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An Indigenous Study on Malassezial Susceptibility Testing by Selected Plant Extracts

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ABSTRACT: The present study was carried out to evaluate the antifungal activity of ten selected plant extracts of *Nelumbo nucifera, Senna auriculata, Salvia officinalis, Tagetes erecta, Psidium guajava, Argemone mexicana, Hibiscus sabdariffa, Hemidesmus indicus, Moringa oleifera*, and *Glycyrrhiza glabra* with ketoconazole as the positive reference standard. Their antimalassezial efficacy was tested using different concentrations of extracts in various solvents. The preliminary screening of plant extracts was determined by Kirby Bauer disc diffusion method and among the ten plant extracts, aqueous extract of *S. officinalis* and ethanol extract of *H. sabdariffa* extract exhibited potent antifungal activity against *Malassezia furfur*. The purification and partial characterization of *H. sabdariffa* and *S. officinalis* were carried out by Thin layer chromatography (TLC) technique. The Minimum Inhibitory Concentration (MIC) of *H. sabdariffa* and *S. officinalis* against *M. furfur* by Kirby Bauer method was 0.312 mg/mL and 0.625 mg/mL respectively against 50 µg/mL of Ketoconazole which was used as the standard. The phytochemical analysis of *S. officinalis* and *H. sabdariffa* revealed the presence of terpenoids, steroids, tannins, and polyphenols of which the concentration of polyphenols and flavonoids were significantly higher in *S. officinalis* and *H. sabdariffa* respectively. Thus, the present study concludes that *S. officinalis* and *H. sabdariffa* have a potent activity against *M. furfur*, thereby reducing the prevalence of dandruff, provided the future perspectives of the study that include characterization and cytotoxicity of the compound.

INTRODUCTION

Dandruff, caused by *Malassezia furfur* is a serious threat due to aesthetic conditions. It is a common scalp disorder affecting almost half of the population at the prepubertal age and all gender and ethnicity [1]. The fungus *M. furfur* is lipophilic dimorphic yeast which is widely known to be the causative agent of dandruff, *Pityriasis versicolor*, *Seborrheic dermatitis*, and *Tinea circinata*. Due to its lipase activity, it releases proinflammatory free fatty acids causing dermal inflammation and tissue damage [2]. The currently available treatment options of chemical origin have various limitations, either due to poor clinical efficacy or compliance issues. Also, these drugs are unable to prevent recurrence, which is one of the main common problems [3, 4]. Due to this drawback of conventional drugs, attention is shifted towards herbal remedies with medicinal plants which are being widely popular for their empirical antifungal properties [5, 6].

Fungi that are harmful to humans are known to cause different pathogenic infections in both normal and immunocompromised hosts. Normally fungi exist as mold and yeast forms, both forms are infectious to humans. Dermatophytic infections are categorized into systemic fungal infections and superficial skin infections [7].

M. furfur is a fungus which belongs to the class exobasidiomycetes characterized with oblong ellipsoidal yeast cells. *M. furfur* is commonly lipid dependent found on human skin particularly on the upper body where sebum secretions are high [8]. *M. furfur* growth is enhanced with the hyper secretion of sebum and hyper proliferation of the stratum corneum. The lipase activity of *M. furfur* hydrolyses the triglycerides and free fatty acids that lead to excess release of oleic acid along with cytokines and as a result it aggravates the scalp by causing dermal inflammation and tissue damage [9].

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Presently many chemical substances are available to treat dandruff on the scalp, but these antidandruff products are known to contain various chemical agents like azoles, zinc pyrithione (ZPTO), salicylic acid, etc, which possess harmful side effects [10,11]. Due to toxic and harmful side effects of synthetic or conventional agents, there is need for affordable, effective and nontoxic alternatives, which has led to the search for compounds from natural sources such as plants [12]. In the current scenario small bioactive constituents from the plants are exploited for therapeutic uses, playing an important role in development of drug discovery of bioactive compounds which ranges from 50 to 70% of molecules in clinical use till date [13]. This indicates that there is a shift in universal trend from synthetic to herbal drugs which can be coated as return to Mother Nature. Various natural plant extracts are known for their antidandruff properties along with conditioning effects. Evaluation of antifungal properties of such plant extracts can be done and they can be used effectively as an alternative to synthetic chemical agents in different anti dandruff formulations [14]. Considering the above said aspects, the aim of this study is to evaluate the antifungal activity of selected plant extracts on *M. furfur*. The MIC of the selected plant extracts was determined and demonstrated with effective activity. The phytochemical analysis was also carried out to identify the bioactive phytocompounds responsible for the inhibition of the dermatophyte.

