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ANTIBACTERIAL ACTIVITY OF MEDICINAL PLANTS AGAINST *CORYNEBACTERIUM MINUTISSIMUM:* A CAUSATIVE ORGANISM OF THE SKIN DISEASE ERYTHRASMA

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Abstract

Erythrasma is an intertriginous infection caused by *Corynebacterium minutissimum* that is most common among patients with diabetes and among people living in warmer climates. The aim of the present study is to explore antibacterial activity of the plants *Cyperus rotundus* tuber, *Michelia champaca* flower bud and *Clerodendrum inerme* leaves against *Corynebacterium minutissimum* (ATCC 23348). The ethanol extract of the selected plants was subjected to preliminary phytochemical analysis and HPTLC analysis. The antibacterial effect of the ethanol extract of the plants was evaluated against *Corynebacterium minutissimum* using agar well diffusion, disc diffusion method. Minimum Inhibitory Concentration (MIC) of *Cyperus rotundus* tuber was determined using 96 well plate method. In disc diffusion method, the tested plants viz., *Cyperus rotundus*, *Clerodendrum inerme* and *Michelia champaca* at higher concentration (100µg) showed maximum zone of growth inhibition 12 mm, 10 mm and 11 mm respectively. In well diffusion method, the plants *Cyperus rotundus*, *Clerodendrum inerme* and *Michelia champaca* at higher concentration (200µg) showed maximum zone of growth inhibition 20 mm, 22 mm and 18 mm respectively against *Corynebacterium minutissimum* when compared with standard. The minimum inhibitory concentration of *Cyperus rotundus*, *Clerodendrum inerme* and *Michelia champaca* for arresting the growth of the tested organism was found to be 63 µg/ml. The phytochemical screening demonstrated the presence of different types of compounds like alkaloids, terpenoids, tannins, glycosides, saponins, phenolic compounds, proteins and flavonoids which may contribute for the antimicrobial action of the above medicinal plants and can be developed into safe and effective topical herbal formulation for the management of erythrasma.

Keywords: Antibacterial, *Clerodendrum inerme*, *Corynebacterium minutissimum*, *Cyperus rotundus*, Erythrasma and *Magnolia champaca*.

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INTRODUCTION

The normal human skin is colonized by huge numbers of bacteria that live harmlessly as commensals on its surface and within its follicles. At times, overgrowth of some of these resident organisms may cause minor disease of skin or its appendages. If the skin is damaged or the immune status of the subject is impaired, bacteria usually regarded nonpathogenic on body surface may assume the role of opportunist pathogens.[1] Erythrasma is a chronic superficial infection of the skin widely prevalent all over the world. The causative agent is an aerobic diphtheroid called *Corynebacterium* minutissimum.[2] *Corynebacterium minutissimum* is the bacteria that leads to cutaneous eruptions of erythrasma and is the most common cause of interdigital foot infections.[3] It is found mostly in occluded intertriginous areas such as the axillae, inframammary areas, interspaces of the toes, intergluteal and crural folds, and is more common in individuals with diabetes mellitus than other clinical patients.[4]

Plant medicines are used on a worldwide scale to prevent and treat infectious diseases. Plant based antimicrobial compounds have great therapeutic potential as they have lesser side effects as compared with synthetic drugs and also little chance of development of resistance.[5] Considering the vast potentiality of plants as sources for antimicrobial drugs with reference to antibacterial agents, a systematic investigation was undertaken to screen the local flora for antibacterial activity of *Cyperus rotundus* tuber, *Michelia champaca* flower bud and *Clerodendrum inerme* leaves for the treatment of erythrasma.

Cyperus rotundus L. (Cyperaceae) is considered one of the most widely distributed plant species in the world, especially in tropical and subtropical regions. In addition, it is commonly used in India, China and Japan in traditional medicine to treat different diseases, including dermatitis and other skin disorders. Ayurvedic doctors make use of nut grass preparations to treat numerous skin conditions such as itching and rashes, as well as to lighten skin and reduce aging effects.[6] The tuber of the plant *Cyperus rotundus* possess anti-diarrheal, antioxidant, anti-inflammatory, anti-mutagenic, antiperiodic, anticonvulsant, anti-saturative, antipyretic, antifungal, antidiabetic, antimalarial, antilipidemic, anti-bacterial, antiviral, anti-tumoral, cardio protective and wound- healing properties.[7] Flower buds of *Michelia champaca* Linn. belonging to the family is traditionally being used in fever, colic, leprosy, post partum protection and in eye disorders and has been reported to possess antipyretic, anti-inflammatory, insecticidal, antimicrobial and leishmanicidal activities.[8,9] *Clerodendron inerme* belongs to the *Verbenaceae* family are used for treating fever, cough, skin rashes and boil.[10] In Indian tribal medicine, leaves of *C. inerme* are used to treat umbilical cord infection and for cleaning the uterus in local medicine.[11] Existing topical antibiotic causes side effects such as rashes, dryness, itching, redness whereas oral antibiotics causes nausea, rash, diarrhea, loss of appetite and vomiting. As the plants are higher in active constituents, safer with less side effects and cost effective, the present study aimed to explore antibacterial activity of the plants *Cyperus rotundus* tuber, *Michelia champaca* flower bud and *Clerodendrum inerme* leaves against *Corynebacterium minutissimum*, a causative organism of a superficial skin disease erythrasma.

