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REGULAR ARTICLE

A COMPARATIVE NOVEL METHOD OF ANTIFUNGAL SUSCEPTIBILITY FOR *MALASSEZIA FURFUR* AND MODIFICATION OF CULTURE MEDIUM BY ADDING LIPID SUPPLEMENT

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SUMMARY

Introduction of new antifungal compounds has increased the demand for method of *in vitro* testing. The present study has proposed a new method of studying antifungal activity of different compounds by using 96 well plates. The infection by different species of *Malassezia* is quite common in tropical country like India. *Malassezia* being a eukaryote, the treatments against it may also adversely affect the patient. Hence, as an alternative, cheap, affordable, ecofriendly, botanicals may be used. In this background, a comparative study on the efficacy of synthetic ingredients (on the basis of their performance in the market) and botanicals was carried out *in-vitro* against *Malassezia furfur* (MTCC 1765).Evaluation of Minimum Inhibitory Concentrations (MICs) of two standard antifungal drugs (Ketoconazole and Fluconazole) available in the market against *Malassezia furfur* and their comparison with botanicals was done using broth microdilution method recommended by Clinical Laboratory Standard Institute (CLSI) with slight modifications. The present work is also an attempt to standardized culture medium for growing *Malassezia* species by overlay of sterile cotton seed oil with principal medium. Cotton seed oil can be used as supplement with principal medium for best growth of *Malassezia*.

Keywords: Botanicals, *Malassezia furfur,* Minimum Inhibitory Concentrations, Synthetics

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1. Introduction

The genus *Malassezia* includes 10 anthropophilic and obligatory lipophilic species (*M. globosa*, *M. restricta*, *M. slooffiae*, *M. obtusa*, *M. furfur*, *M. sympodialis*, *M. japonica*, *M. yamatoensis*, *M. dermatis*, *M. nana*) and 3 zoophilic species *M. pachydermatis*, *M. caprae*, *M. equine*(1). *Malassezia* species have been associated with a number of diseases of human skin, such as pityriasis versicolor, seborrheic dermatitis, dandruff, folliculitis, atopic dermatitis, and psoriasis etc. (2).

Pityriasis versicolor is mild chronic superficial infection of the stratum corneum characterized by patchy and scaly discoloration of the skin.

Seborrhea, dandruff and seborrheic dermatitis are three closely related terms. Seborrhea literally means a state of oiliness of skin. The skin is shiny and oily looking, particularly on the face. Dandruff (also called *Pityriasis capitis*) means scaliness of the scalp skin without signs of inflammation. Dandruff is so common that it can be considered physiological. It represents desquamation of the skin surface, due to separation of layers of stratum corneum, which is a continuous process, in the form of scales. The status of dandruff being amphibious a disease/disorder, and relatively less medical intervention is sought after for the treatment but dandruff is the most commercially exploited skin and scalp disorder/disease by personal care industries(3).

Malassezia formerly called *Pityrosporum* is a yeast causing infection of skin and scalp (4).

The infection of the scalp clinically represents as Dandruff (5). Dandruff is occurring in at least 40-50% and Seborrheic dermatitis (SD) 1-3% of the general population (6, 7).The vast majority of recent data support a direct causal link between *Malassezia* and D/SD. The factors that support are, firstly antifungal treatment are found most effective in treating disease and secondly, an improvement SD/D is accompanied by a reduction in *Malassezia* levels on the scalp (1,8-13). Warm and humid atmosphere, overcrowding and poor personal hygiene are ideally suited for the growth of *Malassezia* (14*).* Magnitude of problem is presumed to be high in Allahabad, India due to warm and moist weather.

Although a variety of antifungal agents are available in the market for the treatment of dandruff, seborrheic dermatitis, pityriasis versicolor, folliculitis, atopic dermatitis, but complete control is far from reach. Most of the available drugs are expensive and have side effects. The treatments that are available have certain limitations, either due to poor efficacies or due to compliance issue.

India being a developing country is losing much of its foreign exchange in buying formulations and readymade drugs from other developed countries. Patients uncomfortable or unsatisfied with synthetic pills and prescriptions may consider turning to herbal remedies extracted from plants, roots, seeds and fruits. Herbal remedies have been used throughout human history. Recent medical evidences support and clearly define the benefits of herbal medicine. Many modern prescriptions contain herbal extracts or a synthesized equivalent. India's rich natural resources and knowledge of traditional medicine have important role in modern health care system (15,16). The Indian System of Medicine has identified 1,500 medicinal plants, of which 500 are commonly used. The main objective is to use medicinal plants against common prevalent diseases.