MATERIALS AND METHODS

Procurement of microbial culture

The test organism *M. furfur* strain no.1374 was procured from Microbial Type Culture Collection (MTCC), Institute of Microbial Technology, and Chandigarh, India.

Media used

Leeming and Notman's agar media

Collection of the plant samples

Plants used were selected based on their ethno-medicinal importance and their antimicrobial, topical therapeutic properties from the literature survey of different research articles. Ten plants were collected from Gandi Krishi Vignana Kendra (GKVK), University of Agricultural Sciences and Jnana Bharathi Campus, Bengaluru.

Preparation of the plant crude extracts

Extraction is the first essential step in which different solvents are utilized under a variety of conditions such as temperature and time of extraction. It is necessary to extract the desired chemical components from the plant materials for further separation and characterization. The basic operations included here for extraction were pre-washing, drying of plant materials and grinding to obtain a homogenous sample.

Aqueous extraction

Aerial parts of each plant were washed thoroughly 4-5 times with running tap water and blot dried. 100 g of each plant material was macerated with 300 ml (1:3 w/v) of sterile distilled water in a blender for 10 min. The macerate was first filtered through double layered muslin cloth and then centrifuged at 8000 rpm for 10 min. The supernatant was filtered through Whatman No. 1 filter paper and sterilized at 120ºC for 30 min, which serves as the mother extract. The mother extract was concentrated on water bath shaker and stored at 4ºC for further use [15].

Solvent Extraction (Soxhlet)

Plant materials were thoroughly cleaned to ensure that it is free from foreign particles like dust, pollens, surface adhered microbes, etc. They were shade dried and then finely ground. About 30 g of the powder was filled in a thimble and sequentially extracted with 200 ml of *n*-hexane, ethanol and methanol using a Soxhlet extractor for 48 hrs. or until the solvent in the extractor remained colorless. All extracts were concentrated using rotary evaporator and stored at 5ºC in an air tight brown bottle until further use [16].

Screening of plants for Antifungal activity

Disc Diffusion method

An antimicrobial activity of selected plant extracts was evaluated by the disc diffusion method. According to the method outlined in M44, NCCLS inoculum suspension of *M. furfur* was adjusted to 0.5 McFarland standard followed by the inoculation of the suspension onto LN media by spread plate method. 10µL of different concentrations of the phyto-extracts were impregnated on to the sterile 6mm discs which were placed equidistantly on inoculum seeded agar plates with sterile forceps and incubated at 32ºC for 48 hrs. Antimicrobial activity was determined by measuring the zone of inhibition around the disc. Experiment was carried out in triplicates and zone of inhibition compared with reference standard ketoconazole 50 µg /ML and DMSO was used as negative control [17].

Determination of MIC of potent plant extracts

MIC is the lowest concentration of the drug that inhibits the growth of microorganisms. The potent plant extracts exhibiting effective antimicrobial activity at 10 mg/mL were used to determine the MIC by using disc diffusion method. Different concentrations of the potent plant extracts were made by 2 fold dilution method or (double dilution). The discs containing different concentrations of the extracts were placed equidistantly on petriplate uniformly inoculated with 0.5 McFarland standard inoculums. The plates were incubated at 32º C for 48 hrs. The zone of inhibition was measured and recorded against the concentrations of the potent plant extracts [18].

Statistical analysis

The results were expressed in mean ± Standard deviation with the level of significance, $P < 0.05$. The statistical analysis was performed using SPSS software. All the assays were performed in triplicates.

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Phytochemical screening of plants

Phytochemical analysis for the identification of the chemical groups of those plants which showed bioactivity was carried out using standard procedures as described in standard books and research articles. The aqueous extract of *S. officinalis* and ethanol extract of *H. sabdariffa* were evaluated for the presence of alkaloids, flavonoids, phenols, tannins, saponins, glycosides and terpenoids [19, 20].

Test for alkaloids: Mayer's test

Crude extract was mixed with 2 ml of 1% HCl and heated gently. Mayer's reagent was then added to the mixture. The presence of alkaloids was indicated by cream precipitate with Mayer's reagent.

Test for flavonoid: **Alkaline reagent test**

Test solution when treated with sodium hydroxide solution, showed increase in the intensity of yellow color which would become colorless on addition of few drops of dilute Hydrochloric acid, indicated the presence of flavonoids.

