ORIGINAL ARTICLE

Susceptibility pattern of *Malassezia* species to selected plant extracts and antifungal agents

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Objective: *Malassezia* is associated with dandruff, seborrhoeic dermatitis, pityriasis versicolor folliculitis and atopic eczema. This study determined the susceptibility pattern of *Malassezia furfur, M. globosa, M. obtusa, M. restricta, M. slooffiae* and *M. sympodialis* isolated from patients diagnosed with dandruff against plant extracts and antifungal agents. **Materials and Methods:** Twenty aqueous plant extracts and five azole drugs were tested against the isolates by well diffusion and broth dilution method. **Results:** Among the plant extracts, *Phyllanthus emblica* (fruits), *Hibiscus rosa sinensis* (flowers) and *Acacia concinna* (pods) have demonstrated significant antidandruff activity. Minimum inhibitory concentration values revealed that ketoconazole as the most effective drug followed by itraconazole. **Conclusion:** *M. furfur* and *M. globosa* were found as the most susceptible organisms against the aqueous extracts of *Phyllanthus emblica* (fruits), *Hibiscus rosa sinensis* (flowers), *Acacia concinna* (pods) and azole drugs.

Key words: Antidandruff, antifungal, dandruff, *Malassezia*

INTRODUCTION

Dandruff, an easily recognizable skin flaking and pruritic scalp condition occurs in humans[1,2] is associated with *Malassezia* species. It is characterized by abnormal flaking of the scalp related to mild inflammatory reaction.[3] In severe cases, dandruff leads to mild scale formation to seborrhoeic dermatitis.[4] *Malassezia* species have been associated with pityriasis versicolor, seborrhoeic dermatitis, dandruff, folliculitis, atopic eczema.[5-8] Fourteen species of *Malassezia* were reported and 13 are lipid dependent.[9] Lipophilic Malassezia is a common mycoflora of human skin, especially the upper sebaceous parts such as hair which has high sebum excretion.[10,11] The absence of fatty acid synthase gene in lipid dependent *Malassezia* species (*M. globosa*) is complemented by multiple lipases to utilize host lipids.[12] These lipases hydrolyse triglycerides and fatty acids which induces scaling and inflammation.[13,14]

Treatment of microbial diseases with available drugs is restricted due to microbial resistance, compliance and cost.[15] The emergence of fungal strains resistant to the available antifungal agents demonstrates the need of new antifungal agents to overcome problems caused by *Malassezia*.[16] Most of the natural drugs are not associated with side effects when compared to commonly used synthetic drugs. Plant based drugs with better antifungal activities are known to produce less or negligible undesirable effects than chemotherapeutic agents. This study aimed at determining the effectiveness of selected aqueous plant extracts as an alternate for the available antifungal agents to treat dandruff. A total of 10 plants namely *Phyllanthus emblica, Aegle marmelos, Ricinus communis, Lawsonia inermis, Hibiscus rosa-sinensis, Trigonella foenum-graecum, Azadirachta indica, Sapindus mukorossi, Acacia concinna* and *Murraya koenigii* were selected based on their ethnomedicinal importance to determine the anti-dandruff activity. Further, it compares the susceptibility pattern of *Malassezia* species to the selected plant extracts and antifungal agents.

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MATERIALS AND METHODS

Selection Criteria

A total of 100 patients from both sexes aged between 15-30 years who were diagnosed as suffering from dandruff were selected in this study.

Collection and Identification of Specimen

Sampling from people was done by scraping the lesions from head using sterile scalpel and tape method after the institutional research ethics committee approval. Direct microscopy with 20% KOH and methylene blue was performed and the samples were inoculated on modified Dixon agar. After incubation at 37°C for

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4-5 days, isolated colonies were identified by catalase reaction, tween assimilation and esculin hydrolysis.[17,18]

Plant Extraction

A total of 10 plants were collected from their natural habitat in Bangalore in October 2013 and identified by a botanist from Bangalore University, India. A voucher specimen (IADC 10/13) has been deposited at the herbarium of Indian Academy Degree College, Bangalore, India. Aqueous plant extracts were prepared by dissolving pulverized different parts of plant materials in sterile distilled water (1:5 w/v) and extracted for 24 hrs at 25°C and 4°C. The extracts were filtered, concentrated in rotary vacuum evaporator and the stock solutions were prepared in sterile distilled water for anti-dandruff assay.

