *Serratia marcescens*, *Salmonella typhi*, *Pseudo-monas aeruginosa*, *Staphylococcus aureus*, and *Salmonella typhi* among the four types of callus extracts.

**Table 2**

**Effect of different concentrations of 2,4-D (mg/l) and Kinetin (mg/l) for induction of callus on leaf explants of *Micrococca mercurialis***

| S. No. | Plant growth regulators (mg/l) | | Productivity of callus in leaf explants of *Micrococca mercurialis* | | |
|---|---|---|---|---|---|
| | 2,4-D | Kinetin | Green friable Callus (Mean±SE) | Yellow friable callus (Mean±SE) | % of explants producing callus (Mean±SE) |
| 1. | 0.2 | 2.0 | 0.58+0.12 | 0.52+0.17 | 0.54+0.08 |
| 2. | 0.4 | 1.8 | 0.60+0.11 | 0.48+0.16 | 0.55+0.08 |
| 3. | 0.6 | 1.6 | 0.62+0.10 | 0.53+0.08 | 0.57+0.09 |
| 4. | 0.8 | 1.4 | 0.60+0.08 | 0.51+0.06 | 0.62+0.09 |
| 5. | 1.0 | 1.2 | 0.57+0.08 | 0.52+0.08 | 0.55+0.10 |
| 6. | 1.2 | 1.0 | 0.58+0.08 | 0.55+0.12 | 0.61+0.10 |
| 7. | 1.4 | 0.8 | 0.61+0.10 | 0.56+0.12 | 0.57+0.12 |
| 8. | 1.6 | 0.6 | 0.56+0.10 | 0.53+0.08 | 0.60+0.10 |
| 9. | 1.8 | 0.4 | 0.57+0.12 | 0.55+0.07 | 0.56+0.07 |
| 10. | 2.0 | 0.2 | 0.56+0.12 | 0.53+0.08 | 0.55+0.08 |

**Table 3**

**Determination of antibacterial activity of callus extracts in *Micrococca mercurialis***

| Test micro-organisms (Bacteria) | Diameter of inhibition zone (mm) in using various solvents | | | | | |
|---|---|---|---|---|---|---|
| | Ethyl acetate | Ethanol | Hexane | Chloroform | Positive Control | Negative Control |
| *Bacillus subtilis* | 0.10+ 0.09 | 0.09+0.08 | 0.27+0.09 | NA | 1.99+0.33 | 0.37+0.17 |
| *Escherichia coli* | NA | 0.30+0.13 | 0.29+0.09 | NA | 2.00+0.33 | 0.31+0.15 |
| *Pseudomonas aeruginosa* | 0.09+0.07 | NA | NA | 0.21+0.08 | 1.98+0.32 | 0.34+0.14 |
| *Proteus vulgaris* | 0.12+0.11 | 0.30+0.13 | NA | 0.19+0.08 | 1.95+0.31 | 0.37+0.12 |
| *Serratia marcescens* | NA | NA | 0.29+0.12 | NA | 1.93+0.33 | 0.40+0.14 |
| *Vibrio cholera* | 0.10+0.08 | 0.25+0.13 | 0.26+0.08 | 0.22+0.07 | 1.86+0.34 | 0.42+0.13 |
| *Salmonella typhi* | NA | 0.26+0.12 | NA | 0.23+0.09 | 1.88+0.33 | 0.39+0.15 |
| *Staphylococcus aureus* | NA | 0.28+0.11 | 0.26+0.09 | NA | 1.84+0.32 | 0.37+0.14 |
| *Klebsiella pneumoniae* | 0.11+0.09 | NA | 0.09+0.08 | 0.22+0.11 | 1.79+0.28 | 0.31+0.16 |
| *Streptococcus mutans* | 0.12+0.10 | NA | 0.24+0.08 | 0.21+0.11 | 1.77+0.24 | 0.35+0.12 |

NA-No activity.

All the results are measured by mean of inhibition zone in mm ± S.D of three replicates.

