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Research Article

Anti-microbial and Phytochemical Studies of *Mussaenda frondosa* Linn. Leaves

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ABSTRACT

Mussaenda frondosa L (Rubiaceae) has been traditionally used in the treatment of White leprosy, eye troubles, skin infections, tuberculosis, jaundice, ulcers, wounds, cough and Bronchitis. The current study investigated antimicrobial effects of *Mussaenda frondosa* L against bacteria and fungus. In addition, Phytochemical profiling of the methanol extract of *Mussaenda frondosa* was done using High Performance Thin Layer Chromatography (HPTLC). The antimicrobial activity of Methanol (MEMF), Ethyl acetate (EEMF), Chloroform (CEMF) and Hexane (HEMF) extracts of *Mussaenda frondosa* leaves were tested against nine bacterial and four fungal strains. The Methanol extract showed significant antibacterial and antifungal activity than hexane, Chloroform, Ethyl acetate extracts which could be attributed to the presence of phenols, flavonoids and the other bioactive compounds identified through phytochemical screening. The findings in the present study offer a scientific support to the ethno medicinal use of the plant by the traditional healers.

Key words: Antibacterial, Antifungal, High Performance Thin Layer Chromatography (HPTLC), *Mussaenda*, Extract.

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INTRODUCTION

Infectious diseases are an important cause of mortality and morbidity, in all regions of the world. The increasing emergence of fantimicrobial resistance worsens the impact.[1,2] Increased prevalence of resistant bacteria, together with lack and high cost of new generation drugs has escalated infection-related morbidity and mortality particularly in developing countries.[3] Antibiotics are crucial in the treatment of several severe infections such as pneumonia, tuberculosis and meningitis. Thus, the emergence of the multi drug resistant strains poses a huge challenge for humanity to combat.[4] Therefore; there is an ever growing demand for the new antibiotics that are cost effective and easily available to the common people.

In recent years, there has been a growing interest in researching and developing new antimicrobial agents from various sources to combat microbial resistance.[5] Therefore, a greater attention has been paid to antimicrobial activity screening from natural source.

Mussaenda frondosa L belonging to the family Rubiaceae has been traditionally used in the treatment of White leprosy, eye troubles, skin infections, tuberculosis, jaundice, ulcers, wounds, cough and Bronchitis. A weak decoction of dried shoots is given to children to relieve cough .[6] Traditional claim indicates the use of the plant against infections. Literature survey revealed that there is no scientific study on antimicrobial efficacy of the plant. Hence an attempt has been made to investigate antimicrobial effects of *Mussaenda frondosa* L against pathogenic bacteria and fungus. The methanol, chloroform, Ethyl Acetate and Hexane extracts of the leaves of the plant *Mussaenda frondosa* L. were subjected to antibacterial studies against Gram positive and Gram negative bacteria and antifungal studies.

The phytoconstituents such as rutin, quercetin, hyperin, singapic acid, Ferulic acid and stigluside which were isolated by droplet counter current chromatography from the methanolic extract of the sepals of *Mussaenda frondosa* L.[7] The petals of *Mussaenda frondosa* Linn was found to have antibacterial activity against *Saccharomyces cerevisiae, Ustilago Mayadis, Escherichia coli, Micrococcus luteus, Bacillus subtilis* and *Bacillus cereus*.[8] The methanolic extract of *M.frondosa* was found to possess hypolipidemic activity in high fat diet fed rats. The aqueous and alcoholic extract of the *Mussaenda frondosa* showed significant hepatoprotective activity in paracetamol induced liver damage model in Wistar rats.[9]

The protective effect and possible mechanism of alcoholic and aqueous extract from *Mussaenda frondosa* Linn was studied on Ethanol induced hepatic injury in Wistar rats.[10]A perusal of literature revealed that no detailed study on the efficacy of the leaves of *Mussaenda frondosa* on pathogenic micro organisms.