MATERIALS AND METHODS

Collection and Authentication

The plant specimen for the proposed study viz., tuber of *Cyperus rotundus* Linn, *Michelia champaca* flower bud and *Clerodendrum inerme* leaves were purchased from commercial source, Chennai, Tamil Nadu and authenticated by Dr. P. Jayaraman, Director, Plant Anatomy Research Center, (PARC) Tambaram and Chennai. Registration number of certificate PARC/2020/4211, PARC/2020/4212 and PARC/2020/4210 respectively.

Extraction

The dried tubers of *Cyperus rotundus, Michelia champaca* flower bud and *Clerodendrum inerme* leaves were taken and coarsely grinded separately. The powdered drugs were macerated with ethanol (100 ml/gm dry weight) for 72 hrs respectively. The yield was noted and its percentage was calculated.

Preliminary Chemical Analysis

The total ethanol extract of three plants were subjected to the chemical tests for the identification of phytochemical constituents as per the standard procedure.[12]

HPTLC Analysis

HPTLC method is a modern sophisticated and automated separation technique derived from TLC. Pre-coated HPTLC graded plates and auto sampler was used to achieve precision, sensitive, significant separation both qualitatively and quantitatively.

High performance thin layer chromatography (HPTLC) is a valuable quality assessment tool for the evaluation of botanical materials efficiently and cost effectively. HPTLC method offers high degree of selectivity, sensitivity and rapidity combined with single-step sample preparation. In addition, it is a reliable method for the quantization of nanograms level of samples. Thus this method can be conveniently adopted for routine quality control analysis. It provides chromatographic fingerprint of phytochemicals which is suitable for confirming the identity and purity of medicinal plant raw materials.[13]

HPTLC Profile

Extracts tested

Ethanolic extract of *Cyperus rotundus* tuber, *Michelia champaca* flower bud and *Clerodendrum inerme* leaves.

Sample application

The samples were dissolved in methanol and 10 µl quantity of sample was applied on the HPTLC Silica Merck 60F 254 graded plate sized 5cm x 10 cm as narrow bands using CAMAG Linomat V injector.

Chromatogram Development

It was carried out in CAMAG Twin Trough chambers. Sample elution was carried out according to the adsorption capability of the component to be analyzed. After elution, plates were taken out of the chamber and dried.

Scanning

Plates were scanned under UV at 254 nm using Camag TLC scanner 3. The data's obtained from scanning were interpreted using WINCATS-4 software. Chromatographic finger print was developed for the detection of phytoconstituents present in each extract and Rf values were tabulated. Mobile Phase Chloroform: Methanol (4:6)

Antimicrobial Activity

The test strain *Corynebacterium minutissimum* (ATCC 23348) was obtained from Himedia in a lyophilized form (Himedia, Mumbai, India). Obtained strain was recovered in a Brain heart infusion broth (Himedia, Mumbai, India).

Disc diffusion method

The antibacterial activity of the ethanolic extract of *Cyperus rotundus* tuber, *Michelia champaca* flower bud and *Clerodendrum inerme* leaves were tested against *Corynebacterium minutissimum* by disc diffusion methods in accordance with CLSI (2012). About 25 mL of molten Mueller Hinton Agar was poured into a sterile Petri plate (Himedia, Mumbai, India). The plates were allowed to solidify, after which 18 h grown (OD adjusted 0.6)100 µl of above said pathogenic bacteria cultures were transferred onto plate and made culture lawn by using sterile L-rod spreader. After five min setting of the bacteria, the test samples were dissolved in ethyl acetate at various concentrations (i.e. 25, 50, 75 and 100 µg/disc) impregnated onto the sterile disc (Himedia, Mumbai, India). The drug loaded discs were deposited onto the plate between 24 mm diameter distance. The solvent ethyl acetate loaded disc served as control. The plates were incubated at 37°C in a bacteriological incubator for 24h. The antibacterial activity was determined by measuring the diameter of the zone of inhibition around the well using antibiotic zone scale (Himedia, Mumbai, India).[14,15]

Well diffusion method

The antibacterial activity was determined by well diffusion methods.[16] About 25 mL of molten Mueller Hinton agar was poured into a sterile Petri plate (Himedia, Mumbai, India). The plates were allowed to solidify, after which 18 h grown (OD adjusted to0.6)100 µl of above said pathogenic bacteria were transferred onto plate and made culture lawn by using sterile L-rod spreader. After five min setting of the pathogenic microbes, a sterile cork borer was used to make 5 mm well on the agar. The test samples were loaded into wells with different concentration of 50 µg/well, 100 µg/well, 150 µg/ well, 200 µg/well. The plates were incubated at 37°C in a bacteriological incubator for 24 h. The antibacterial activity was determined by measuring the diameter of the zone of inhibition around the well using antibiotic zone scale (Himedia, Mumbai, India).

