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INVESTIGATION ON BIOCHEMICAL CHARACTERISATION AND *IN VITRO* ANTIFUNGAL EFFICACY OF PLANT EXTRACTS ON *MALASSEZIA FURFUR*

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ABSTRACT

Malassezia furfur, a lipophilic yeast belongs to the resident flora of human skin and has been responsible for the pathogenesis of several skin diseases such as dandruff, pityriasis versicolor, folliculitis, seborrhoeic dermatitis and some forms of atopic dermatitis. The synthetic drugs available were proved to be toxic and expensive when used in prolonged treatment. The present study was aimed to evaluate the inhibitory effects of ten plant extracts on *M. furfur*. The antifungal activity was assayed at various concentrations under *in vitro* conditions by radial agar disc diffusion assay and broth microdilution. The extracts which conferred antifungal activity were also tested for its active constituents using standard phytochemical tests. The strain was initially studied for biochemical properties such as, the presence of catalase, ability to use Tween 80 and splitting of esculin. Our studies revealed that hexane extract of *Syzygium aromaticum* L. showed highest activity with a Minimum Inhibitory Concentration (MIC) of 0.390 mg mL^{-1}, followed by methanol extract of *Lawsonia inermis* L. with MIC 0.781 mg mL^{-1}. The results of phytochemical analysis of these two plants indicated the presence of tannins, quinones, glycosides, flavonoids and terpenoids. Following the preliminary phytochemical analysis, separation of the extracts on silica gel 60F254 plate was achieved through high performance thin layer chromatographic analysis, followed by scanning of the spots at 254 nm and 366 nm using a UV detection mode and derivatization was made to establish the phytochemical profile of these two plants. The hexane extract of *S. aromaticum* and methanolic extract of *L. inermis* have potential to be used as ideal anti-malassezial agents. The antifungal activity of these plants could be due to the presence of these detected metabolites and can be used directly or considered as a source for developing better anti-malassezial agents.

KEY WORDS : *Malassezia furfur,* dandruff, phytochemicals, medicinal plant extracts, antifungal activity.

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INTRODUCTION

Malassezia furfur, an opportunistic lipophilic yeast is a member of the normal skin flora, associated with dandruff and several chronic skin diseases such as, pityriasis versicolor [1], folliculitis [2], seborrhoeic dermatitis [3], some forms of atopic dermatitis, psoriasis and confluent and reticulate papillomatosis [4]. They inhabit seborreheic areas such as, the upper trunk, head and face [5]. Dandruff (D) is generally characterized by the flaking on the scalp with pruritis and experienced by greater than 50% of the global population instigating from post-puberty period [6, 7]. Seborrhoeic dermatitis (SD), a highly pruritic chronic inflammatory skin disorder which is dandruff-related, is generally considered as an advanced form of dermatitis and occurs in about 1–3% of the general population [8, 9]. Pityriasis versicolor is a chronic scaly hypo- or hyperpigmented lesions with minimal pruritus characterized by the appearance of round to oval lesions, most commonly found on the trunk and upper arms [10, 11]. Etiological factors contributing to the pathogenicity of *M. furfur* is due to its lipase activity which induces an irritation response due to free fatty acids released from sebaceous triglycerides, thus leading to severe infections [12]. The therapy is contingent on imidazolic drugs such as ketoconazole, fluconazole, miconazole and bifonazole and other drugs such as ciclopiroxolamine, zinc pyrithione, terbinafine *etc.* [13]. These drugs are proven to be toxic when used for prolonged treatments and others present certain limitations, either due to poor clinical efficacy or due to compliance issues [14]. These negative health trends necessitated the drive to find alternative antifungal agents with versatile features such as, less toxicity, good functionality and compatibility. As reported by the World Health Organization (WHO, 2011), about 70–95% of the world's population in developing countries rely primarily on plants for their primary health care [15]. Antimicrobials of plant origin have a very high prospective as antimicrobial drugs for treating various skin diseases and represent a long history of human interactions with the environment [16]. The medicinal value of these plants lies in phytoconstituents that produce definite physiological action on the human body [17]. They are employed as popular folk medicines, whereas some have gained popularity in the form of finished products and are considered as safe and cost effective with fewer side-effects and better tolerance levels. Through ethnobotanical surveys and literature reviews, close to a hundred plants were found to be used in Guatemala for the treatment of dermatophytoses [18]. The active principles are present in different parts of the plant such as root, stem, bark, heartwood, leaf, flower and fruit can be used in various crude dosage forms like pills, powders, essential oils, infusions or poultices [19]. Information on the phytochemical profile of the plants is essential to determine the effect of therapeutic treatments [20]. Hence, we have developed a simple high performance thin layer chromatographic method for the accurate and rapid analysis of the active plant extracts. The method was found suitable for rapid screening of plant materials for their antifungal activities and can be performed without any special sample pre-treatment. Taking these data into account, the present study was aimed to evaluate the effect of aqueous and solvent extracts of selected plants on the growth of *M. furfur.*