MATERIALS AND METHODS

Collection and authentication

The leaves of *Mussaenda frondosa* L. were collected from Kodai hills, Tamil Nadu. The plant was identified, confirmed and authenticated by comparing with a Herbarium by a botanist. Dr. Jayaraman, Plant anatomical Research Centre (PARC), Tambaram, Chennai.

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Extraction

The freshly collected plant materials were cut into small pieces, shade dried and coarsely powdered. The powdered material was successively extracted with n- hexane (HEMF), chloroform (CEMF), ethyl acetate (EEMF) and methanol (MEMF) in an aspirator bottle by cold percolation method for 72 hrs. The extracts were filtered and concentrated in rotary evaporator. These extracts were subjected to antimicrobial activity, phytochemical screening and HPTLC analysis.[11]

High performance thin layer chromatography

The MEMF, EEMF, CEMF and HEMF extracts under study, each (1 g) were dissolved in the solvents such as methanol, ethylacetate, chloroform, n-hexane individually on a water bath, filtered and made upto 10ml in a standard flask. Samples (10 µl) were applied using Aluminium sheets precoated with silica gel merck 60F $_{254}$, 0.2 mm layer thickness (10 x 10 cm) as a stationary phase. Toluene: Ethyl Acetate: Formic acid [7: 2.5: 0.5] was used as a mobile phase and the scanning was performed at 254nm using `CAMAG' densitometer scanner.[12]

Antibacterial activity

The organisms used were clinical samples of Madras Medical College which was identified and maintained in the Department of microbiology. The antibacterial activity was done by determining the Minimum Inhibitory Concentration (MIC) and disc diffusion assay according to the method recommended by Clinical and Laboratory Standards Institute (CLSI).[13]

Microorganisms

The organisms used were Gram positive organisms (*Coagulase negative Staphylococcus, Staphylococcus aureus)* and Gram negative organisms (*Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa, Salmonella typhi, Salmonella paratyphi A, Salmonella paratyphi B* and *Vibrio cholerae).* The organisms were maintained on Nutrient agar slopes after confirmation by biochemical tests and stored at 4°C.

Preparation of the bacterial suspension for inoculation

Few colonies of the pathogenic strains were picked and inoculated into 4ml of peptone water. These tubes were incubated for 2 to 5 hours to produce a bacterial suspension. The suspension was then diluted, if necessary with saline solution to density visually equivalent to that of standard (0.5ml of 1% Barium chloride to 99.5ml of 1% sulphuric acid (0.36N)). This suspension was then used for seeding.

Determination of Minimum Inhibitory Concentration (MIC)

The extracts were prepared by dissolving the Methanol, Ethyl acetate, Chloroform and Hexane extract residue of the leaves of *Mussaenda frondosa* L. in dimethyl sulphoxide (DMSO). Mueller Hinton agar (MHA) was prepared and sterilized by autoclaving at 121°C at 15lb pressure for 15 min and used for the sensitivity tests.

The extracts were incorporated into Mueller Hinton agar (MHA) such that the final concentration of extract being 66.67, 100, 133.33 and 166.66 µg / ml in the plates. The plates were prepared using agar and different extracts of various dilutions, allowed to solidify and dry. Then a loopful of the cultures was inoculated at the labeled spots. The plates were then incubated at 37°C for 24 hours and observed for the presence (or) absence of growth of the organism.

Agar disc diffusion method

The discs of 6mm diameter were prepared from whatmann filter paper number 1 and were sterilized in hot air over at 160°C for one hour. The Discs were then impregnated with the MIC of the extracts and the solvent Dimethyl Sulphoxide (DMSO). Ciprofloxacin discs (5µg) were used as standard.[14,15]

Seeding was done on Mueller Hinton agar (MHA) plates with the help of a sterile swab from the peptone water culture and allowed to dry. The extract, standard antibiotic and Dimethyl sulphoxide discs were placed on the seeded plates and kept at 4°C for 30 min to allow the prediffusion of the antibiotic, extract and DMSO. The plates were then incubated at 37°C for 24 hours. The results were read by measuring the zone of inhibition around the discs.