Microbroth dilution assay

Muller Hinton Broth for antibacterial screening was also tested using the 96-well microtiter plate with lid. The extract was prepared in a concentration twice the desired final concentration as it will be diluted with an equal amount of bacteria in broth. Briefly, 100 μL of the prepared extract in broth was introduced into the first wells in row A–C (in column 1). Column 2–10 in rows A–C had 100 μL of broth alone while rows A–C in column 11 had 100 μL of broth and 50 μL of broth was in A–C in column 12. Twofold serial dilutions using a micropipette was done systematically down the columns 1–10 (from rows A–C). 100 μL was removed from the starting concentrations (columns 1–10 in rows A-C) and transferred to the next column with the 100 μL broth, properly mixed, and the procedure was repeated up to the last column (10) where the last 100 μL was discarded. This brings the final volume in all the test wells with the extracts and the standard drugs to 100 μL except the 11th column which had 200 μL of the broth that served as sterility control. An equal volume (100 μL) of the 1×10^6 CFU/mL bacterial [*Corynebacterium minutissimum* (ATCC 23348)] inoculum was transferred into all the wells except the 11th column to give us the desired final inoculum load of 5×10^5 CFU/mL. Column 12 served as growth control (drug-free).[17]

The ethanol extract of *Cyperus rotundus* tuber with the concentrations ranged from 1000 µg/mL to 2 µg/mL and microtiter plate was incubated at 37°C for 18–20 hrs. The Minimum Inhibitory Concentrations was determined visually in the broth dilutions as the lowest concentrations of the extract at which no bacterial growth was visible on naked eyes.

RESULTS AND DISCUSSION

Extraction

The percentage yield values of ethanolic extract *of Cyperus rotundus, Michelia champaca* and *Clerodendrum inerme* were found to be 8.5%w/w, 12.5%w/w and 5.71%w/w respectively.

Qualitative Phytochemical Analysis

The qualitative phytochemical analysis of *Micheliachampaca, Cyperus rotundus* and *Clerodendrum inerme* revealed the presence of several secondary metabolites.

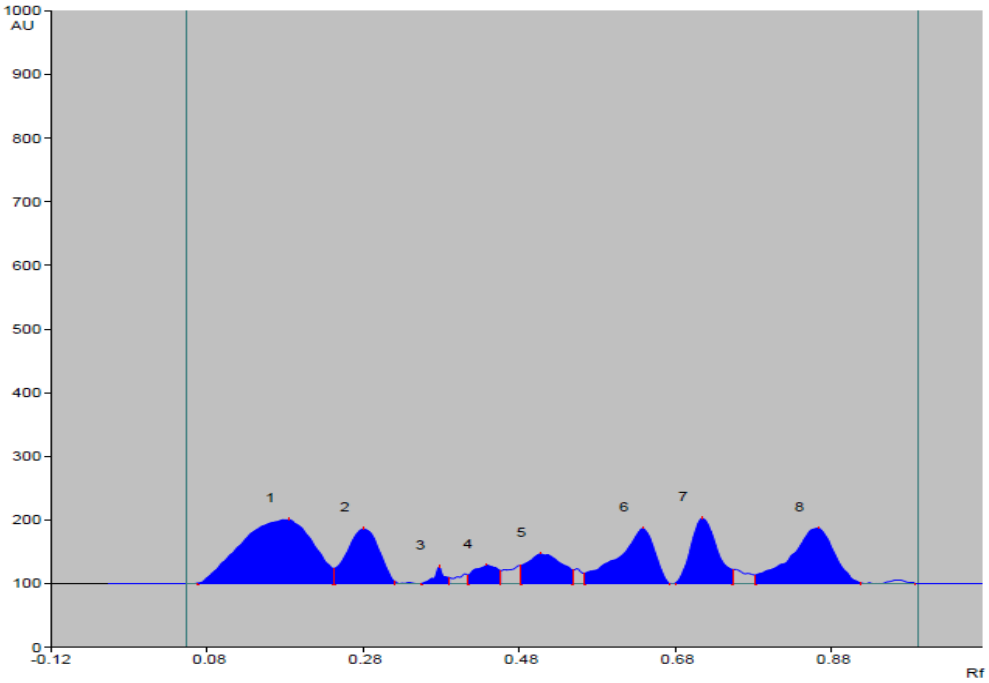
Preliminary phytochemical analysis of the ethanol extract of *Michelia champaca* flower indicates the presence of various secondary plant metabolites like phenols, tannins, flavonoids, terpenoids, steroids and glycoside, ethanol extract of *Cyperus rotundus* tuber indicates the presence of terpenoids, steroids, saponins, phenols, tannins, flavonoids, and protein and ethanol extract of *Clerodendrum inerme* leaves indicates the presence of alkaloids, steroids, flavonoids, flavones and anthraquinone glycoside.