MATERIALS AND METHODS

1. *Tested microorganism*

M. furfur (MTCC strain no: 1374) was obtained from Microbial Type Culture Collection Centre and Gene bank (MTCC), Chandigarh, India. The culture was maintained in modified Emmon's agar medium and Saborauds Dextrose Agar (SDA) supplemented with milk. Leeming–Notman agar (LNA) medium was used for carrying out antimicrobial studies. Phenotypic characteristics of the strain were understood by studying its morphological and biochemical characteristics.

1.1. Morphological characteristics of M. furfur

The strain was cultured in Leeming-Notman(LN) medium [21]. The plates were incubated at 32±2 ℃ for four days. After four days, macroscopic characters of the colonies such as shape, size, elevation, surface and

colour were recorded. Further, microscopic characters were studied by Gram staining method [22].

1.2. Biochemical characteristics of M. furfur

The following tests were carried out to understand the physiological identification of *M. furfur*

1.2.1. Test for lipase by tween-80 opacity test

The tween 80 opacity medium was prepared with 10.0 g of peptone, 5.0 g of NaCl, 0.1 g of $CaCl_2$, 15.0 g of agar and 1,000 mL of distilled water. The media was left to stand for approximately 10 min and then adjusted to pH 6.8 by adding 1M HCl. After the medium was autoclaved at 121˚C for 15 min and cooled to about 60 ˚C, 5 mL of Tween 80 (warmed at 60 ˚C to 70 ˚C before being added to the medium) was added to the medium. The medium was dispensed into sterile 90 mm - diameter petridishes and set for solidification. A loopfull of 18 h culture of *M. furfur* was spread approximately 10 mm in diameter. The inoculated agar plates were incubated aerobically at 32 ˚C and were examined for 6 days. The test was carried out in triplicate [23].

1.2.2. Test for catalase

A colony was placed as a small spot on a slide without making a suspension and dried. 1 to 2 drops of 3% - H_2O_2 was placed on the spot with the yeast material and examined immediately for the evolution of gas, which indicates catalase activity [24].

1.2.3. Test for β- glucosidase

The β-glucosidase activity of *M. furfur* was assessed by Bile Esculin hydrolysis test following the procedures of Kaneko *et al,* 2007 [25]. Bile-Esculin agar medium was prepared with 5.0 g of peptone, 3.0 g of beef extract, 20.0 g of oxbile, 1.0 g of esculin, 0.5 g of ferric citrate, 14.0 g of agar and 1000 ml distilled water; boiled for a minute until complete dissolution. The final pH was maintained to 6.6 ± 0.2 and autoclaved at 121 ˚C for 15 min. The tubes were allowed to solidify in slanted position. A loopful of 18 h culture of *M. furfur* was inoculated deeply in the bile esculin agar slant and incubated for 4 to 6 days at 30 ˚C. A positive reaction was indicated by blackening of the medium, whereas absence of blackening indicates lack of β-glucosidase activity.

2. Selection and collection of the plants based on ethnobotanical survey

Plants used were selected based on their ethnobotanical survey for their antimicrobial and topical therapeutic properties. Specific part of each plant was taken according to Ayurvedic system of medicine [26]. Authenticated medicinal plants were collected from Gandhi Krishi Vignan Kendra, Bengaluru, Karnataka and Agricultural University, Trivandrum, Kerala. A voucher specimen of the plant was deposited in the Department of Microbiology and Biotechnology, Bangalore University, Bengaluru. The names of selected plants and their reported bioactivities [27] are listed in Table 1

2.1. Preparation of plant extract

2.1.1. Aqueous extraction

Fifty gram of thoroughly washed plant materials were blot-dried and macerated with 150 mL of distilled water (1:3 w/v) in a homogenizer. The suspension was filtered through a muslin cloth and then centrifuged at 8000 rpm for 10 min. The supernatant was further filtered through Whatman No. 1 filter paper and sterilized at 121℃ for 30 min, which served as the mother extract [28]. Some extracts were concentrated on water bath and stored at 4 ˚C for further use.

