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Original Article

Screening and Evaluation of Potential Antifungal Plant Extracts against Skin Infecting Fungus *Trichophyton rubrum*

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ABSTRACT

Dermatophytosis is mainly caused by *Trichophyton rubrum*. The use of presently available conventional antifungal agents is limited due to various side-effects, requirement of long-term application, emergence of resistant organisms and cost-effectiveness. In the present study, methanolic extract of 12 medicinal plants was screened for antifungal activity against *T. rubrum*. To identify the phytochemicals in the extract qualitative and quantitative analysis was performed along with minimum inhibitory concentration (MIC). By analysing the samples using gas chromatography-mass spectrometry (GC-MS), the chemicals that were present were identified. Among the various plants studied, the methanolic extracts of *Aegle marmelos* leaves (AML-ME) and *Pimpinella anisum* seed (PMS-ME) showed high zone of inhibition of 16 and 22 mm, respectively. MIC of both the extracts was found to be 100 µg/ml. Some major compounds identified in AML-ME with alkaloids are N, N,-dimethyl-o-(1-methyl-butyl)-hydroxylamine, 13-tetradece-11-yn-1-ol (15.21%), n-hexadecanoic acid, Propanal, 2,3-dihydroxy-, (s)-(5.22%) and 1,3-butanediol, and in PAS-ME, hexamethylene chloroiodide, cyclopentene,3-hexyl, E-2-octadecadecen-1-ol, n-hexadecanoic acid and 2-cyclopentene-1-undecanoic acid, ethyl ester. Both AML-ME and PAS-ME showed dose-dependent *in vitro* antioxidant activity against the radicals such as DPPH and ABTS. Also, FRAP activity was found in both the extracts. Altogether, the presence of antifungal and antioxidant activity in the AML-ME and PAS-ME might be advantageous in exploring these extracts as either alternative or complementary therapy to the available antifungal agents further. The phytochemicals present in these extracts has acted synergistically against the *T. rubrum* and affected its viability.

Keywords: Antifungal activity, *Pimpinella anisum*, *Aegle marmelos*, Bioactive compounds, Dermatophytosis.

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INTRODUCTION

Dermatophytes causes dermatophytosis, which is the most common fungal infection worldwide (20–25% of the world population are affected).[1] Keratin is the main substrate for dermatophytes hence it affects mostly the keratinized tissues. The fungus, *Trichophyton rubrum*, belongs to the genus, Trichophyton is the major causative organism of the dermatophytes throughout the World.[2] In India, the incidence of dermatophytes has increased in recent years with *T. rubrum* as major causative organism.[3] *T. rubrum* is capable of causing tinea corporis, tinea pedis, tinea manus, tinea capitis and tinea cruris.[4] In addition, *T. rubrum* also causes onychomycosis, a clinical condition of chronic fungal infection of toe or finger nails, which results in thickening, discoloration and separation of nail from nail bed.[5] Present treatment for dermatophytes greatly depends on azole and allyamine. However, side-effects (if taken orally mostly caused hepatotoxicity) like, recurrent infections, frequent adverse reactions, requirement of prolonged treatment period, limitation to superficial infection (in case of onychomycosis, the drug is required to penetrate the nail plate), appearance of multi-drug resistant (MDR) fungus and associated bacterial infection has challenged the use of these agents with unsatisfactory clinical outcome.[2] Fungal infections can be fatal for patients who are receiving anticancer or immunosuppressive agents, since these patients are vulnerable to infections. When compared to oral treatment, tropical treatment could be advantageous because it could be applied directly on the infected sites and reduced toxicity due to elimination of hepatic metabolism.[6] The fungal infections require long-term therapy, which involves several weeks of treatment however; the patients tend to discontinue the treatment due to cost factor, which results in reoccurrence of the infections.[3] Hence, all these facts point the need to search for alternative or complementary agent against dermatophytes. Due to long-term traditional use and increased scientific recognition

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in recent years, extracts prepared from medicinal plants could be advantageous in this milieu to avoid the synthetic drugs usage.[7]

Although plant-derived compounds are gaining importance as an alternative medicine to treat various diseases, the extract or concoction prepared from medicinal plants are considered advantageous due to presence of various medicinally-active constituents in the preparation.[8] The plant extracts may contain a variety of secondary metabolites, including carotenoids, lignans, and polyphenols (phenolic acid, stibins, flavonoids, tannins and coumarins) which may work in concert to produce a wide range of biological effects, including antioxidant, antimicrobial, anti-inflammatory, antidiabetic, anticancer and anti-atherogenic. Some of the phytochemicals present in the plant extract preparation might provide therapeutic potential and others might curb the possible side-effects.[9] In case of dermatophytes treatment, the major problems such as MDR fungal development as well as the long time-period of treatment could be averted by the use of plant extracts that contain various medicinally-active compounds. Hence, in the present study, the methanolic extract of either leaf or seeds of some least explored medicinal plants for dermatophytes such as *Hypericum scabum, Terminalia chebula, Anagalis arvensis, Zanthoxylum americanum, Juglans regia, Syzygium jambolanum, Mentha piperita, Pimpinella anisum, Achillea millefolium, Xanthium strumarium, Rhoicissus digitata* and *Aegle marmelos* were prepared. The extracts screened for anti-fungal activity against *T. rubrum*. Further, using gas chromatography-mass spectrometry (GC-MS), the phytochemicals included in the extracts that shown strong anti-fungal action were measured and identified. Further, the extracts *in vitro* antioxidant properties were also examined.