Anti fungal activity

Microorganisms

The fungi studied were *Trichophyton simii, Trichophyton mentagrophytes, Aspergillus niger, Rhizopus* and *Candida albicans.* The fungi were maintained on Sabouraud's Dextrose Agar (SDA) Slopes and stored at 4°C.

Determination of Minimum Inhibitory Concentration (MIC)

A fungus is large cells with more variation in size than bacterial preparation of a standard suspension of known CFU and is difficult to perform fungal susceptibility testing. It is much less standardized than the bacterial methods. Although possible for yeast, establishing a standardized inoculum for filamentous fungi is beyond the capability of most laboratories.[16].Disc diffusion tests for testing antifungal antibiotics have been described.[17] but they are not widely accepted by shadomy *et al*., 1991.[18] The fungi used were filamentous in nature except *candida* hence the inoculum could not be standardized. The fungi, hence forth were seeded directly on to the media.

The extracts were prepared by dissolving the MEMF, EEMF, CEMF and HEMF extract residue of the leaves of *Mussaenda frondosa* L. in Dimethyl sulphoxide (DMSO). Sabouraud's Dextrose agar (SDA) was prepared and sterilized by autoclaving at 121°C at 15lbs for 15min.[19]

Four fungal cultures were taken up for the study. Four slants of SDA media along with extract were prepared for each concentration (100, 150 and 200 µg / ml) and allowed to set. The different fungi were inoculated into SDA slant and incubated at 37°C for 1 to 4 weeks. The results were read by noting the presence (or) absence of growth in the slant.

RESULTS AND DISCUSSION

Mussaenda frondosa L (Family: Rubiaceae) has been traditionally used in the treatment of White leprosy, eye troubles, skin infections, tuberculosis, jaundice, ulcers, wounds, cough and Bronchitis. Based on the traditional claim the plant was evaluated for antimicrobial activity by *invitro* methods. The different extracts of *Mussaenda frondosa* L. were subjected to preliminary phytochemical investigation which shows the presence of flavonoid, saponin, Glycoside, sugar, steroid, mucilage, phenol and protein (Table 1).

HPTLC finger prints

HPTLC analyses of extracts were performed at 254 nm and the fingerprints were shown in Figure 1. MEMF showed 10 peaks at 0.11, 0.16, 0.23, 0.27, 0.37, 0.50, 0.58, 0.70, 0.81 and 0.88. The peak at 0.11 was found to have 32.65% and 0.50 was 4.07%.

EEMF indicated the presence of 8 peaks with Rf value 0.07, 0.16, 0.23, 0.27, 0.39, 0.69, 0.80 and 0.84. The Rf value 0.07 was found to have 27.15% and 0.39 was 2.15%.

CEMF showed the presence of 9 peaks at 0.06, 0.09, 0.14, 0.21, 0.24, 0.41, 0.46, 0.55 and 0.62. The peak at 0.55 was found to have 23.21% and the peak at 0.24 was 4.02%.

Table 1: Preliminary Phytochemical Analysis of Different Extracts of *Mussaenda Frondosa* Linn.

Sl.No.	Phytoconstituents	HEMF	CEMF	EEMF	MEMF
1.	Flavonoids	-	+	+	+
2.	Steroids	-	+	+	+
3.	Glycosides	-	-	+	+
4.	Carbohydrates	-	+	+	+
5.	Phenol	-	-	+	+
6.	Tannin	-	-	-	+
7.	Saponin	-	-	-	+
8.	Alkaloid	-	-	-	-
9.	Terpenoids	-	-	-	-
10.	Anthraquinones	-	-	-	-
11.	Quinones	-	-	-	-
12.	Proteins & Amino acids	-	-	-	+
14.	Mucilage	-	-	-	+

MEMF-Methanol extract of *Mussaenda frondosa,* EEMF-Ethyl acetate extract of *Mussaenda frondosa*, CEMF-Chloroform extract of *Mussaenda frondosa,* HEMF-Hexane extract of *Mussaenda frondosa.*
+ indicates presence, - indicates absence