2.1.2. Solvent extraction

Thoroughly washed plant materials were shade-dried and then powdered with the help of a pulverizer. About 65 g of the powder was filled in a thimble and sequentially extracted with 200 mL of hexane, chloroform and methanol using a Soxhlet extractor for 48 h. All extracts were concentrated using rotary evaporator and stored at 5 ℃ in an air tight brown bottle until further use. All the extracts were subjected to antifungal studies [29].

3. Antimicrobial susceptibility test

3.1. Disc diffusion assay

The disc diffusion assay was used to screen the antifungal activity of all plant extracts following the procedure of NCCLS [30]. LNA plates were prepared by pouring 15 mL of molten media into sterile petriplates and the

plates were allowed to solidify for 10 min. 100 µL of inoculum suspension previously adjusted to 0.5 McFarland standard was swabbed evenly over the sterile LNA agar medium plates set for the disc diffusion assay [31]. Inoculum was allowed to dry for 10 min. Sterile 6 mm diameter discs were placed equidistantly around the margin of the petridish. 10 µL of extracts (of concentration 25 mg mL^{-1} and 12.5 mg mL^{-1} each) was dispensed onto the discs. The standard reference used in this study was ketoconazole at 0. 125 mg mL^{-1} and Dimethyl sulphoxide (DMSO) served as a negative control. Plates were kept for 2 h in the refrigerator to enable pre-diffusion of the extracts into the agar without microbial growth. Then the inoculated plates were incubated at 32 ± 2℃ for 48 h. At the end of the incubation period, the antimicrobial activity was recorded as the width (diameter of inhibition zone plus diameter of the disc) of the inhibition zone after incubation. Each test was performed in triplicate and the values were averaged [32].

3.2. Determination of MIC by microdilution technique

The broth microdilution was performed as described in M27-A2, CLSI [33]. The plant extracts were prepared and diluted in 10 % DMSO by two-fold serial dilution ranging from 25 mg mL^{-1} to 0.097 mg mL^{-1}. The cell suspension was prepared in 1X phosphate buffer saline of pH 7.3, with an optical intensity equivalent to 0.5 McFarland standard which will give a final concentration of 4.5 x 10^6 cfu mL^{-1}. This suspension was inoculated in each well of the microdilution plate previously prepared with double dilution of plant extracts. The plates were incubated with agitation at 34 ± 2˚ C for 48 h. After 48 h of incubation, 15 µL of *p*-iodonitrotetrazolium chloride dye (INT) was added in each well and further incubated for 4 h for a colour change from colourless to purple. MICs were determined as the lowest concentration of sample tested that prevented the colour change from colourless to purple. The standard reference used in these studies was ketoconazole at the concentration of 0.156 mg mL^{-1}. A well was reserved in each plate to study DMSO inhibitory effect which served as negative control. The procedure was performed in octuplicate under stringent aseptic conditions.

4. Qualitative phytochemical screening

Phytochemical tests were carried out to detect the chemical groups of those extracts which showed bioactivity using standard procedures as described by Rashid, Harborne and Raaman [34, 35, 36]. The extracts were evaluated for the presence of alkaloids, terpenoids, naphthoquinones, tannins, phenols, flavonoids, steroids, glycosides, proteins and carbohydrates.

4.1. Test for alkaloids

One gram of extract was warmed with 10 mL of 1% HCl and filtered using Whatman filter paper. 2 mL of filtrate was mixed separately with a) Mayer's reagent and b) Dragendorff's reagent. The presence of alkaloids was indicated by yellow / brown / orangish precipitate.

4.2. Test for terpenoids

Five hundred milligram of the extract was mixed with 2 mL of chloroform, followed by the addition of 2 mL of conc. H_2SO_4. A reddish brown colouration indicated the presence of terpenoids.

4.3. Test for naphthoquinones

Treat 2 mL of chloroform extract and 2 mL of ethyl ether with dilute ammonia (NH_3) solution. Formation of pink colour indicates the presence of naphthoquinones.

4.4. Test for tannins and phenols

One gram of the extract was mixed with 15 mL of water in a test tube and filtered. The filtrate was subjected to ferric chloride test and lead acetate test. To the 4 mL of filtrate, 2 to 3 drops of 0.1% $FeCl_3$ was added. Formation of blue, black or brownish green colour indicated the presence of tannins and phenols. To the 3 mL of above filtrate, 1 mL of 10% lead acetate solution was added. The presence of bulky white precipitate indicated the presence of tannins.