MATERIALS AND METHODS

Materials

Tannic acid (TA), gallic acid (GA), quercetin, 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2′-azino-bis(3-e thylbenzothiazoline-6-sulfonic acid) (ABTS) and Folin's phenol reagent were purchased from the SRL chemicals, India. Atropine, polyvinylpolypyrrolidone (PVPP) and 2,4,6-Tri(2-pyridyl)-s-triazine (TPTZ) were purchased from Sigma Aldrich, India. Diosgenin was purchased from TCI, India. Potato Dextrose Agar (PDA) was purchased from Himedia, India.

Plant sample collection

Fresh, mature and healthy leaves and seeds of *Hypericum scabum, Terminalia chebula, Anagalis arvensis, Zanthoxylum americanum, Juglans regia, Syzygium jambolanum, Mentha piperita, Pimpinella anisum, Achillea millefolium, Xanthium strumarium, Rhoicissus digitata* and *Aegle marmelos* were collected from Hosur and its associated Ghats region, Krishnagiri District, Tamil Nadu, India. The collected plant samples were subjected to Botanical identification and authentication at the Department of Horticulture, Lalbagh, Bangalore, India. The seeds and leaves were cleaned, airtight containers were used to store them in the dark after being pulverized and shade dried.

Preparation of sample extracts

About 10 g of powdered air-dried leaves or seed samples were extracted using hot continuous Soxhlet extraction for 8 hr with methanol as a preferable solvent based on the literature survey. The methanol was evaporated at 50°C using rotary evaporator. The extracts obtained were stored at 4°C.

Microbial Strain

The test organism, *Trichophyton rubrum* (MTCC 7859) from Microbial Type Culture Collection (MTCC), India was purchased and revitalized at 27°C on PDA for 7 days as per the MTCC recommended growth parameters.

Antifungal activity assay

The punch well diffusion method was used to test the plant extracts anti-fungal properties.[10] 25 ml of PDA medium was added to the sterile petri dishes and allowed to solidify. About 200 µL inoculum (*Trichophyton rubrum*) was poured into the PDA plates and spread thoroughly using a plate spreader. Utilizing a sterile cork borer, 4 mm wells in the medium were made. About 200 µL of various plant extracts were added and then incubated at 27°C for 7 days. The plates were observed for its antifungal activity by measuring the clear zone around the well using caliber and recorded. The standard antifungal drug ketoconazole and 200 µl of methanol (98%) were used as negative and positive controls, respectively. The experiment was carried out three times, and the results are presented as means.

Minimum Inhibitory Concentration (MIC) determination

The methanol extract of *A. marmelos* leaf (AML-ME) and *P. anisum* seed (PAS-ME) that showed significant antifungal activity were selected for MIC determination using micro titer plate method with minor modifications.[11] Sterilized PDB broth (100 µl) was added in the testing wells. In the remaining surrounding wells of micro titer plate, 300 µl of deionized water was added to prevent the sample from drying. Methanol was added in the wells, B1, C1, E1 and F1 as blank. About 0.1% (w/v) of resazurin dye was added to the wells B2, C2, E2 and F2 as color blank. The wells B3, C3, E3 and F3 are control containing test organism and dye. About 100 100 µl of serially diluted samples (125, 100, 75, 50, 25, 10, 5 and 2 µg/ml) of AMME and PAME were added in duplicates in the row B/C and E/F from the well 4 to 11, respectively. The samples were incubated along with 100 µl of test organism and dye at 25°C for 24 hr. The wells were examined for visual color development by the lowest concentration of the extract for MIC determination. Presence of blue color indicates inhibition of the

organism growth whereas appearance of pink color indicates no inhibition.

Qualitative and quantitative analysis of phytochemicals

AML-ME and PAS-ME extracts were subjected to qualitative and quantitative analyses of the total phenolic content, total flavonoid content, alkaloids, saponins and tannins using the methods previously reported.[12-14]

Phenolics

The Folin's reagent was used to determine TPC. The reaction mixture consisted of the 10% Folin-Ciocalteau reagent and 0.4 ml of sample solution. After incubation for 5 min, 10% sodium carbonate was added, and the mixture was then left to sit for an additional 1 hour in the dark. The blue colour formed was read at 700 nm using UV-visible microplate reader (BioTek Epoch 2, Agilent, USA). The TPC were expressed as µg GAE/g of extract in terms of GA equivalent.