High Performance Thin Layer Chromatography

The Rf value of the phytoconstituents of the ethanol extract of *Michelia champaca* flower, *Cyperus rotundus* tuber and *Clerodendrum inerme* leaves were shown in Table 1 and HPTLC chromatogram in Fig. 1-3.

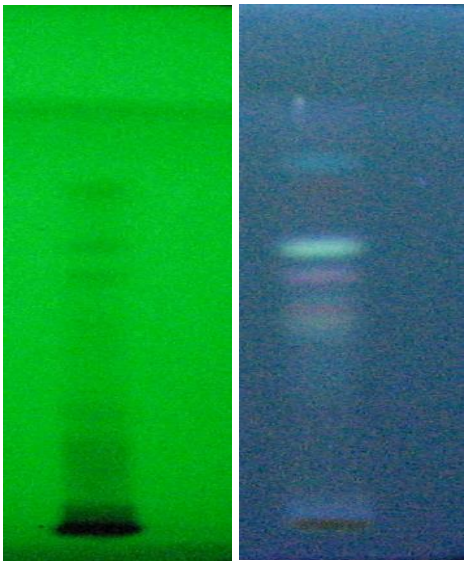
Table 1. Rf value of the Phytoconstituents of the ethanol extract of *Cyperus rotundus*, *Clerodendrum inerme* and *Michelia champaca*

Detecting Wavelength	*Cyperus rotundus* tuber		*Clerodendrum inerme* leaves		*Michelia champaca* flower	
	No. of Spots	Rf Value	No. of Spots	Rf Value	No. of Spots	Rf Value
254	8	0.19 0.28 0.38 0.44 0.51 0.64 0.72 0.87	7	0.42 0.50 0.57 0.61 0.65 0.72 0.86	6	0.30 0.40 0.57 0.69 0.76 0.86



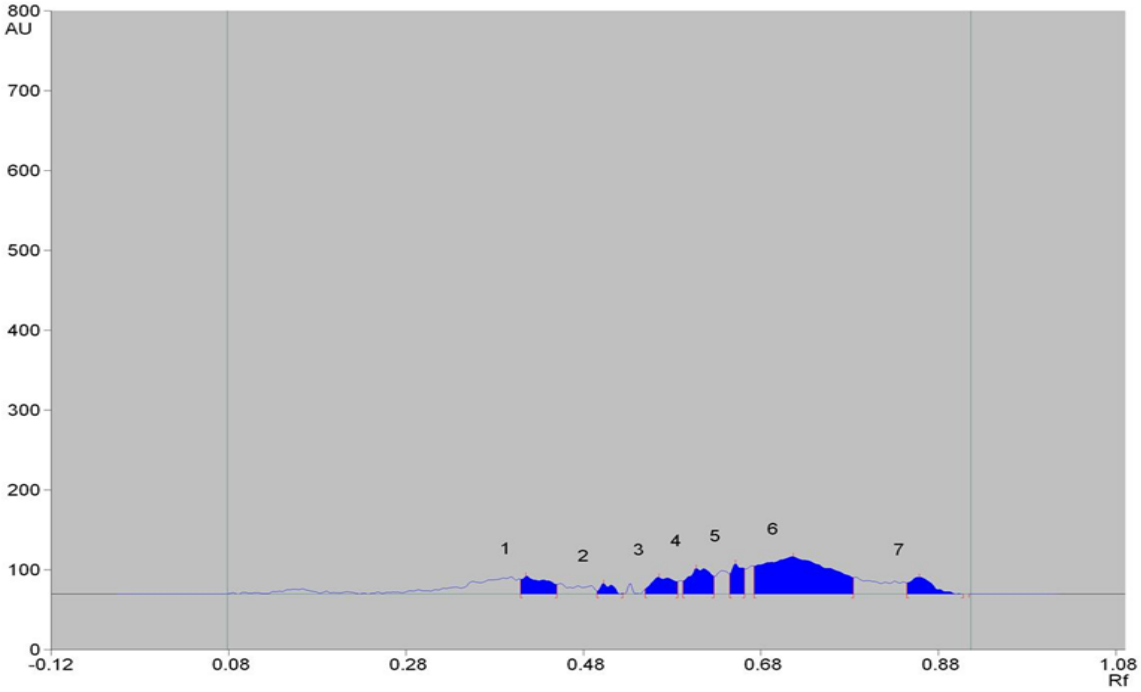
Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	0.07	0.9	0.19	100.5	17.83	0.24	23.9	7188.9	32.68	unknown *
2	0.24	24.2	0.28	86.0	15.27	0.32	3.5	2720.1	12.36	unknown *
3	0.36	0.4	0.38	26.0	4.62	0.39	9.4	247.4	1.12	unknown *
4	0.42	13.4	0.44	28.4	5.05	0.46	20.5	677.9	3.08	unknown *
5	0.48	28.5	0.51	46.6	8.27	0.55	21.1	1654.9	7.52	unknown *
6	0.57	16.4	0.64	86.5	15.36	0.68	0.4	3112.6	14.15	unknown *
7	0.68	0.6	0.72	102.2	18.14	0.76	22.5	2641.8	12.01	unknown *
8	0.79	13.6	0.87	87.1	15.45	0.92	1.2	3754.8	17.07	unknown *