4.5. Test for flavonoids

Five hundred milligram of the extract was mixed with 10 mL of ethanol in a test tube and filtered. To the filtrate, few fragments of magnesium ribbon and conc. HCl (drop wise) were added. The appearance of pink or red colour indicated the presence of flavanol glycosides.

4.6. Test for phytosteroids

The extract (50 mg) was dissolved in 2 mL of acetic anhydride. To this, 1 or 2 drops of conc. H_2SO_4 were added slowly along the sides of the test tubes. Dark green colouration indicated the presence of steroids.

4.7. Test for glycosides

Fifty milligram of the extract was dissolved in pyridine, sodium nitroprusside solution was added and made alkaline using 10% NaOH. The pink colour indicated the presence of glycosides.

4.8. Test for proteins

500 microlitre of ninhydrin solution (100 mg of ninhydrin in 100 mL of acetone) was added to 2 mL of aqueous filtrate. The appearance of pink/purple colour indicated the presence of proteins and aminoacids.

4.9. Test for carbohydrates

Hundred milligram of the extract was dissolved in 5 mL of water and filtered. 1 mL of filtrate was boiled with 1 mL of each of Fehling solution A and B. The formation of red coloured precipitate indicated the presence of sugars.

5. High-performance Thin Layer Chromatographic (HPTLC) analysis of Lawsonia inermis and Syzygium aromaticum

HPTLC was performed on pre coated thin layer silica plate 60 F_{254} 10 × 10 cm, E-Merck. HPTLC plates (10 X 10 cm; 0.25 mm layer thickness; Merck). Samples were applied as 10 mm wide bands, positioned 12 mm from the bottom of the pre coated thin layer silica plate, using a Camag (Mutten, Switzerland) Linomat IV automated TLC applicator. These vital parameters were maintained same for both the analyses. TLC plate development was performed using a Camag twin trough glass chamber pre-saturated with mobile phase of volume 20 mL for 20 min. Solvent was allowed to run to a height of 8.5 cm. A mixture of toluene: ethyl acetate: formic acid (6 : 3 : 1) (v/v/v) was optimized as solvent system for both the plant extracts under the laboratory conditions of 25 ± 5℃ and relative humidity of 40-50 %. After development, the chromatograms were dried and the components were visualised by UV irradiation at 254 and 365 nm. They were also derivatised in vanillin-sulphuric acid reagent [1 % vanillin solution (w/v) in methanol and 10 % sulphuric acid (v/v) in methanol; mixed in the ratio of 1:1] and Rf values were recorded [37, 38].

6. Statistical Analysis

Statistical analysis was performed using SPSS software: Version 16.0. The results were expressed as mean ± SE (n=3). Multivariate analysis was applied for statistical analysis with the level of significance set at $P<0.05$. Results with $P<0.05$ were considered to be statistically significant.

RESULTS

1. Cultural and biochemical characteristics

The identity of the strain was confirmed by studying its morphological features (macroscopic and microscopic) and biochemical characteristics like Tween 80 opacity test, catalase test and β-glucosidase test. Direct macroscopic observation revealed that colonies appeared to be creamish to yellowish colonies around 3 to 4 mm in diameter, with smooth texture, entire margin and umbonate elevation. Microscopic observation showed small pink ovoid cells with monopolar budding as a charactristic feature. *M. furfur* showed positive, catalase reaction which was characterized by the formation of effervescence due to the evolution of free O_2 (Figure 1a). It also showed a positive β-glucosidase activity by blackening of the Bile esculin agar medium (Figure 1b). The results of Tween 80 opacity test showed that *M. furfur* produced a positive halo response that circumscribed inoculation site after six days post inoculation. This confirms the lipase activity of the strain which was responsible for its pathogenicity (Figure 1c).

2. Anti-malassezial activity of plant extracts

Among ten plants tested, two plants viz., *Lawsonia inermis* L. and *Syzygium aromaticum* L. showed significant activity against *M. furfur* (Table 2). The initial screening results revealed that *S. aromaticum* extracts showed inhibition zones of [(18.36±0.32) mm] and [(13.9±0.3) mm] and *Lawsonia inermis* extracts showed inhibition

zones of [(13.8±0.4) mm] and [(10.63±0.55) mm] at 25 mg mL^{-1} and 12.5 mg mL^{-1} (Figure 2a and 2b). The results of MIC determination showed that hexane extract of *S. aromaticum* was found to be very active with MIC 0.391 mg mL^{-1} which was succeeded by methanolic extract of *L. inermis* with MIC 0.781 mg mL^{-1} (Table 3). Chloroform and methanolic extract of *L. inermis* also showed activity with relatively higher MIC (6.25 mg mL^{-1} and 6.25 mg mL^{-1} respectively) whereas the same extracts of *S. aromaticum* did not show anti-malassezial activity.