2. Materials and Methods

Collection and maintenance of the culture

Pure culture of *Malassezia furfur* (MTCC-1765) was obtained from Institute of Microbial technology, Chandigarh, India. The Sabouraud-Dextrose Agar (SDA) medium and Dutta and Dikshit Modified culture medium were selected and prepared for sub culturing of *Malassezia* (17*).*

These strains were routinely cultured on Sabouraud Dextrose Agar (SDA) slants and modified culture medium at 35^0 C and the pH was adjusted to 5.8 prior to autoclaving at 15 lbs, 121^{o}C for 15 min. All the cultures were maintained at 34 ± 2^{o}C in incubator for four days. *In vitro* investigation of *Malassezia furfur* by culturing (medium + oil as lipid source) was carried out in Biological Product Lab, Department of Botany, and University of Allahabad for standardization of medium. Coconut oil, Cotton seed oil, Til oil and Olive oil used as carbon source. Morphological and physiological structure on the basis of literature as well as microscopic examination of colony was also done.

Collection of plant and extraction of essential oil

Plants were selected on the basis of their ethno-medicinal importance and literature surveyed from various libraries in Allahabad, NBRI, Lucknow and internet sites. The selected plants were identified with the help of flora (Hooker, 1872-1892; Bailey 1958; Srivastava, 1976 and Singh, 1989) and authentic herbarium of Botanical Survey of India, Allahabad and the *Duthie* Herbarium, Department of Botany, University of Allahabad.

Micromeria biflora Benth. collected from Kinnor, Himanchal Pradesh during botanical excursion tour was selected for the antifungal testing, identified by Botanical survey of India (BSI) Allahabad, and extraction of oil was done using Clevenger type apparatus(18). *Syzygium aromaticum* oil was extracted and on the basis of GC-MS analysis found that major component was Eugenol and it was taken for antifungal susceptibility testing. Eugenol was purchased from Central drug house (P) LTD, New Delhi (Batch No 01077, Product No.028374).

Antifungal susceptibility testing

For determination of Minimum Inhibition Concentrations (MICs), media and their components were prepared as per

recommendation of NCCLS; 2002(19) {currently known as CLSI} with slight modification

- Inoculum = $0.5x10^3$-$2.5 x10^3$ CFU/ml
- Medium = RPMI 1640
- pH = 7
- MOPS = 0.165M
- Temp = 35°C
- Duration of incubation = 72H

In 96 well plates four drugs were taken, each have 2 replicate Fluconazole, Ketoconazole, Eugenol and *Micromeria biflora* Benth essential oil respectively. All dilutions were prepared in DMSO (Dimethyl sulfoxide), the final concentration of DMSO per inoculum was as described by the CLSI. The antimalassezia activity of drugs was determined by broth microdilution method recommended by Clinical Laboratory Standards Institute (CLSI) using BPL Modified medium Broth. Culture was maintained on BPL modified medium and Modified Lemming Notman agar medium (MLNA). The 96-well microtiter plates were used for twofold serial dilution. 20 µl of stock sol. of sample drugs was added into 4th well of microtiter plate horizontally having 180µl RPMI 1640 medium. So the maximum concentration of the sample drug was 2.5mg/ml. From here the solution was serially diluted up to 4th well to 11th well resulting into the half of the concentration of the test essential oil.

The yeast inoculum was prepared at 0.5 McFarland standards; the absorbance was equal to the inoculum suspension containing $1x10^6$. Then standard yeast inoculum was added and kept for incubation at 32°C in a moist chamber. The Minimum Inhibitory Concentration (MIC) and Inhibitory Concentration at 50% (IC_{50}) was recorded spectrophotometrically at 530 nm using SpectraMaxplus^{384} after 72 hrs incubation.

For synthetic drug, stock solution was made 1mg/ml and for natural drug, stock solution was made 40mg/ml. In the case of natural 40mg oil/active constituents have been taken and dissolved in 960 µl DMSO and for synthetic 1mg dissolved in 999 µl DMSO.

Fig.1. 96 well plate format for drug testing by serial dilution method.AB drug -1,CD drug-2 EF drug-3,GH drug 4

The MIC end-points for each antifungal agent were defined as the first concentration where spectrophotometrically 80% or more reduction was measured.

