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Antifungal activity of *Ventilago maderspatana* on *Trichophyton rubrum* - An in vitro study

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ABSTRACT

The aim of the study was to contribute a single drug for specific dermatophyte species to encourage diagnosis specific treatment. The study had two arms one clinical part consisting of cross-sectional study of 80 participants and other part *invitro* study. The survey was conducted to know the prevalence of dermatophyte species among cutaneous dermatophytosis patients in Kottakkal population there by collecting the skin scrapings of the patients. As the second part of the study these skin scrapings were cultured in sabouraud dextrose agar (SDA) and the species were inoculated from their and identified. Thus from 80 participants 2-genus and 6 different species were obtained. Trichophyton and microsporum were the genus and among Trichophyton 4 species like T.rubrum, T.mentagrophytes, T.tonsurans and T.verrucossum and Microsporum genus consists of M.canis and Microsporumaudouinii. First MIC was calculated and after that antifungal activity was done through the agar well diffusion method using *ventilago maderaspattana* extracts against fluconazole. Three different extracts were used chloroform, methanol and ethanol. In this article the action of easily available low polarity chloroform extract against the most prevalent species T. rubrum is highlighted because it shows the highest mean zone of inhibition in agar well diffusion method.

Key words: *Cutaneous dermatophytosis, T. rubrum, Ventilago maderaspattana, agar well diffusion.*

INTRODUCTION

In the present scenario environmental pollution is at a peak rate and due to ozone depletion, global warming is increasing. This increased heat and humid climate give rise to the emergence of many fungal infections.

Superficial fungal infections are among the most common diseases seen in our daily practices. These infections affect outer layer of skin, hair, and nails. In contrary to many of the other infections affecting the other organ systems in humans, the fungi may cause dermatological condition that do not include tissue invasion. However the habit of some of the fungi is on skin surface that is liable to environmental contamination.[1],[2]

Mainly there are three genera of fungi like dermatophytes, Moulds and Yeast. The most common cause of superficial fungal infections is dermatophytes. By convention Dermatophytes are a group of fungi that grow on the substrate of keratin. Cutaneous means something related to skin. *Dineśavallītvakcūrṇa* which is mentioned in the *Kīṭaviṣaprakaraṇa* of *Kriyākaumudi* for chronic itchy round lesions was tested for the antifungal activity against fluconazole.[3]

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MATERIALS AND METHODS

Inclusion criteria

- KOH mount positive
- Age group 16-60 years, both sexes irrespective of caste, religion and economic status.

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- Those who are willing to give consent.

Skin scraping examination for fungal Dermatophytes was carried out for confirmation of diagnosis. All the patients were subjected to this examination and registered, if found to be positive for the presence of fungal mycelium or pseudo mycelium.

Skin scraping examination

This was carried out in Toxicological lab, Department of Agad Tantra, VPSV Ayurveda College, Kottakkal. In this procedure, the skin of the patients from affected area was scraped with a blunt scalpel (no: 20). Scales were placed on center of microscope slide, swept in to a small pile, and covered with a cover slip. Capillary action draws solution under cover slip. The preparation was gently heated with a match or lighter until bubbles begin to expand, clarifying the preparation. Excess KOH solution was blotted out. Then stained with Lactophenol cotton blue, slide was then examined by direct microscopy for the presence of fungal hyphae.[4]

Exclusion criteria

- Other cutaneous lesions associated with Dermatophytosis
- Patients undergoing other systemic and topical application
- Immuno-compromised patients
- Vulnerable group
- Bleeding disorders

Research techniques and tools

A Case record form was made to record the details of the case. Consent form in Malayalam language was prepared and prior consent of all the participants were obtained on the consent form. A pamphlet containing the details of the research was given to the participants. The whole plan of study was approved by Institutional Ethics Committee (IEC) prior to starting of work (IEC NO:-IEC/CL/02/17 Dated on 27/04/2017) and an interim report on the status of research was also got approved in due course.

Clinical study

The method used is cross-sectional survey

Experimental study

Scrapings will be taken from each sample and mounted with KOH and followed by Lactophenol cotton blue staining to identify the microorganism

- Culture of fungi will be done using SDA agar
- Identification done using the microscopic features
- Phytochemical screening and HPTLC will be done for *Dineśavallitvakcūrṇa extract*
- Antifungal activity against identified species by well diffusion method
- MIC determination by serial agar dilution plate technique

KOH Mounting

In the case of mycological diagnostic approach there are two diagnostic categories traditional methods and advanced molecular diagnostic tools. Traditional methods like specimen direct microscopy and fungal culture medium are used for the study.[4]