Test for tannins and phenols: Ferric Chloride test & Lead acetate test

1g of the extract was mixed with 15 ml of water in a test tube and filtered. To the 4 ml of filtrate, 2-3 drops of 0.1% $FeCl_3$ was added. Formation of blue, black or brownish green color indicated the presence of tannins and phenols. To the 3 ml of the above filtrate, 1 ml of 10% lead acetate solution was added. The presence of bulky white precipitate indicated the presence of tannins.

Test for saponins: Foam test

Crude extract was mixed with 5 ml of distilled water in a test tube and was shaken vigorously. The formation of stable foam was taken as an indication for the presence of saponins.

Test for terpenoids: Salkowski test

Crude extract was dissolved in 2 ml of concentrated H_2SO_4. A yellow coloration at the lower layer indicated the presence of terpenoids.

Test for glycosides: Liebermann's test

Crude extract was mixed with each of 2 ml of chloroform and 2 ml of acetic acid. The mixture was cooled in ice. Concentrated H_2SO_4 was added carefully. A color change from violet to blue to green indicated the presence of steroidal nucleus i.e., glycone portion of glycoside.

Thin Layer Chromatography

Thin layer chromatography (TLC) technique was performed for the separation and identification of bioactive constituents using silica gel 60 F254,20×20cm(Merck). 10 mg of dried plant extract was dissolved in 1 ml ethanol and water and 10 µL of both solutions were applied to TLC plate and developed in different mobile phase systems. Solvent system tried as a mobile phase was Chloroform: Hexane: Methanol (7:3:1 v/v/v). The plate was dried at room temperature. The developed chromatogram was air dried and observed for the bands under ultraviolet (UV) light at long wave (366 nm). They were also visualized under visible light (600 nm). The movement of analyte was expressed by its Retention factor (R_f) and intended using below formula [21, 22].

R_f = Distance travelled by the analyte / Distance travelled by the solvent front

RESULTS AND DISCUSSION

Screening of plants for antimalassezial activity

Among the ten plants screened for antimalassezial activity (**Table 1**), assessed at two different concentrations 20 mg/mL and 10 mg/mL by disc diffusion method, the highest zone of inhibition was exhibited by ethanolic extract of *H. sabdariffa* 18±0.45 mm and aqueous extract *S. officinalis* of 16.3±0.03 mm at 20 mg/mL concentration (**Figure 1** and **2**).

Table1. List of plant species selected for antifungal activity

Sl. No	Name of the plant	Family
1	*Argemone mexicana*	Papaveraceae
2	*Hemidesmus indicus*	Apocyanaceae
3	*Psidium guajava*	Myrtaceae
4	*Nelumbo nucifera*	Nelumbonaceae
5	*Salvia officinalis*	Lamiaceae
6	*Tagetes erecta*	Asteraceae
7	*Moringa oleifera*	Moringaceae
8	*Hibiscus sabdariffa*	Malvaceae
9	*Glycyrrhiza glabra*	Fabaceae
10	*Senna auriculata*	Fabaceae

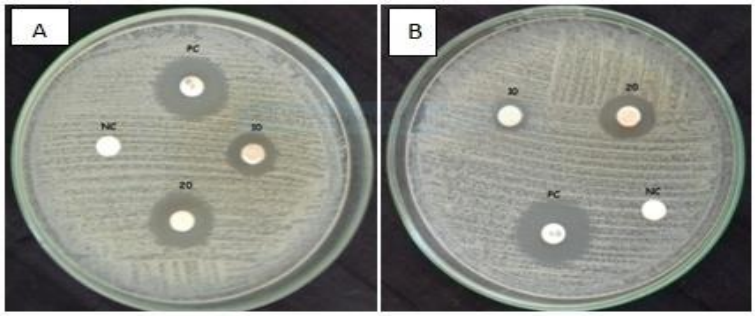

Fig. 1: Antifungal activity of plant extracts on *M. furfur* by disc diffusion method. A.*Hibiscus sabdariffa* **B**. *Salvia officianlis* [1-20, 2-10 mg/mL, **PC**-Positive Control (ketoconazole); **NC**-Negative Control (DMSO)]

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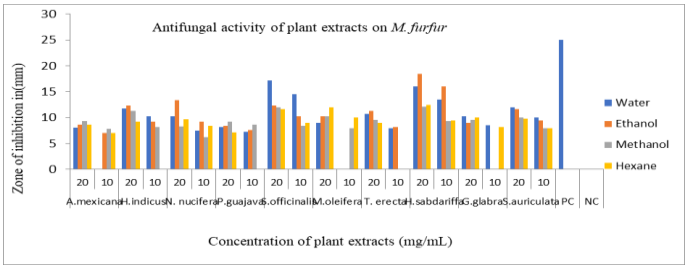

Fig. 2: Screening of ten plant extracts for antimalassezial activity

Determination of Minimum Inhibitory Concentration (MIC) of selected potential plant extracts

The MIC of ethanol extract of *H. sabdariffa* and aqueous extract of *S. officinalis* against *M. furfur* by Kirby Bauer method exhibited potent activity when compared against 50 µg/mL of Ketoconazole which was used as the standard (**Figure 3**).