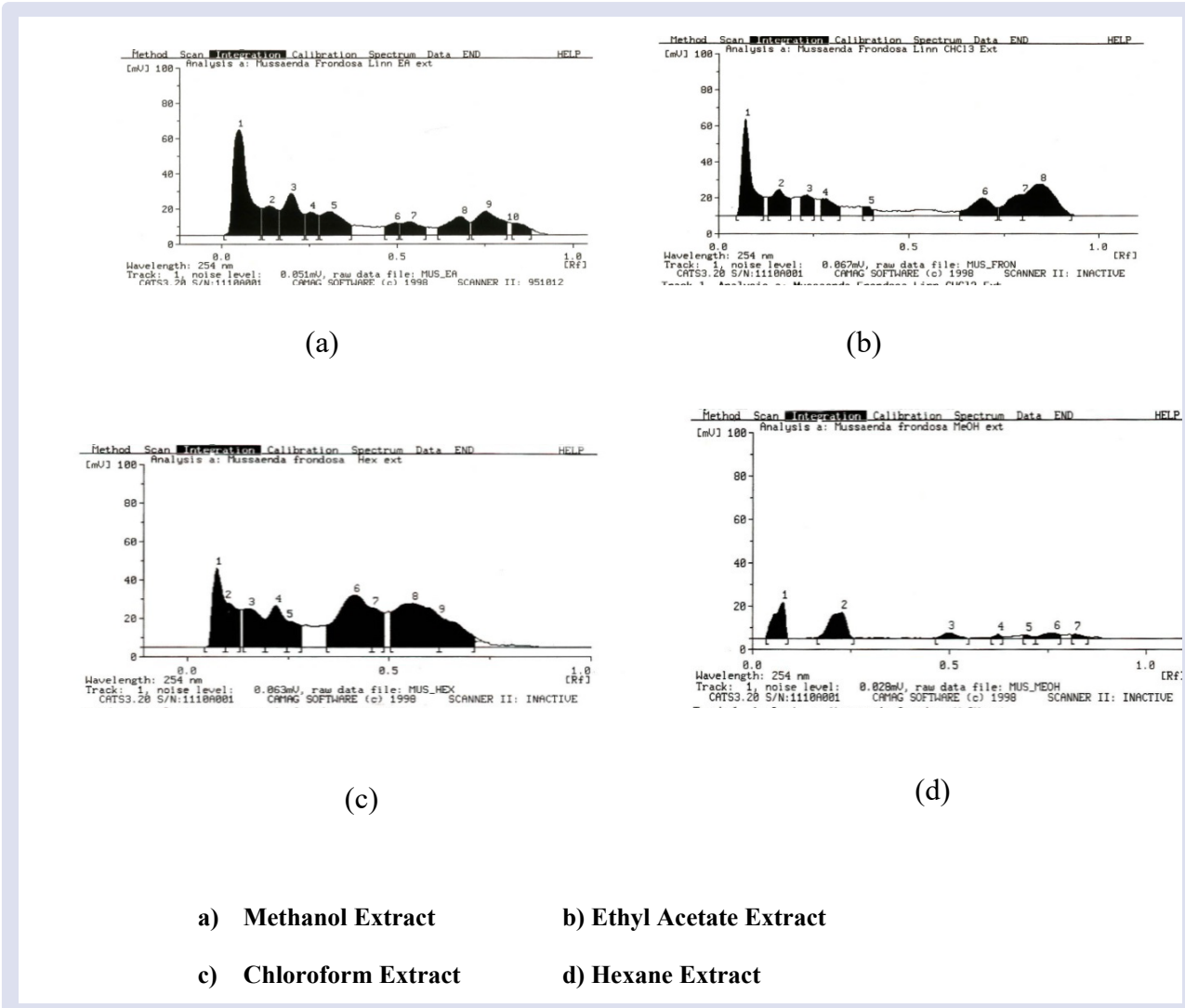

Figure 1: HPTLC Fingerprint Profile of Various Extracts.