Specific direct microscopy - Direct microscopic observation of fungi in clinical samples is obviously a cheap and short-time diagnostic method. Clinical specimen must be prepared by scalpel, moving to a clean glass slide with a drop of 10%-20% KOH. Mild heat may help to increase the lytic activity of KOH on fungal α-(1,3) and α-(1,4) cross linkages in cell wall glucan polymers to have a clear and transparent vision of Dermatophyte fungal elements including filamentous, septate, and branched hyphae with or without conidia among the specimen. Providing suitable samples is an important part of direct microscopy. Accuracy in isolation of scale from peripheral border of suspected lesion, obtaining infected hair shafts or hair follicles, scraping from infected nails are the primary procedures for preparing valuable samples to have a successful observation to confirm the presence or absence of Dermatophyte fungi. According to previous studies,

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sensitivity and the specificity of direct KOH microscopy are ~65% and >45%.

Fungal culturing using SDA Agar

It is the most direct and conclusive methods for diagnosing the fungal infections. Samples were collected and processed through centrifugation, softening or liquidisation method. Common media used is SDA Agar. [5]

SDA Agar comprised of enzymatic digest of casein and animal tissues. It provides a nutritious source of amino acids and nitrogenous compounds for the growth of fungi and yeasts.

Table 1: Composition of media

Ingredients	Gms/liter
Mycological peptone	10
Dextrose	40
Agar	15
pH adjusted to 5.6 at 25°C	

Procedure of preparation of media

1. Suspend 65gm of media in 1 liter of distilled water.
2. Heat with frequent agitation and boiled for 1 minute to completely dissolve the medium.
3. Autoclave at 121°C for 15 minutes
4. Cool to 45-50°C and pour in to petridishes[5]

Process of culturing

After confirming that skin scrapings are positive for fungus using KOH mounting and staining with lacto phenol cotton blue the remaining scrapings are spreaded on petridishes containing media. After 3 weeks fungal growth were obtained. For processing of specimens, streak the specimen on to the medium with a sterile inoculating loop in order to obtain isolated colonies. Incubate the plates at 25-30˚ in an inverted position (agar side up) with increased humidity.[6]

Identification of genus and species

Based on growth characters rapidity

1. Rapidity of growth
2. Color
3. Morphology of the colony

Morphology studied in teased mounts or slide cultures

1. Hyphae diameter
2. Presence /absence of septa
3. Conidia morphology – diagnostic importance

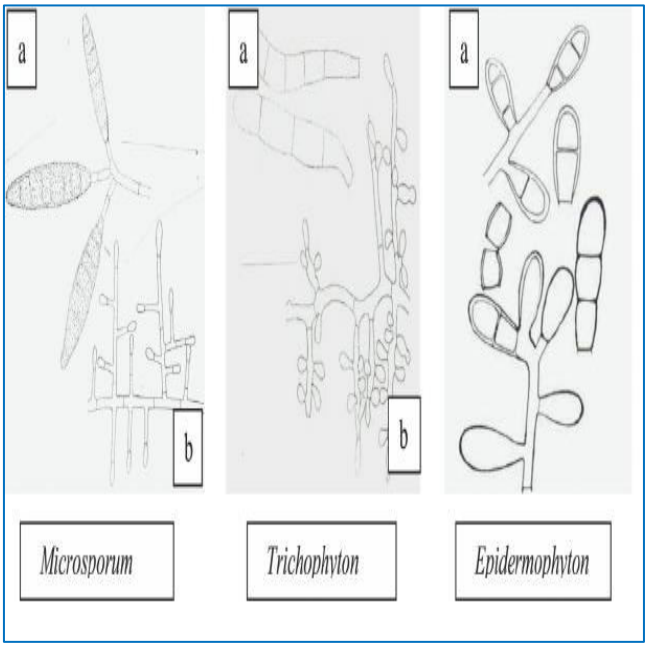

Fig. 1: Picture of three genera of dermatophyte species[7]

In lesions Dermatophytes appear as hyphae and arthrospores. In cultures of sabouraud's agar, they form characteristic colonies consisting of septate hyphae and two types of asexual spores, micro conidia and macro conidia. Differentiation in to the three genera is based mainly on the nature of macro conidia.

Trichophyton

Colonies may be powdery, velvety or waxy, with pigmentation characteristic of different species. Microconidia are abundant and are arranged in clusters along the hyphae or borne on conidiophores.