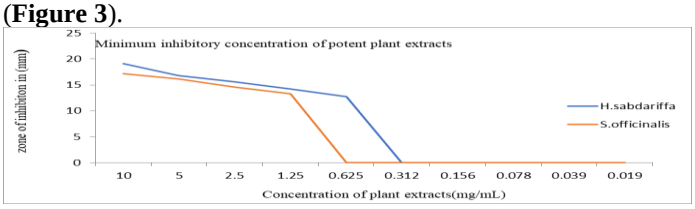

Fig. 3: MIC of *Salvia officinalis* and *Hibiscus sabdariffa*

Phytochemical Screening of the selected plant extracts

The results of phytochemical analysis of *Hibiscus sabdariffa and Salvia officinalis* and revealed the presence of phytochemical bioactive compounds like phenols, alkaloids, flavonoids, terpenoids, tannins, saponins and glycosides (**Table 2**).

Table 2. Phytochemical screening of potential plant species

Phytochemical constituents	***Hibiscus sabdariffa*** **(Ethanol extract)**	***S. officinalis*** **(Aqueous extract)**
Phenols	+	+
Alkaloids	+	–
Flavonoids	+	+
Tannins	+	–
Saponins	–	+
Terpenoids	+	–
Glycosides	–	–

Thin Layer Chromatography of potential plant extracts

TLC exhibited a remarkable result that directed towards the presence of different types of phytochemicals with different R_f values in the solvent system. The ethanol extract and aqueous extract of potential plants were resolved using the mobile phase Chloroform: Hexane: Methanol (7:3:1 v/v), and observed under ultraviolet (UV) light at long wave (366 nm) and visible light (600 nm) (**Fig. 4**).

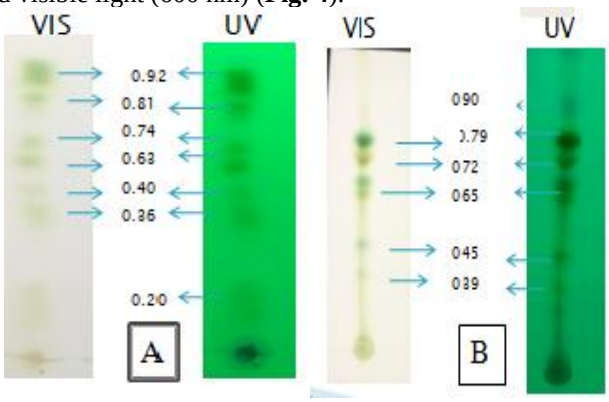

Fig. 4: TLC of phytoextracts (A) *S. officinalis* (B) *H. sabdariffa*

Due to certain limitations of the chemical products on skin increased the demand to alternative potentially effective and safer natural products. From the present study it can be concluded that the plant extracts *viz.*, aqueous extract of *S. officinalis* and ethanol extract of *H. sabdariffa* exhibited significant antidandruff activity against *M. furfur*. Phytochemical screening showed the presence of secondary metabolites. The antimicrobial activity may be due to the presence of bioactive compounds such as flavonoids, terpenoids, alkaloids, tannins and phenols. The results presented in the study suggests that *H. sabdariffa* and *S. officinalis* extracts can be used as an antimalassezial agents for the effective management of dermatological pathologies reported by *Malassezia furfur*.

CONCLUSION

To conclude, the present study of ours was initiated in finding a suitable herbal remedy against *M. furfur* which is the causative agent of dandruff. Our primary phase of study showed up with promising antidandruff properties of *S. officinalis* and *Hibiscus sabdariffa.* The future perspective lies in the characterization of the active compound in these plant extracts along with the cytotoxicity evaluation.

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AUTHOR'S CONTRIBUTION

All the authors have contributed substantially in the work, read the final version of the manuscript and approved the same.

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ETHICS STATEMENT

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"This article does not contain any studies with human participants or animals performed by any of the authors."

CONFLICTS OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY

Not declared.

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No external funding source has been disclosed.

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