3. *Phytochemical analysis of the active extracts*

The extracts were prepared according to the procedure mentioned earlier. Phytochemical analysis was done only with the extracts which showed positive anti-malassezial activity *viz*., *L. inermis* and *S. aromaticum*. The extract yield of these plants was not consistent with any particular solvent. The methanol extract of *L. inermis* leaves yielded 18.1% based on dry weight of the leaves whereas aqueous extract, chloroform extract and hexane extract yielded 16.9%, 11.3% and 2.6% respectively. The hexane extract of *S. aromaticum* yielded 11.64% based on dry weight of the flower buds wherein percentage quantities of the aqueous extract, methanol extract and chloroform extract were 8.98%, 8.30% and 4.90% respectively. It is understood that the bioactivity of plant extracts can be explained on the basis of their phytoconstituents which confers medicinal value to the plant. The results of the phytochemical analysis were tabulated in Table 4. The phytochemical analysis indicated the presence of phenols, tannins, quinone, flavonoids, carbohydrates and proteins in *L. inermis* extracts and that of *S. aromaticum* showed the presence of terpenoids, phenolics, tannins, flavonoids, glycosides and proteins.

4. *High-performance Thin Layer Chromatographic (HPTLC) analysis of L. inermis and S. aromaticum*

Different compositions of the mobile phase for HPTLC were tried and the fine separation of the extracts was achieved by using the combination of toluene: ethyl acetate: formic acid (6 : 3 : 1 v/v/v) as the mobile phase for all the extracts (Fig. 1). The results from HPTLC finger print of methanol extract of *L. inermis* scanned at 254 nm and R_f values were obtained in the range between 0.082 and 0.705 whereas hexane extract of *S. aromaticum* showed R_f in the range between 0.232 and 0.842. The R_f values of hexane, chloroform and methanol extracts of both the plants were recorded and tabulated in Table 5.

Table 1
Plants used in the present investigation and their reported medicinal uses.

Name of the plant	Family	Part used	Bioactivities of plant extracts
Emblica officinalis Gaertn.	Euphorbiaceae	Leaves	Used in treatment of skin disorders, i.e., scabies, dry skin, and wrinkled skin.
Vitex negundo L.	Verbenaceae	Leaves	Leaf- anti-inflammatory. Oil- applied to scrofulous sores.
Thespesia populena L.	Malvaceae	Leaves	Specific for skin diseases. Root, fruit and leaf- used in psoriasis, scabies and other cutaneous diseases. Leaf- anti-inflammatory.
Allium cepa L.	Liliaceae	Bulb	Antibacterial, anti-inflammatory and antidermatophytic.
Lawsonia inermis L.	Lythraceae	Leaves	Leaves- Used externally to treat skin infections (tinea); also as a hair conditioner
Anacardium occidentale L.	Anacardiaceae	Shells	Leaves and bark- fungicidal, vermicidal, protozoicidal, antimicrobial (used for toothache, sore gums).
Cassia alata L.	Caesalpiniaceae	Leaves	Leaf- used in skin diseases like herpes, blotch, eczema, mycosis (washerman's itch). Dried leaves- in leprosy. A strong decoction is used for ringworm, eczema and herpes.
Mangifera indica L.	Anacardiaceae	Leaves	Unripe fruit- astringent. Leaves- anti-inflammatory, antibacterial. Used externally in burns and scalds. Kernel-astringent, anti-inflammatory, antibacterial, antifungal. Stem bark- astringent.
Syzygium aromaticum L.	Myrtaceae	Flower buds	Flower buds- anti-inflammatory, antibacterial, antifungal.
Sapindus laurifolius Vahl.	Sapindaceae	Leaves	Fruit- astringent, emetic, detergent, antihelmintic.

Table 2

Antifungal activity of selected plant extracts against M. furfur by disc diffusion method.