HEMF indicated the presence of 7 peaks with Rf 0.09, 0.23, 0.55, 0.63, 0.72, 0.78, and 0.85. The peak at 0.23 was found to have 39.71% and 0.72 was 4.05%. HPTLC fingerprinting will help to authenticate the plant in future.

Anti bacterial studies

The extracts viz. HEMF, CEMF, EEMF and MEMF were tested at various dose levels on the pathogenic bacteria. The results of MIC extracts of *Mussaenda frondosa* L. against the pathogenic bacteria were reported in Table 2. Sensitivity test was done by agar disc diffusion method and zone of inhibition were measured (Table 3). The results revealed that the extracts were found to be effective against all the pathogenic bacteria such as *coagulase negative staphylococcus, staphylococcus aureus, salmonella typhi, salmonella paratyphi A, salmonella paratyphi B, pseudomonas aeroginosa, klebsiella pneumoniae, vibrio cholerae* and *Escherichia coli.*

The MEMF inhibits the growth of *Pseudomonas aeruginosa, Salmonella typhi, Salmonella paratyphi B* at the concentration of 100µg/ml whereas *coagulase negative Staphylococcus, Klebsiella pneumoniae, Vibrio cholerae* were inhibited at 133.33 µg/ml. The EEMF, CEMF and HEMF extract showed moderate inhibition against bacterial strains. The maximum zone of inhibition was obtained with MEMF extract against *Pseudomonas aeruginosa* (18 mm), *Salmonella typhi* (19mm) and *Salmonella paratyphi A* (18 mm).

Antifungal studies

Antifungal activity of MEMF, EEMF, CEMF and HEMF were observed in Sabouraud Dextrose Agar slants. The extracts were tested at different dose level against *Trichophyton mentagrophytes, Trichophyton simii, Aspergillus niger & Rhizopus* which cause Tinea cruis, Tinea pedis, Tinea capitis, systemic aspergillosis, Invasive aspergillosis and Mucormycosis.

The extracts shows significant inhibition in growth of fungi in the agar slants. The MIC of extracts was shown in the Table 4. The MEMF inhibits *T.simii* & *T.mentogrophytes* at 150 µg/ml and *A.niger* & *Rhizopus* at 100 µg/ml. The MEMF also showed significant inhibition against *T.simii, T.mentogrophytes, A.niger* and *Rhizopus* whereas EEMF showed moderate inhibition.

CONCLUSION

The MEMF showed significant antibacterial and antifungal activity than HEMF, CEMF, EEMF extracts which could be attributed to the presence of phenolics, flavonoids and the other bioactive compounds identified through phytochemical screening. The findings in the present study offer a scientific support to the ethno medicinal use of the plant by the traditional healers. This plant can be suggested in the treatment of skin infection, enteric fever, cholera, urinary tract infection, wound infection, Nosocomial infection, Respiratory infection, Aspergillosis and Mucormycosis.

Table 2: Minimum Inhibitory Concentration of the Extracts of *Mussaenda Frondosa* Linn.

Extracts	MINIMUM INHIBITORY CONCENTRATION									
	1	2	3	4	5	6	7	8	9	10
MEMF	133.33	166.66	133.33	133.33	100	100	133.33	100	133.33	100
EEMF	100	166.66	166.66	133.33	133.33	133.33	133.33	133.33	166.66	100
CEMF	133.33	166.66	133.33	133.33	133.33	133.33	166.66	100	133.33	100
HEMF	133.33	166.66	133.33	133.33	133.33	133.33	166.66	133.33	133.33	100

Coagulase negative staphylococcus[1], *Staphylococcus aureus*[2], *Escherichia coli*[3], *Klebsiella pneumoniae*[4], *Pseudomonas aeruginosa*[5], *Salmonella typhi*[6], *Salmonella paratyphi A*[7], *Salmonella paratyphi B*[8], *Vibrio cholerae*[9], *Candida albicans*[10].
MEMF-Methanol extract of *Mussaenda frondosa,* EEMF-Ethyl acetate extract of *Mussaenda frondosa*, CEMF-Chloroform extract of *Mussaenda frondosa,* HEMF-Hexane extract of *Mussaenda frondosa*

Table 3: Zone of Inhibition (Mm) of the Extracts of *Mussaenda Frondosa* Linn.