**Table 4**

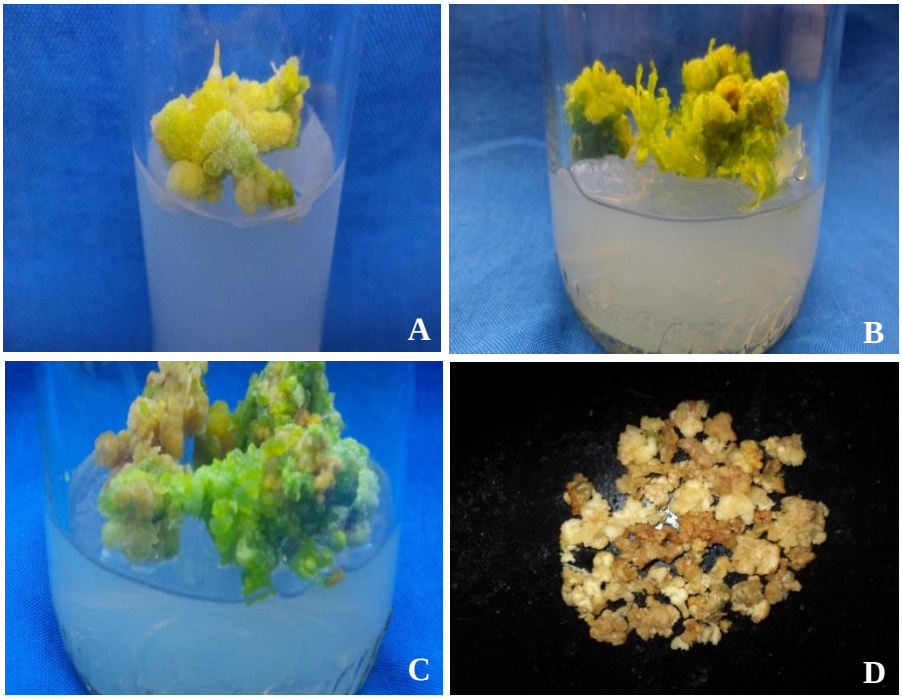
**Determination of antifungal activity of callus extracts *Micrococca mercurialis***

| Test micro-organisms (Fungi) | Diameter of inhibition zone (mm) in using various solvents | | | | | |
|---|---|---|---|---|---|---|
| | Ethyl acetate | Ethanol | Hexane | Chloroform | Positive Control | Negative Control |
| *Aspergillus flavus* | 0.21+0.07 | 0.26+0.12 | 0.21+0.09 | NA | 1.62+0.17 | 1.04+0.46 |
| *Candida albicans* | NA | 0.22+0.10 | 0.18+0.07 | NA | 1.67+0.17 | 1.13+0.38 |
| *Trichophyton mentagrophytes* | 0.21+0.07 | NA | 0.19+0.07 | 0.25+0.11 | 1.75+0.14 | 1.24+0.14 |

NA-No activity.

All the results are measured by mean of inhibition zone in mm ± S.D of three replicates.

**Fig.1: Showing different types of callus culture developed on MS medium using leaf explant of *Micrococca mercurialis.***




A. Yellowish green nodular callus expansion on MS after three weeks of culture.
B. Initiation of shoot buds from the same callus on MS after 7$^{th}$ weeks of culture.
C. Showed that the shoot differentiation from callus on MS medium after 10$^{th}$ weeks of culture.
D. Fresh callus was used for antimicrobial studies.

The antifungal studies clearly exhibit minimum inhibition zone against the four types of callus extracts. The hexane extracts was showed a significant activity against all the fungal species. The *Trichophyton mentagrophytes* was showed moderate activity against the extracts i.e., ethyl acetate, hexane and chloroform except ethanol extract. The *Candida albicans* also exhibited least inhibition zone activity against ethanol and hexane extracts. This broad spectrum of antimicrobial activity may be due to the presence of some novel secondary metabolites in the callus culture. Callus of *Micrococca mercurialis* is found to possess pharmaceutically important bioactive principles which can be commercially exploited for the benefit of mankind.

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