In Vitro Susceptibility Testing

Aqueous extracts of different plant parts in the concentration range between 256-16 µg/ml were prepared and used to determine the anti-dandruff activity against various *Malassezia* species. Final concentrations of antifungal drugs ranged between 16-0.125 µg/ml for ketoconazole, 32-0.25 µg/ml for miconazole, 16-0.125 µg/ml for clotrimazole and 16-0.125 µg/ml for itraconazole were prepared in 100% dimethyl sulfoxide (DMSO). 32-0.25 µg/ml for fluconazole was prepared in distilled water. Stock inoculum suspensions were prepared from 7-days-old colonies developed on modified Dixon agar at 37°C. The fungal inocula suspension prepared as per 0.5 McFarland standards (corresponding to a CFU of 1.5×10^7 cells/ml) for performing the susceptibility testing by disc diffusion method. Growth and sterility control wells were also prepared for each isolate tested. Standardized inoculum was used as positive control. After 5 days of incubation at 37°C, the plates were observed for the growth of each strain at various concentrations of plant extracts and drugs. Zone diameters were measured at the point where the growth significantly decreased and were recorded to the nearest millimeter. Minimum inhibitory concentrations were recorded following the M27-A3 protocol.[19] The lowest concentration of extract and or drug that inhibits the growth of test organism in broth dilution susceptibility test was determined as MIC.

Data Analysis

Zone of inhibition values of plant extracts and antifungal drugs for different *Malassezia* species were compared by repeated measure and one-way analysis of variance (ANOVA) using SPSS version 16 software. P values < 0.05 were considered statistically significant.

RESULTS

Among the 100 patients selected, 62 were males and 38 were females. Of 100 samples tested, 78 were reported 10% KOH positive. Biochemical characterization of positive samples revealed 35 isolates were *M. globosa* (44.8%), 22 were *M. sympodialis* (28.2%), 16 were *M. furfur* (20.5%), three were *M. obtusa* (3.8%), one each of *M. restricta* and *M. slooffiae* (1.3%) [Table 1 and Figure 1].

Statistical analysis of *in vitro* susceptibility testing using plant extracts and antifungal agents revealed significant differences in susceptibility pattern among the isolates [Table 2]. Fruits of *P. emblica* were exhibited significant inhibitory activity (21.7 mm) than the leaves (13.9 mm). Highest inhibition was observed with *M. furfur* (23.7 mm) followed by *M. sympodialis* (22.8 mm). Lowest inhibition was recorded with *M. slooffiae*. Mean value of 13.9 mm was observed with *P. emblica* leaves. Aqueous extract of *A. marmelos* has significant mean activity (12.33 mm) against the isolates than the leaves (10.25 mm) with *M. furfur* as the most sensitive organism (13.5 mm). Leaves and seeds of *R. communis* had no significant difference and were less effective against the most of the isolates. *L. inermis* leaves were effective against *M. furfur* (18.6 mm) and exhibited mean inhibition value of 17.8 mm against the isolates whereas 15.45 mm inhibition was recorded with the seeds. Flowers of *H. rosa sinensis* were effective against *M. sympodialis* and a mean inhibition of 18.45 mm and 20.95 mm was observed for leaves and flowers respectively. A mean inhibition value of 13.48 mm and 11.4 mm were recorded for *T. foenum* seeds and leaf extracts. Leaves of *A. indica* were more effective (17.78 mm) than fruits (12.43 mm) with *M. furfur*