Organisms	Zone diameter of ciprofloxacin (mm)	Zone diameter of MEMF (mm)	Zone diameter of EEMF (mm)	Zone diameter of CEMF (mm)	Zone diameter of HEMF (mm)
Coagulase negative Staphylococcus	30	17	11	11	9
Staphylococcus aureus	19	15	12	11	9
Escherichia coli	29	17	14	11	11
Klebsiella pneumoniae	28	17	13	14	11
Pseudomonas aeruginosa	30	18	13	13	14
Salmonella typhi	38	19	16	12	12
Salmonella paratyphi A	34	18	12	16	11
Salmonella paratyphi B	23	16	11	13	9
Vibrio Cholerae	22	12	10	11	10

MEMF-Methanol extract of *Mussaenda frondosa,* EEMF-Ethyl acetate extract of *Mussaenda frondosa*, CEMF-Chloroform extract of *Mussaenda frondosa,* HEMF-Hexane extract of *Mussaenda frondosa*.

Table 4: Minimum Inhibitory Concentration of the Different Extracts of Mussaenda *Frondosa* Linn.

Extracts	Trichophyton simii (µg/ml)	Trichophyton mentagrophytes (µg/ml)	Aspergillus niger (µg/ml)	Rhizopus (µg/ml)
MEMF	150	150	100	100
EEMF	200	200	100	150
CEMF	200	200	150	150
HEMF	200	200	150	200

MEMF-Methanol extract of *Mussaenda frondosa,* EEMF-Ethyl acetate extract of *Mussaenda frondosa*, CEMF-Chloroform extract of *Mussaenda frondosa,* HEMF-Hexane extract of *Mussaenda frondosa.*

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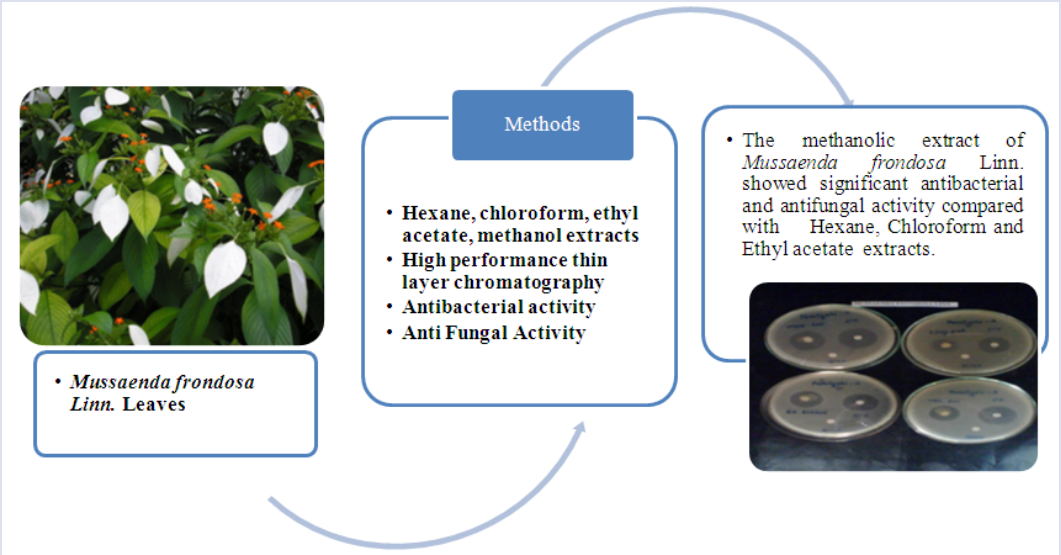
GRAPHICAL ABSTRACT



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