3. Results

The recorded results are presented in the form of Tables 1 and 2, Fig. 1, 2, 3 and 4. *Malassezia furfur* (MTCC 1765) developed as white to cream colour and smooth pasty colony on modified medium (as shown in fig.-3). Excellent growth was reported in modified culture medium. Growth of *Malassezia* on the basis of different carbon sources (cotton seed oil, coconut oil, til oil, olive oil) was also observed and was found best growth on cotton seed oil supplemented with principal medium. Based on morphological characters the purified colonies were identified. *Malassezia* is able to exist both in yeast and mycelial forms. The fungus is dimorphic, occurring as a saprophytic yeast form and a parasitic mycelial form. Yeast is the prime form isolated *in vitro* from the culture media. Yeast like conidia are predominant structures.

Malassezia colonies grow rapidly and mature in five days at 30-37˚C. It undergoes asexual reproduction by enteroblastic budding from a characteristic broad base. The daughter cell separate by fission, leaving a bud scar or collarette through which daughter cell emerge. These cells are globose to ellipsoidal in shape. Sexual spores do not exist.

MICs of Fluconazole, Ketoconazole, Eugenol and *Micromeria biflora* essential oil against *Malassezia furfur* were found 10.538 mg/ml, 6.438 mg /ml, 6.956 mg/ml, and 8.928 mg/ml respectively.

Table-1 MICs of Fluconazole, Ketoconazole, Eugenol and *Micromeria* essential oil against *Malassezia furfur* MTCC-1765

Minimum Inhibitory Concentration				
STRAIN	Fluconazole	Ketoconazole	Eugenol	*Micromeria*
MTCC-1765	10.538 mg/ml	6.438 mg /ml	6.956 mg/ml	8.928 mg/ml

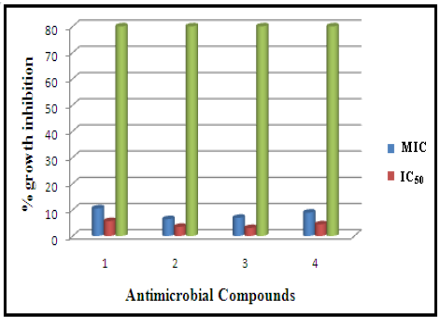


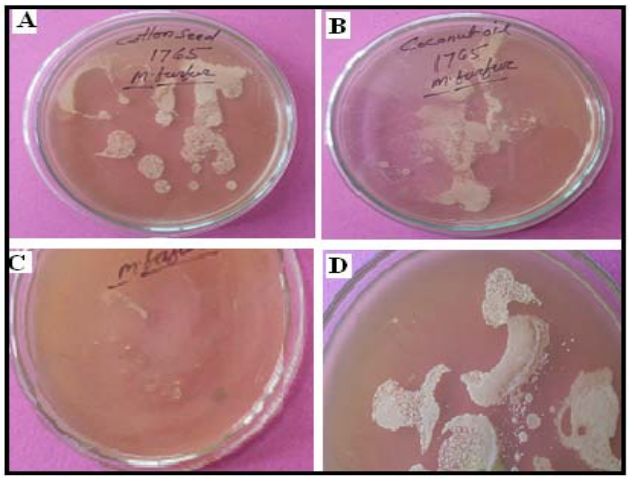
Fig.-2 Graphical representation of antimicrobial activity of candidate compounds against *Malassezia furfur* MTCC-1765

Table -2 Table show IC-50 of four drugs

IC 50 Determinations of four Drugs				
STRAIN	Fluconazole	Ketoconazole	Eugenol	*Micromeria*
MTCC-1765	5.689 mg/ml	3.515 mg /ml	3.044 mg/ml	4.466 mg/ml

Fig. 3: Growth of *Malassezia furfur* (MTCC, 1765) on Dutta and Dikshit Modified Culture Medium Dorsal and ventral view of colony

Fig. 4: Growth of *Malassezia furfur* on Dutta and Dikshit modified culture medium with different Lipid supplement (A) Cotton seed oil (B) Coconut oil (C) Til oil (D) Cotton seed oil .Excellent growth reported on principal medium supplemented with cotton seed oil



4. Discussion

The method used is economical, accurate, rapid, cheaper and comparative for four drugs, one organism and vise-versa in single plate (Fig.-1). The modified susceptibility method can be also used for unicellular yeast and other *Malassezia* species.