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Macroconidia are relatively scanty. They are generally elongated, with blunt ends. Macroconidia have distinctive shapes in different species and are of importance in species identification. Some species possess special hyphal characters, such as spiral hyphae, racquet mycelium and favic chandeliers. Trichophyton infect skin, hair and nails. *Trichophyton rubrum* is the most common species infecting human beings. It often causes chronic, treatment resistant lesions. In *Trichopyton mentagrophytes* colony will be seen as white to tan colored and its texture will be like cottony or powdery appearance and its morphology is cigar shaped rat tail end. Another species is *Trichophyton tonsurans* in this type colony will be like cream or yellow colored with central furrows and irregular macro conidia with thick wall is seen. In *Trichophyton schoeleinii* colony will be smooth, waxy, and brownish the morphology will be seen like hyphalswelling, chlamydospores, favic chandelier. In *Trichophyton violeceum* very slow growing. Waxy, violet to purple pigment colony will be there and distorted hyphae and rare conidia are found.[8]

Serial agar dilution technique

Commonly used technique to determine the minimal concentration of antimicrobial agents that kill or inhibit the growth of microorganisms. In the agar dilution method, different concentrations of antimicrobial agent, by direct addition in to the diluted agar medium. After the required time period the growth at different concentrations streaked against the agar plate and kept incubated. Note the time period for attaining the colony growth and find out at which concentration the growth had inhibited, that concentration is taken as the MIC- minimal inhibitory concentration.[9]

Agar well diffusion method

It was adopted for the antifungal study. Firstly the agar plate surface is inoculated by spreading a volume of fungal growth inoculum over the entire agar surface. Then a hole with a diameter of 6-8mm is punched aseptically with a sterile cork borer and a volume of 100µL of the extract solution at desired concentration is introduced into the well. Then agar plates are incubated under suitable conditions depending upon the test microorganism. The antifungal agent diffuses in the agar medium and inhibits the growth of the microbial strain tested.[10]

Results and Discussion

Table 2: Results on clinical study

Species identified	Present study (%)	Previous study (%)
TRR	31.25	27.13
TRM	22.5	38.75
TRT	8.75	6.9
TRV	3.75	-
MA	3.75	-
MC	1.75	-

Table 3: Minimum inhibitory concentration of *Dinesavalli Tvakchurna* against the most prevalent species *Trichophyton rubrum*.

Organism	Drug	chloroform extract	Methanol extract	Ethanol extract
Trichophyton rubrum	*Dinesavalli Tvakchurna*	60µg	240µg	240µg

Table 4: The mean inhibition zone of various doses of CHL extract of VM against TRR with std. fluconazole.

TRR CHL	N	Mean	Std. Deviation	Std. Error
480mcg	3	3.00	0.00	0.00
240mcg	3	6.66	1.15	0.66
120mcg	3	7.33	0.57	0.33
60mcg	3	11.33	0.57	0.33
30mcg	3	11.33	0.57	0.33
Std	3	3.00	0.00	0.00
Total	18	7.11	3.54	0.83

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The highest mean inhibition was shown by 60mcg and 30mcg with SD±0.57 while the std. only showed 3mm inhibition.

Table 5: Group wise comparison of CHL extract of VM against TRR with std. Fluconazole using one way ANOVA

TRR CHL	Sum of squares	Df.	Mean square	F	Sig.
Between groups	209.11	5	41.82	107.54	0.000
Within groups	4.66	12	0.38		
Total	213.77	17			

The statistical test single factor ANOVA showed significance at p<0.001.

Table 6: Multiple comparison of CHL extract of VM against TRR with std. Fluconazole using Post-Hoc test

Groups (I)	Groups (J)	Mean difference (I-J)	Std. Error	Sig.
480mcg	Std	0.00	0.50	1.00
240mcg	Std	3.66	0.50	0.00
120mcg	Std	4.33	0.50	0.00
60mcg	Std	8.33	0.50	0.00
30mcg	Std	8.33	0.50	0.00

On Post-Hoc comparison test the different doses except 480mcg showed significance at p<0.001 and the 480mcg dose was found to be insignificant at p>0.05.

Table 7: The mean inhibition zone of various doses of METH extract of VM against TRR with std. fluconazole.

TRR METH	N	Mean	Std. Deviation	Std. Error
480mcg	3	3.25	0.25	0.14
240mcg	3	3.09	0.08	0.04
120mcg	3	3.16	0.20	0.12
60mcg	3	3.05	0.04	0.02
30mcg	3	3.14	0.12	0.07
Std	3	3.05	0.06	0.03
Total	18	3.12	0.14	0.03

All the concentrations and std. showed lowest inhibition zone that is around 3mm inhibition.

Table 8: Group wise comparison of METH extract of VM against TRR with std. Fluconazole using one way ANOVA

TRR METH	Sum of squares	Df.	Mean square	F	Sig.
Between groups	0.08	5	0.01	0.74	0.605
Within groups	0.26	12	0.02		
Total	0.35	17			

All the concentrations and std. lowest inhibition zone that is around 3mm inhibition.