Name of the plant	Solvent extract	Zone of inhibition in millimetres		
		Concentration of plant extracts (mg mL^{-1})		Ketoconazole (0.125 mg mL^{-1})
		25	12.5	
Lawsonia inermis L.	Chloroform	8.86 ± 0.06^{b}	8.6 ± 0.1^{c}	16.06±0.15
	Hexane	10.0 ± 0.13^{b}	9.46 ± 0.233^{b}	
	Methanol	13.8 ± 0.4^{b}	10.63 ± 0.55^{c}	
Syzygium aromaticum	Chloroform	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	
	Hexane	18.36 ± 0.32^{a}	13.9 ± 0.3^{b}	
	Methanol	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	

The data were the means of triplicate analysis ± Standard error, (n = 3). The values followed by different alphabets differ significantly when subjected to Tukey HSD test, P value ≤0.05

Table 3

MICs of the crude extracts of L. inermis and S. aromaticum against M. furfur by 96-well microdilution technique.

Plant material	Minimum Inhibitory Concentration(mg mL^{-1})			
	Chloroform	Hexane	Methanol	Water
L. inermis L.	6.25	6.25	0.781	-
S. aromaticum L.	-	0.391	-	-

(-); Activity not observed. Data are the means of octuplicate analysis (n = 8)

Table 4

Qualitative phytochemical screening of different extracts of Lawsonia inermis L. and Syzygium aromaticum L.

Sl. No:	Phytochemical tests	Aqueous extract		Chloroform extract		Hexane extract		Methanol extract	
		Li	Sa	Li	Sa	Li	Sa	Li	Sa
1.	Alkaloids								
	a)Mayer's test	+	+	-	-	-	-	+	+
	b)Dragendorff's test	+	+	-	-	-	-	+	+
2.	Terpenoids	+	+	-	+	+	+	+	+
3.	Quinones	+	+	+	+	+	+	+	+
4.	Tannins and phenols	+	+	+	-	+	-	+	+
5.	Flavonoids	-	-	+	+	-	-	-	+
6.	Phytosteroids	+	+	-	+	-	+	+	-
7.	Glycosides	+	+	+	+	+	-	+	+
8.	Proteins	+	+	-	-	-	-	+	+
9.	Carbohydrates	+	+	+	+	-	-	+	+

(+: present; - : absent)
(Li: Lawsonia inermis; Sa: Syzygium aromaticum)

Table 5

R_f values of HPTLC fingerprint of S. aromaticum and L. inermis extracts under UV (254 nm and 366 nm) and after derivatization of chromatogram.

Plant extract used	R_f values		
	Short wave UV(254nm)	Long wave UV(366nm)	After derivatization in Vanillin -H_2SO_4
L. inermis			
a. Chloroform extract	0.505, 0.553, 0.647, 0.705, 0.882	0.082, 0.259, 0.541, 0.6, 0.705, 0.753, 0.824	0.624, 0.753
b. Hexane extract	0.505, 0.553, 0.705, 0.753	0.553, 0.6, 0.705, 0.812	0.553, 0.624, 0.776, 0.824
c. Methanol extract	0.082, 0.482, 0.553, 0.647, 0.705	0.106, 0.565, 0.705	0.553, 0.623, 0.776, 0.823
S. aromaticum			
a. Chloroform extract	0.063, 0.421, 0.842, 0.905	0.063, 0.295, 0.379, 0.474, 0.505, 0.547, 0.632, 0.684, 0.779	0.232, 0.305, 0.537, 0.632, 0.684, 0.842
b. Hexane extract	0.842, 0.905	0.474, 0.526, 0.6, 0.737, 0.905	0.232, 0.263, 0.358, 0.453, 0.537, 0.632, 0.705, 0.779, 0.842
c. Methanol extract	0.063, 0.421, 0.842, 0.905	0.063, 0.379, 0.474, 0.505, 0.632, 0.684, 0.779	0.232, 0.305, 0.537, 0.632, 0.684, 0.842