One study reported from India, showed MIC of Ketoconazole 2.5 µg/ml, Fluconazole 2.5 µg/ml, Clove oil 1000 µg/ml, Coleus oil 25 mg/ml, and Basil oil 10 mg/ml was effective against *Malassezia furfur* by disc diffusion method (20). Another study done by Miranda *et al.*,in which MIC ranges were <0.03-4 microg/ml for Ketoconazole and <0.125 to >64 microgram/ml for Fluconazole against *Malassezia* (21*)*.In our current study, Eugenol and *Micromeria biflora* oil recorded a very good activity among the herbal ingredients. MIC of Eugenol is very close to Ketoconazole which is a popular synthetic antifungal.

The three major groups of drugs in clinical use are polyenes, azoles & pyrimidines. With the exception of 5-FC, the azoles and polyene antifungal drugs in common usage are directed in same way against Ergosterol, the major sterol in fungal plasma membrane. Ergosterol in fungal membrane contributes to a variety of cellular functions. It is important for the fluidity & integrity of the membrane and for the proper function of membrane bound enzymes including chitin synthetase, which is important for proper cell growth and division. For azole drugs mode of action several lines of evidences suggest that the primary target of azoles is heme protein which co-catalyses cytochrome P450 14α-demethymation of lanosterol. Inhibition of 14α-demethylase leads to depletion of Ergosterol and accumulation of sterol precursors, including 14α-methylated sterols resulting in the formation of a plasma membrane with altered structure and function. However, a significant proportion of patients have experienced azole treatment failure due to the development of drug resistance in *Malassezia* species.

Ketoconazole is an imidazole derivative, broad spectrum antimycotic agent that is active against *Pityrosporum ovale* and is effective against many fungi both *in-vivo* and *in-vitro* (22, 23). It is also effective in many dermatomycoses, including pityriasis versicolor (24).

However, in very severe cases of dandruff, Ketoconazole based shampoos are preferred despite their relatively higher costs.

Herbal ingredients like tea tree oil, rosemary oil, coleus oil, clove oil, pepper extract, neem extract, and basil extract also recorded anti-pityrosporum activity, but their MIC are much higher than the synthetic ingredients.

Synthetic drugs have several side effects and resistance to antifungal drugs .The synthetic antifungals are very popular and drug of choice due to their easy availability, but the reports of increasing number of side effects on the patients, cannot be ignored in anyway. It is estimated that there are over 7800 medicinal drug manufacturing unit in India, which are estimated to consume about 2000 tons of herbs annually (25). According to a recent estimate of World Health Organization (WHO), 60-80% of the world population especially in developing countries relies on traditional medicine or plant based drugs for their primary health care needs (26, 27). The main objective is to use medicinal plants against popular prevalent diseases. In tropical countries like India, fungal infections are of common occurrence. Identifying yeast as the causative agent creates the potential for being able to control this condition with antifungal agents. Dandruff and seborrheic dermatitis patients may require regular, long-term use of therapeutic agents. It is important that the treatments be formulated so as to be aesthetically and cosmetically acceptable to the patient. As such, the study was done mainly to discover the potential active ingredients from the selected plants active against the test pathogen. Eugenol has highest potential as antimalassezia activity and *Micromeria biflora* essential oil also possess significant activity against *Malassezia* causing dandruff, seborrheic dermatitis, pityriasis versicolor, folliculitis, and atopic dermatitis.

One of the important achievement of the present work is standardization of culture medium for growing *Malassezia* species by adding Cotton seed oil as lipid source in medium composition. Hence, it is suggested that modified medium can be further modified by supplementing cotton seed oil. Use of Cotton seed oil for culturing *Malassezia* is a new report and Cotton seed oil can be used as lipid source for excellent growth of *Malassezia*. This work is also important due to the first report of antimalassezia activity of Eugenol and *Micromeria biflora* essential oil. These ingredients can be exploited for its antimalssezia activity individually or in combination.

Eugenol and *Micromeria biflora* essential oil can be used as antifungal drugs against *Malassezia* spp. causing Dandruff, Seborrrheic Dermatitis, and Pityriasis versicolor in Humans. After undergoing detailed bimolecular characterization, formulation of dosages, clinical studies, safety and efficacy is in progress.

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