Table 9: Multiple comparison of METH extract of VM against TRR with std. Fluconazole using Post- Hoc test

Groups (I)	Groups (J)	Mean difference (I-J)	Std. Error	Sig.
480mcg	Std	0.19	0.12	0.41
240mcg	Std	0.04	0.12	0.99
120mcg	Sd	0.11	0.12	0.83
60mcg	Std	0.00	0.12	0.10
30mcg	Std	0.08	0.12	0.92

Multiple comparison by Post-Hoc test showed that all the concentrations of drug and the std. showed insignificance at p>0.05

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Table 10: The mean inhibition zone of various doses of ETH extract of VM against TRR with std. fluconazole.

TRR ETH	N	Mean	Std. Deviation	Std. Error
480mcg	3	4.66	0.57	0.33
240mcg	3	3.00	0.00	0.00
120mcg	3	3.00	0.00	0.00
60mcg	3	3.00	0.00	0.00
30mcg	3	5.00	0.00	0.00
Std	3	3.00	0.00	0.00
Total	18	3.61	0.91	0.21

The group comparison showed significance with highest mean inhibition 4.6 with SD±0.57at p<0.001

Table 11: Group wise comparison of ETH extract of VM against TRR with std. Fluconazole using one way ANOVA

TRR ETH	Sum of squares	Df.	Mean square	F	Sig.
Between groups	13.6	5	2.72	49.0	0.000
Within groups	0.66	12	0.05		
Total	14.2	17			

On single factor ANOVA shows significance at p<0.001

Table 12: Multiple comparison of ETH extract of VM against TRR with std. Fluconazole using Post- Hoc test.

Groups (I)	Groups (J)	Mean difference (I-J)	Std. Error	Sig.
480mcg	Std	1.66	0.19	0.000
240mcg	Std	0.00	0.19	1.00
120mcg	Std	0.00	0.19	1.00
60mcg	Std	0.00	0.19	1.00
30mcg	std	2.00	0.19	0.00

The multiple comparison test Post-Hoc showed significance at the level p<0.001 for the concentrations 480mcg and 30mcg but all other doses showed insignificance at p>0.05.

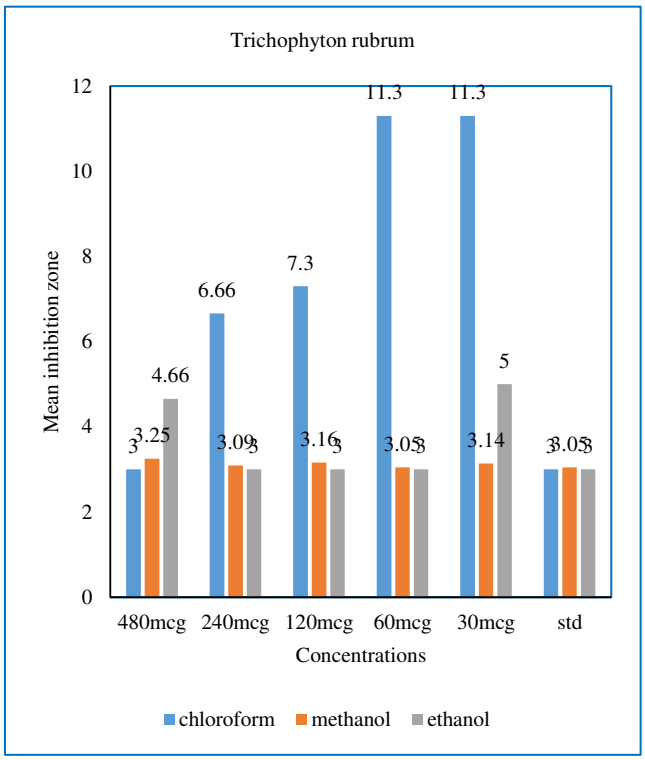

Fig. 2: Graph showing the comparison effects of CHL, METH and ETH extracts of VM against TRR with std. Fluconazole.

CONCLUSION

The *Tvakchurna* extract of *Dinesavalli* against the most prevalent dermatophyte species with standard fluconazole showed significance for both chloroform and ethanol extract. First the MIC was determined using serial agar dilution technique. Among chloroform extracts the doses 60mcg and 30mcg showed a better result at 0.001 level of high significance. Among methanol extracts all the concentrations and the standard showed only minimum inhibition zone and statistically it was found to be insignificant. Among ethanol extracts 480mcg showed a better result than standard and other concentrations a little more inhibition than minimum inhibition at 0.001 level of high significance.

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