Table 1: Species identification of *Malassezia*

Organism	Catalase reaction	Tween 20	Tween 40	Tween 60	Tween 80
Malassezia furfur	+	+	+	+	+
M. globosa	+	–	–	–	–
M. obtusa	+	–	–	–	–
M. restricta	+	–	–	–	–
M. slooffiae	+	+	+	+	–
M. sympodialis	+	–	+	+	+

+ – Positive; – – Negative

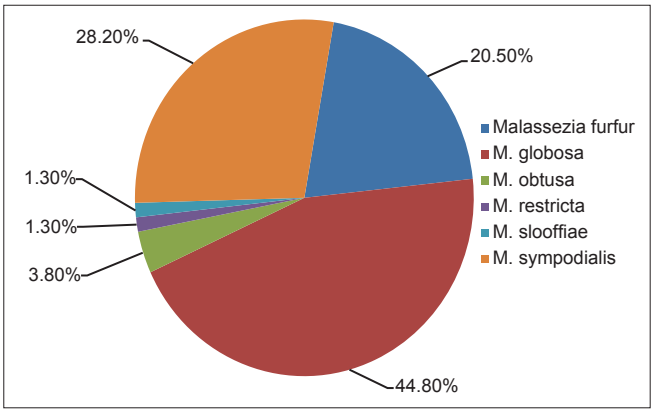

Figure 1: Distribution of *Malassezia* species

Table 2: Antimalassezial activity of plant extracts and antifungal agents

Plant	Parts	*M. furfur* (16)	*M. globosa* (35)	*M. obtusa* (3)	*M. restricta* (1)	*M. slooffiae* (1)	*M. sympodialis* (22)	*M. furfur* MTCC 1374
Phyllanthus emblica L.	Leaves	13.5±0.2	14.1±0.3	16.6±0.8	14.5±0.6	13.8±0.1	11.4±0.6	10.8±0.1
	Fruits	23.7±0.1	22.1±0.7	20.1±0.4	22.6±0.4	19.3±0.6	22.8±0.1	18.7±0.3
Aegle marmelos (L.) CORREA EX. SCHULTZ	Leaves	11.1±0.4	8.9±0.1	10.2±0.6	9.6±0.2	10.3±0.7	11.4±0.1	8.2±0.5
	Fruits	13.5±0.7	11.0±0.4	12.8±0.3	12.1±0.2	11.5±0.3	13.1±0.2	11.3±0.6
Ricinus communis L.	Leaves	9.0±0.2	7.7±0.6	10.1±0.4	8.3±0.5	8.8±0.1	9.3±0.7	7.3±0.2
	Seeds	9.1±0.6	9.0±0.0	10.5±0.2	8.8±0.5	10.5±0.0	9.6±0.1	7.9±0.4
Lawsonia inermis L.	Leaves	18.6±0.5	17.3±0.2	17.2±0.7	18.1±0.3	17.6±0.1	18.0±0.9	15.2±0.1
	Seeds	16.1±0.8	16.8±0.4	15.8±0.0	14.3±0.2	15.1±0.9	14.6±0.7	14.0±0.8
Hibiscus rosa-sinensis L.	Leaves	19.1±0.4	19.9±0.8	18.7±0.3	18.0±0.1	17.2±0.1	17.8±0.8	16.5±0.7
	Flowers	20.5±0.1	21.1±0.3	21.7±0.7	19.6±0.3	20.8±0.4	22.0±0.2	20.1±0.2
Trigonella foenum-graecum L.	Leaves	12.8±0.3	10.7±0.8	11.1±0.2	10.8±0.3	12.1±0.6	11.3±0.8	10.6±0.2
	Seeds	14.6±0.7	12.1±0.3	12.6±0.2	13.7±0.3	14.0±0.4	13.9±0.1	11.3±0.4
Azadirachta indica A. JUSS	Leaves	17.1±0.0	16.2±0.4	18.0±0.8	18.7±0.2	17.5±0.3	19.2±0.9	16.7±0.1
	Fruits	13.2±0.7	11.9±0.4	12.2±0.9	11.8±0.3	13.1±0.0	12.4±0.7	10.8±0.8
Sapindus mukorossi GAERTN.	Leaves	8.7±0.6	8.0±0.3	9.1±0.4	8.3±0.0	9.2±0.4	9.7±0.3	8.2±0.1
	Nut shells	10.8±0.2	11.6±0.5	10.1±0.9	9.6±0.3	9.0±0.5	10.7±0.7	9.3±0.9
Acacia concinna (WILLD.) DC.	Leaves	15.1±0.5	18.5±0.2	16.5±0.1	16.7±0.9	17.4±0.4	15.9±0.0	15.8±0.5
	Pods	22.9±0.8	24.0±0.0	23.3±0.2	21.6±0.2	22.3±0.6	23.0±0.7	20.1±0.7
Murraya koenigii (L.) SPR.	Leaves	11.8±0.7	10.5±0.3	10.1±0.0	9.7±0.5	11.1±0.0	10.3±0.1	9.0±0.5
	Seeds	9.3±0.7	9.1±0.4	8.9±0.4	9.9±0.2	9.0±0.1	8.3±0.2	8.1±0.8
Fluconazole		24.1±0.3	24.6±0.2	23.3±0.8	23.8±0.3	24.2±0.7	23.1±0.9	20.3±0.2
Ketoconazole		27.4±0.7	26.9±0.3	27.1±0.2	27.0±0.0	26.5±0.5	26.7±0.2	21.3±0.0
Miconazole		23.3±0.0	23.9±0.3	22.5±0.7	22.8±0.4	23.1±0.0	23.6±0.1	19.2±0.0
Clotrimazole		22.8±0.5	23.0±0.6	22.7±0.4	23.2±0.1	22.2±0.6	22.6±0.4	19.5±0.4
Itraconazole		21.9±0.6	22.3±0.4	22.1±0.6	21.6±0.5	23.0±0.1	23.6±0.1	21.0±0.7