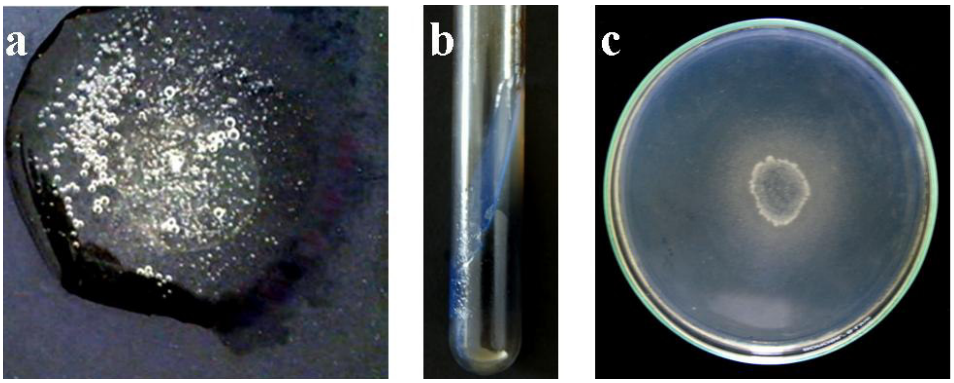

Figure 1

M. furfur exhibiting positive a. Catalase activity; b. Esculin hydrolysis and c. Tween 80 hydrolysis activity.

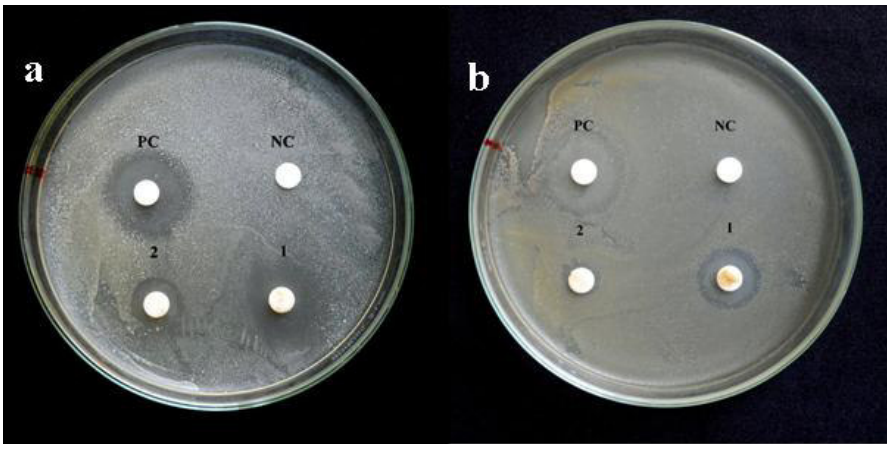

Figure 2

Antifungal activity of plant extracts against Malassezia furfur. a. Hexane extract of Syzygium aromaticum; b. Methanol extract of Lawsonia inermis PC- Positive control; NC- Negative control; 1 - 25 mg mL^{-1} and 2 - 12.5 mg mL^{-1}

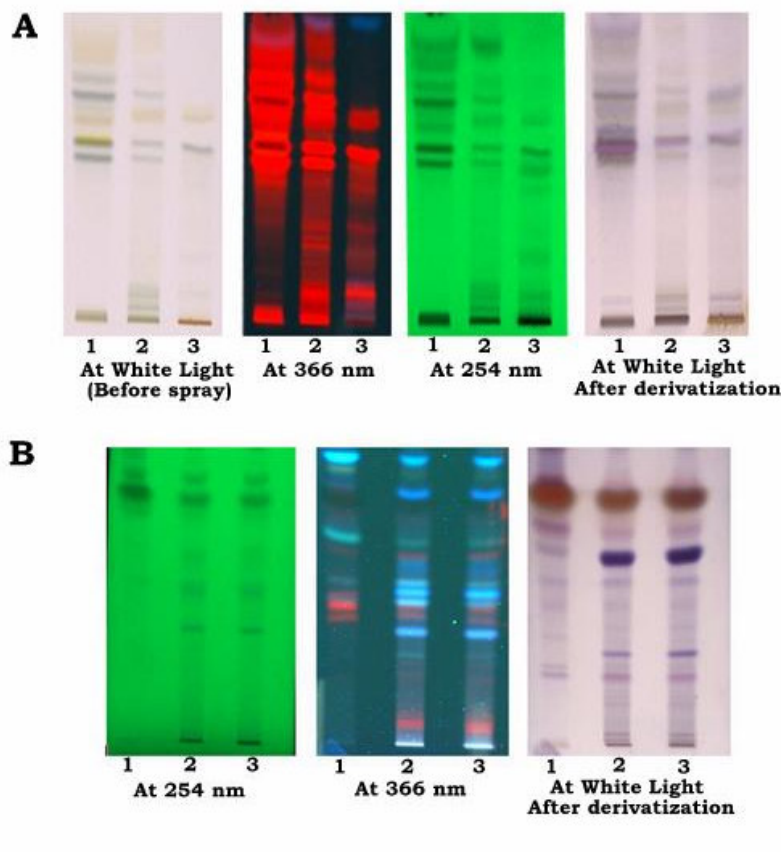

Figure 3
HPTLC fingerprint of A. Lawsonia inermis and B. Syzygium aromaticum
1. Hexane extract; 2. Chloroform extract and 3. Methanol extract
Mobile phase: Toluene: ethyl acetate: formic acid (6:3:1) (v/v)
Derivatization: Vanillin-H_2SO_4 reagent