Figure 1a: HPTLC Chromatogram of *Cyperus rotundus*tuber



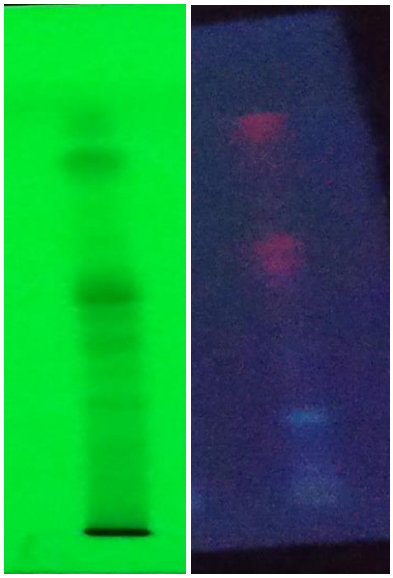
254 nm **366 nm**

Figure 1b: HPTLC fingerprint of *Cyperus rotundus*tuber



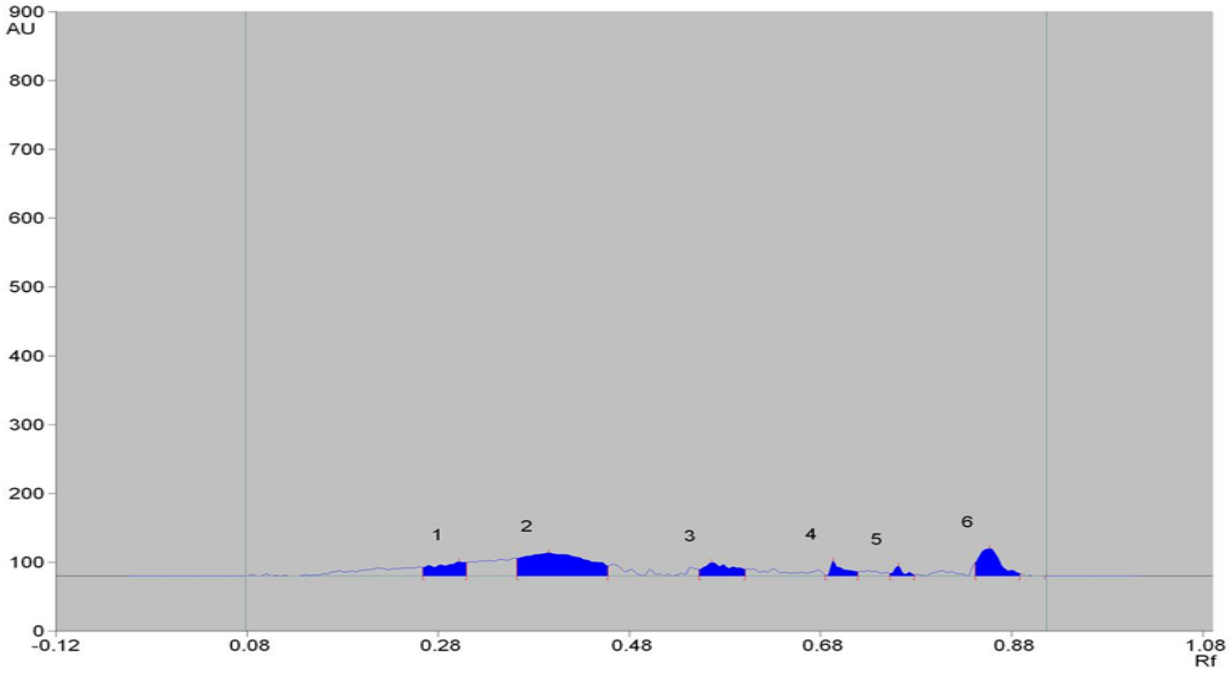
Peak	Start Position	Start Height	Max Position	Max Height	Max %	End Position	End Height	Area	Area %	Assigned substance
1	0.41 Rf	8.6 AU	0.42 Rf	12.3 AU	1.46 %	0.45 Rf	2.2 AU	487.7 AU	9.34 %	unknown *
2	0.50 Rf	3.5 AU	0.50 Rf	3.3 AU	6.81 %	0.52 Rf	0.8 AU	140.9 AU	2.70 %	unknown *
3	0.55 Rf	6.4 AU	0.57 Rf	10.8 AU	0.69 %	0.59 Rf	5.7 AU	413.3 AU	7.91 %	unknown *
4	0.59 Rf	6.1 AU	0.61 Rf	12.4 AU	6.65 %	0.63 Rf	1.8 AU	623.5 AU	1.94 %	unknown *
5	0.65 Rf	14.9 AU	0.65 Rf	18.0 AU	9.53 %	0.66 Rf	1.9 AU	392.6 AU	7.51 %	unknown *
6	0.67 Rf	15.0 AU	0.72 Rf	16.8 AU	14.05 %	0.78 Rf	0.7 AU	749.0 AU	12.62 %	unknown *
7	0.85 Rf	4.0 AU	0.86 Rf	11.0 AU	0.80 %	0.91 Rf	0.1 AU	416.9 AU	7.98 %	unknown *