MTCC – Microbial Type Culture Collection; SPR – SPRENGEL

as the most sensitive organism (17.1 mm) among the isolates. Leaves of *S. mukorossi* have recorded the lowest inhibitory activity (8.83 mm) among the extracts tested. Pods of *A. concinna* have exhibited maximum mean inhibitory activity (22.85 mm) and were effective against all the isolates throughout the study. *M. koenigii* leaves exhibited moderate activity (10.58 mm) against the *Malassezia* species and a mean inhibition zone of 9.08 mm was recorded by the seed extracts. Various susceptibility pattern were exhibited by the isolates with ketoconazole recorded highest anti-*Malassezial* activity against *M. furfur* (27.4 mm), *M. obtusa* (27.2 mm) and *M. restricta* (27 mm) with a mean zone diameter inhibition of 26.9 mm. Fluconazole had significant activity against *M. globosa, M. slooffiae* and *M. furfur* and exhibited mean value of 23.85 mm. Miconazole was effective against *M. globosa, M. sympodialis* and *M. furfur* with a mean inhibition value of 23.5 mm. Clotrimazole was effective against *M. restricta* (23 mm) and *M. globosa* (23.2 mm). Itraconazole was effective against *M. sympodialis* (23.6 mm) and *M. slooffiae* (23 mm).

Among the 78 isolates tested, *M. furfur* was found as the most sensitive organism against the seven of the total 20 extracts tested. Ketoconazole was the effective drug (27.4 mm) and *P. emblica* fruits had significant inhibitory activity (23.7 mm) against *M. furfur*. Fluconazole and miconazole were effective against *M. globosa* with 24.6 m mm and 23.9 mm of inhibition. Five of the twenty plant extracts were exhibited significant activity against *M. globosa* with *A. concinna* pods (24 mm) recorded maximum inhibition. Leaves of *P. emblica* had an inhibitory action of 16.6 mm against *M. obtusa* and the other effective extract was *R. communis*. *M. koenigii* seed extract was effective against *M. restricta* (9.9 mm) and clotrimazole had highest activity (23.2 mm) against the isolate. *M. slooffiae* was tolerant to most of the plant extracts and antifungal agents in the study. *M. sympodialis* was sensitive to *A. marmelos, H. rosa sinensis, T. foenum graecum* and *S. mukorossi*. The antifungal agent itraconazole has highest inhibitory action (23.6 mm) on *M. sympodialis* than the other isolates.