DISCUSSION

The hydrolysis of tween opacity medium is owing to the presence of lipolytic enzymes produced by *M. furfur*. Liberated fatty acids bind with calcium and appear as insoluble crystals around the inoculation site [39]. Esculin supplemented in the medium is hydrolysed to esculetin and dextrose. Esculetin reacts with ferric citrate to form a dark brown or black complex, visualized as a black precipitate around the colonies which indicates the positive reaction [40]. The plants and different parts chosen for the study were based on ethnobotanical survey. Taking into account of their traditional uses and reported bioactivities, we carried out *in vitro* antimycotic activity of ten botanicals against *M. furfur*. In the present investigation, plant materials were subjected to sequential extraction using solvents in order of their increasing polarity to reduce the synergistic effect of the phytoconstituents [41]. Methanolic extract of *L. inermis* showed good activity over chloroform and hexane extracts inferring that concentration of the bio-actives are relatively higher in methanol compared to chloroform and hexane extracts. In the case of *S. aromaticum*, the antifungal activity must be attributed to the fact that active compounds exhibiting anti-malassezial activity are extracted into hexane. These findings agreed with previously published results that the substances responsible for the antimicrobial activity were mainly non-polar in nature [42]. The phytochemical profile findings of *L. inermis* herein presented are in line to other reports where glycosides, flavonoids, quinone and tannins have been detected [43]. The presence of quinone compound in methanolic extract of *L. inermis* evidenced in phytochemical tests confirm the presence of Lawsone, a naphthoquinone which is an active ingredient of the leaf (0.5 to

1.0%) [44]. The therapeutic functions other than dyeing properties of Lawsone and the use of methanol as extractant for lawsone from the leaves of Lawsonia has been studied [45,46]. It has been reported that antimicrobial mechanism of naphthoquinone is to alter mitochondrial respiration of the microbes, due to the similarity of its structure to Coenzyme-Q10 [47]. The other speculated fact is that generation of Reactive Oxygen Species (ROS) from naphthoquinone and their subsequent oxidative damage to cellular structures evincing antimicrobial property [48]. The results of phytochemical screening of *S. aromaticum* was agreeing to the previous reports [49] and the presence of terpenoid compound, eugenol engendered anti-malassezial activity in the extract which corresponds to the reported literature by Kumar [50]. Eugenol was reported to have antibacterial [51], antifungal [52], antiyeast [53], antioxidant[54], anti-inflammatory and cardiovascular properties [55] and is a very promising candidate for designing new drugs based on its pharmacological reactions. It was reported that these compounds were related to the inactivation of cellular enzymes, which depended on the rate of penetration of the substance into the cell or caused by membrane permeability changes [56]. Increased membrane permeability is another major factor in the mechanism of antimicrobial action, where compounds may disrupt membranes and cause a loss of different layers of polysaccharides, fatty acids and phospholipids and eventual cell death. It is also reported that it acts on cell membrane by a mechanism that seems to involve the inhibition of ergosterol biosynthesis. The lower ergosterol content interferes with the integrity and functionality of the cell membrane [57]. Anti-malassezial activities of these plants reported here are coordinating with the reports of Prabhamanju and Fariba [58, 59]. These observations obviously indicate that these extracts can be exploited as antifungal agents in the management of superficial fungal infections caused by *M. furfur*. From the HPTLC studies, it has been found that hexane, chloroform and methanol extracts contain mixture of compounds but not a solitary compound. This fact was considered noteworthy in establishing their positive bioactivity against this dermatologically prevalent yeast *M. furfur* and also ascertains that the pharmacological activity is due to the summative effect of all phytochemicals in the extracts.

CONCLUSION

In conclusion, the results of this study signify the prospects of *L. inermis* and *S. aromaticum* for the development of herbal formulation against superficial infections caused by *M. furfur.* With the increasing interest to combat multiple drug resistance in microbes, increasing cost of prescription drugs, toxicity of drugs, and for the maintenance of personal health, medicinal plant extracts are increasingly gaining acceptance over synthetics as therapeutic agents. The outcome of this investigation provides scientific justification of traditional uses of the plant. Furthermore, detailed toxicological studies are needed to understand the nature of these extracts.

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Conflict of interest statement

We declare that we have no conflict of interest.

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