Figure 2a: HPTLC Chromatogram of *Clerodendrum inerme* leaves



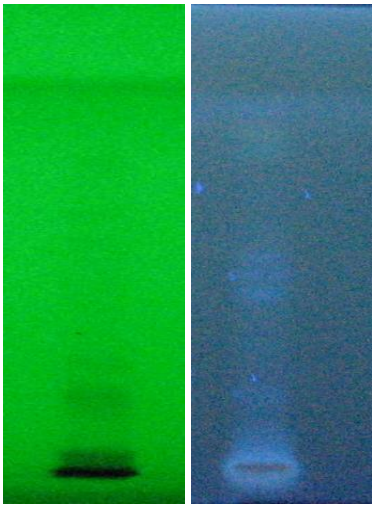
254 nm **366 nm**

Figure 2b: HPTLC Fingerprint of *Clerodendrum inerme* leaves



Peak	Start Position	Start Height	Max Position	Max Height	Max %	End Position	End Height	Area	Area %	Assigned substance
1	0.26 Rf	2.5 AU	0.30 Rf	10.8 AU	3.91 %	0.31 Rf	9.6 AU	504.6 AU	3.49 %	unknown *
2	0.36 Rf	15.6 AU	0.40 Rf	63.7 AU	12.50 %	0.46 Rf	4.5 AU	743.4 AU	16.63 %	unknown *
3	0.55 Rf	9.6 AU	0.57 Rf	9.4 AU	2.95 %	0.60 Rf	0.0 AU	453.5 AU	2.13 %	unknown *
4	0.68 Rf	2.4 AU	0.69 Rf	21.9 AU	4.61 %	0.72 Rf	3.2 AU	227.7 AU	6.09 %	unknown *
5	0.75 Rf	3.6 AU	0.76 Rf	4.3 AU	9.54 %	0.78 Rf	1.6 AU	112.3 AU	3.00 %	unknown *
6	0.84 Rf	9.1 AU	0.86 Rf	39.7 AU	16.50 %	0.89 Rf	3.8 AU	697.5 AU	8.65 %	unknown *

Figure 3a: HPTLC Chromatogram of *Michelia champaca* flower



254 nm **366 nm**

Figure 3b: HPTLC Finger print of *Michelia champaca* flower

The construction of chromatographic fingerprints plays an important role in the quality control of complex herbal medicines. Chemical fingerprints obtained by chromatographic techniques are strongly recommended for the purpose of quality control of herbal medicines, since they might represent appropriately the "chemical integrities" of the herbal medicines and therefore be used for authentication and identification of the herbal products. HPTLC was scanned at 254 nm with the best solvent to detect the maximum number of components and peak abundance qualitatively.

The HPTLC chromatogram of ethanol extract of *Cyperus rotundus* tuber showed 8 spots in the solvent system with the Rf values ranges from 0.19 to 0.87, ethanol extract of *Clerodendrum inerme* leaves showed 7 spots in the solvent system with the Rf values ranges from 0.42 to 0.86and ethanol extract of *Michelia champaca* flower showed 6 spots in the solvent system with the Rf values ranges from 0.30 to 0.86. HPTLC fingerprint is one of the versatile tools for qualitative and quantitative analysis of active constituents. It is also a diagnostic method to find out the adulterants and to check the purity.