Minimum inhibitory concentrations (MIC) of plant extracts and antifungal agents were determined by broth dilution method. *P. emblica* fruits exhibited a MIC value of 32 µg/ml against *M. furfur* and *A. concinna* showed 32 µg/ml as MIC value against *M. globosa*. *H. rosa sinsensis* had an MIC value of 64 µg/ml against *M. sympodialis*. *L. inermis* was having an MIC value of 64 µg/ml against *M. globosa* and *A. indica* recorded MIC value in the range of 64 µg/ml against *M. sympodialis*. MIC of antifungal agents revealed that 1 µg/ml of fluconazole for *M. globosa* and *M. sloofiae*, 0.25 µg/ml of ketoconazole for *M. furfur*, 1 µg/ml for *M. obtusa*

and *M. slooffiae*, 2 µg/ml of miconazole for *M. globosa and M. sympodialis*, 1 µg/ml of clotrimazole for *M. restricta* and *M. globosa*, and 0.5 µg/ml of itraconazole for *M. sympodialis* and *M. slooffiae*.

DISCUSSION

This study was focused on people in age between 15-30 years as the increased production of sebum at puberty[20] triggers *Malassezia* proliferation which leads to dandruff. A total of 78 *Malassezia* isolates were identified by morphological, catalase reaction and tween assimilation properties. Lipid composition of skin, presence of competitive microbiota and immune nature of individuals influences the distribution of *Malassezia* species.[21,22] Six species of *Malassezia* namely *M. furfur, M. globosa, M. obtusa, M. restricta, M. slooffiae* and *M. sympodialis* were identified in this study irrespective of gender and age. *M. globosa, M. sympodialis* and *M. furfur* were found as predominant population on people having dandruff in this study where as Gemmer *et al*.,[23] reported *M. globosa* and *M. restricta* as most common fungi on scalp. Clavaud *et al*.[24] and Paulino *et al*.,[25] has observed *M. restricta* as the major species in their study.

Faergeman *et al*.,[26] has determined the *in vitro* antidandruff activity of ketoconazole against *Malassezia* species. Among the antifungal agents tested, ketoconazole was found as most effective drug against the six *Malassezia* species tested revealing *M. furfur, M. obtusa* and *M. restricta* as highly sensitive for ketoconazole. Sancak *et al*.,[27] and Nakamura *et al*.,[28] has reported itraconazole as the most effective drug against *Malassezia* species. In this study, itraconazole was most effective against *M. sympodialis* (23.6 mm) and *M. slooffiae* (22.5 mm). Gupta *et al*.,[29] has reported *M. sympodialis* as the most sensitive organism against azole drugs.

From the results, it was found that *Malassezia globosa* was the predominant species followed by *M. sympodialis* among the selected study population. Most of the anti-dandruff compounds are barely soluble in water[30] which reduces their efficacy of antifungal activity. One of the highlights of this study was to test the aqueous plant extracts rather than other organic solvent extracts against the *Malassezia* species to reduce the use of organic solvents thereby promoting green pharmacy while identifying new drugs from plant origin. In this study, aqueous extracts from *Phyllanthus emblica* (fruits), *Hibiscus rosa-sinensis* (flowers), *Acacia concinna* (pods) and azole drugs have demonstrated significant anti-dandruff activity against the isolates tested. Further, characterization of active constituents from the extracts would help to identify the potential antidandruff compounds for further use.

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