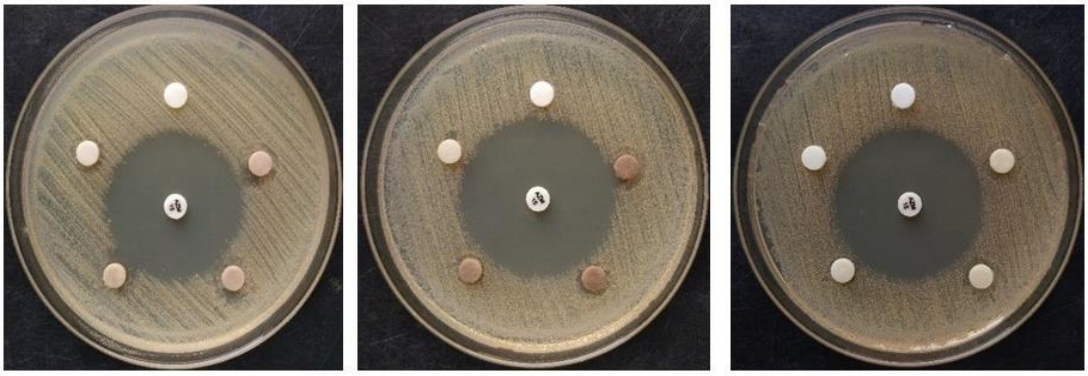
Antimicrobial activity

Disc Diffusion Method

The antibacterial activity of the plant extracts studied was compared with standard antibiotics. The antibacterial activity of the flower, leaf and tuber samples were tested against *Corynebacterium minutissimum*. In the study, the plant extracts *Michelia champaca* flower, *Cyperus rotundus* tuber and *Clerodendrum inerme* leaves were demonstrated to inhibit the growth of all tested bacteria at highest concentration (100 µg) in disc diffusion method (Table 2). The zone of inhibition of *Michelia champaca* flower bud, *Cyperus rotundus* tuber and *Clerodendrum inerme* leaves was 11 mm, 12 mm and 10 mm at the concentration of 100µg in disc diffusion method. It was observed that, tuber extract showed high activity followed by flower and leaf samples (Fig. 4).

Table 2: The antibacterial activity of the flower, leaf and tuber samples against *Corynebacterium minutissimum* by Disc Diffusion Method

Name of the sample	**Antibacterial activity (Zone of Inhibition in mm)**				
Concentration	25 µg	50 µg	75 µg	100 µg	15 µg (AZM)
Cyperus rotundus	**8**	**9**	**10**	**12**	**30**
Clerodendruminerme	**-**	**6**	**8**	**10**	**30**
Micheliachampaca	**-**	**7**	**9**	**11**	**30**



Tuber Extract **Leaves Extract** **Flower Extract**

a: 0 µg/disc; b: 25µg/disc; c: 50 µg/disc; d: 75µg/disc; e: 100 µg/disc;

AZM (Azithromycin): 15µg/disc

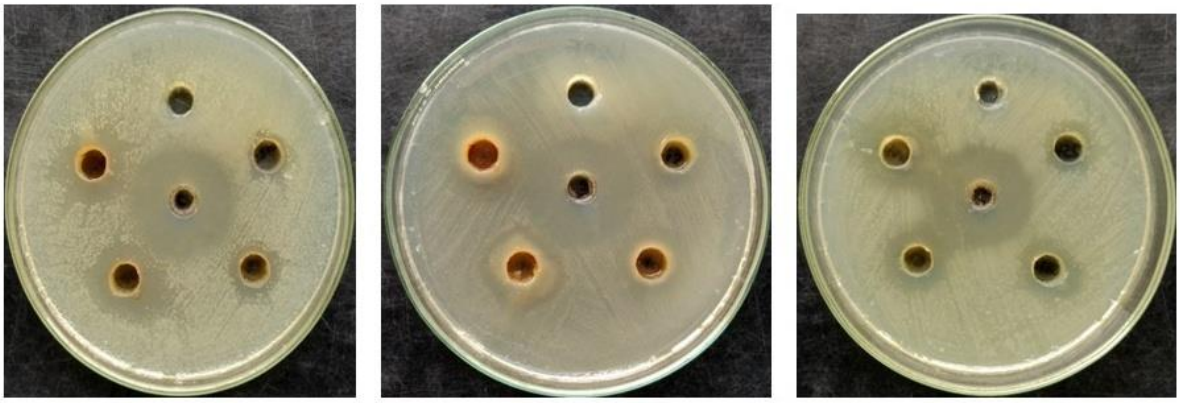
Figure 4: Antibacterial activity of *Cyperus rotundus, Clerodendrum inerme* and *Michelia champaca* against *Corynebacterium minutissimum* by Disc Diffusion Method

Well Diffusion Method

The antibacterial activity of the test samples showed an antibacterial activity towards the tested bacterial strain (Table 3). In the study, the plant extracts *Michelia champaca* flower, *Cyperus rotundus* tuber and *Clerodendrum inerme* leaves were demonstrated to inhibit the growth of all tested bacteria at highest concentration (200 µg) in agar - well diffusion method, The zone of inhibition of *Michelia champaca* flower bud, *Cyperus rotundus* tuber and *Clerodendrum inerme* leaves was 18 mm, 22 mm and 20 mm at the concentration of 200µg in well diffusion method. Among the sample tested, tuber extracts showed highest activity followed by leaves extract towards the tested bacteria (Fig.5).

Table 3: Antibacterial activity of test samples against *Corynebacterium minutissimum* by well diffusion method

Name of the sample	**Antibacterial activity (Zone of Inhibition in mm)**				
Concentration	50 µg	100 µg	150 µg	200 µg	Standard 35 µg (AZM)
Cyperus rotundus	11	15	18	20	27
Clerodendruminerme	14	16	19	22	28
Micheliachampaca	11	14	17	18	27



Tuber Extract **Leaves Extract** **Flower Extract**

a: 0 µg/disc; b: 25µg/disc; c: 50 µg/disc; d: 75µg/disc; e: 100 µg/disc;

AZM (Azithromycin): 15µg/disc

Figure 5: antibacterial activity of *Cyperus rotundus, Clerodendrum inerme* and *Michelia champaca* against *Corynebacterium minutissimum* by Well Diffusion Method

Minimum inhibitory concentration

The minimum inhibitory concentration of tuber extract against *Corynebacterium minutissimum* (ATCC 23348)was tested on microbroth dilution method in a 96 well microtiter plate clearly revealed that there is no growth at the column 1-5 in all the rows A-C contained 1000-125 µg/mL (Fig. 6). There was visible growth can be seen from column 6-10 and 12 (Table 4). Hence, the lowest concentration that inhibited the tested bacteria was found to be 63 µg/mL.

Table 4: Minimum Inhibitory Concentration of test samples against *Corynebacterium minutissimum*

	Concentrations at µg/mL											
Rows	C1	C2	C3	C4	**C5**	C6	C7	C8	C9	C10	C11 (control)	C12 (Drug control)
Cyperus rotundus	1000	500	250	125	**63**	31	16	8	4	2	0	0
Clerodendrum inerme	1000	500	250	125	**63**	31	16	8	4	2	0	0
Michelia champaca	1000	500	250	125	**63**	31	16	8	4	2	0	0

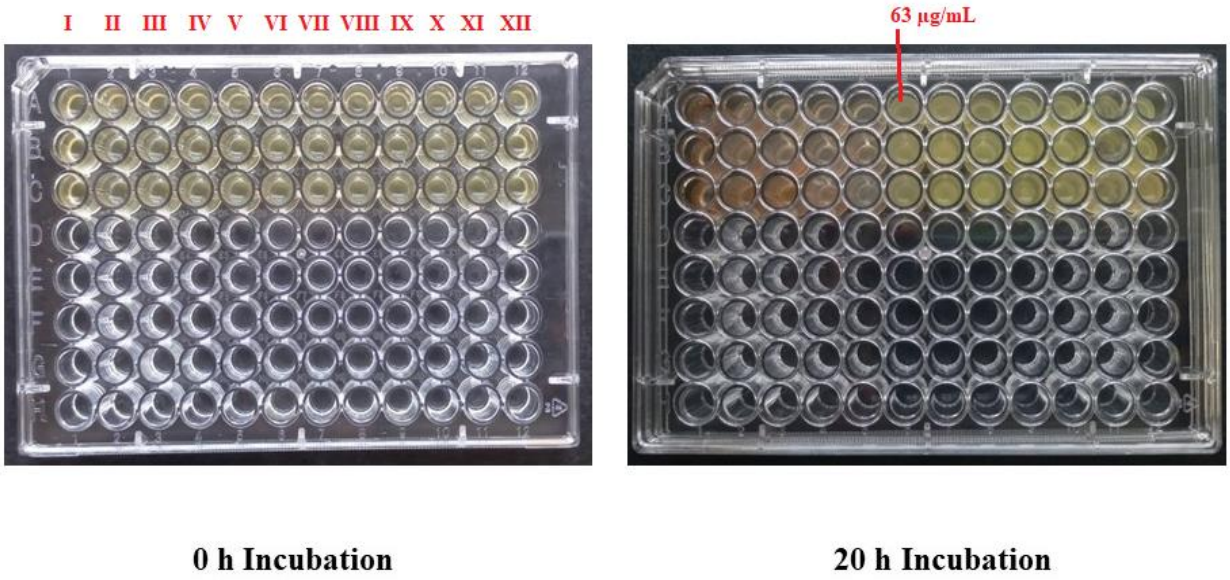


Figure 6: Microbroth dilution assay of *Cyperus rotundus, Clerodendrum inerme* and *Micheli achampaca* tuber for MIC determination against *Corynebacterium minutissimum* (ATCC 23348)

CONCLUSION

Among the tested plants, *Cyperus rotundus* tuber *and Clerodendrum inerme* leaves displayed a potent antibacterial activity with maximum zone of growth inhibition when compared with standard. The study thus reveals the effectiveness of the tested plant extracts against *Corynebacterium minutissimum*, the causative organism of erythrasma. Thus, the significant activity against *Corynebacterium minutissimum* may be due to their phytochemical or secondary metabolites. Further studies are needed to identify the biologically active compounds and to evaluate the efficiency of the compound against *Corynebacterium minutissimum* associated with erythrasma. These plants can be used as potential source for the development of a phytomedicine to combat the skin disease erythrasma caused by bacterial infections.

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