Flavonoids

TFC was determined by aluminum chloride reagent. Briefly, the reaction mixture consisted of 2 ml of 1 M sodium hydroxide and 0.3 ml of 10% aluminium chloride. For 40 min, the solution was incubated at room temperature in the dark. Using a UV-visible multi well plate reader, the absorbance was measured at 520 nm. The TFC were expressed as mg QE/g of extract in terms of quercetin equivalent.

Tannins

For tannin quantification, to 1 ml of sample 100 mg of PVPP was added and kept for agitation for 60 min then centrifuged. The phenolics content in samples without PVPP treatment and sample obtained after PVPP treatment were determined as described above. Total tannin was calculated using absorbance obtained by subtracting the absorbance of non-absorbed polyphenols with absorbance obtained for total polyphenols. In terms of tannic acid equivalent, the total tannin was calculated as mg TAE/g of dried extract.

Alkaloids

Bromocresol green was used to calculate the alkaloids' concentration. Sample solution, 1 ml of 0.2 M sodium phosphate buffer (pH 4.2), 1 ml of 2N HCl, and 5 ml of bromocresol green made up the reaction mixture (0.1 mM). 3 ml of chloroform was added to this solution and thoroughly shaken. The Yellow colour present in chloroform was measured at 430 nm. Alkaloids were measured in milligrammes of atropine equivalent as mg AE/g of dry extract.

Saponins

Vanillin reagent (8%, w/v in 99.9% ethanol) was added to the sample in 0.25 ml, and tubes were submerged in an ice-cold water bath. Slowly adding 2.5 ml of sulfuric acid at a 72% (v/v) concentration to the reaction mixture. The tubes were warmed at 60°C for 10 min after three minutes. At 544 nm after cooling down, absorbance was recorded. Diosgenin equivalents (mg DE/g of dried extract) were used to express the saponin content.

The results of each experiment were performed in triplicate and are presented as mean standard deviation.

GC-MS analysis

Using Clarus 680 GC (PerkinElmer, USA), the extracts of AML-ME and PAS-ME were analyzed. In order to separate the components, a fused silica column packed with Elite-5MS (5% biphenyl, 95% dimethylpolysiloxane, 30m x 0.25mm ID x 250m df) was used. Helium was used as carrier gas, flowing at a constant rate of 1 ml/min. About 260°C was employed as injector temperature, with the instrument received an injection of about 1µl of sample, and the oven was kept at 300°C.

The component spectrums were compared to a database of component spectrums maintained in the NIST (2008) library.

In vitro antioxidant assay

DPPH radical scavenging assay

The reaction mixture for the DPPH scavenging experiment included a 100 µM DPPH solution, various extract concentrations, and methanol to dilute the mixture to 1 ml. The reaction mixture was incubated at room temperature for 30 min in the dark. The extracts ability to scavenge free radicals was demonstrated by the initial purple color disappearance at 517nm.[15]

$$\text{Scavenging activity (\%)} = \left[\frac{\text{Absorbance (Control)} - \text{Absorbance(Sample)}}{\text{Absorbance (Control)}}\right] * 100$$

ABTS radical scavenging assay

The reaction of ABTS with potassium persulfate led to the creation of the ABTS radical cation (ABTS•+). The mixture was equilibrated at 30°C after being diluted with ethanol to an absorbance of 0.70 at 734 nm. The reaction mixture for the scavenging experiment included 900 l of ABTS solution, various extract concentrations, and ethanol to get the solution up to 1 ml. The reaction mixture was incubated at room temperature for 30 min in the dark. The loss of the original blue color served as an indicator of the extract capacity to scavenge free radicals.[16]

$$\text{Scavenging activity (\%)} = \left[\frac{\text{Absorbance (Control)} - \text{Absorbance(Sample)}}{\text{Absorbance (Control)}}\right] * 100$$

Ferric reducing antioxidant power assay (FRAP)

The reaction mixture is made up of 300 µl of FRAP reagent (300 mM acetate buffer, 10 mM TPTZ in 40 mM HCl, and 20 mM

$FeCl_3.6H_2O$) and various sample concentrations, and 1000 µl of distilled water. For 15 min, the reaction mixture was incubated at 37°C. At 593 nm, the blue colour that generated was detected.

The results of each experiment were performed in triplicate and are presented as mean standard deviation.

RESULTS

Screening for antifungal activity by various extracts

Figure 1 depicts an illustration of the zone of inhibition (ZOI) by different plant extracts against *T. rubrum*. Table 1 provides the diameter of ZOI by various extracts. Among the various extracts tested, AML-ME and PAS-ME showed ZOI of 16 mm and 22 mm, respectively.

Determination of Minimum inhibitory concentration (MIC)

The MIC of AML-ME and PAS-ME were calculated using resazurin-mediated microtiter plate assay. Incubation of *T. rubrum* along with AML-ME and PAS-ME up to 75 µg/ml concentration did not affect the growth of the organism (Figure 2). While *T. rubrum* growth was suppressed by AML-ME and PAS-ME concentrations of 100 g/ml and 150 g/ml, respectively. Hence, the MIC of both AML-ME and PAS-ME was found to be 100 µg/ml concentration (Table 2).

Phytochemical analysis of AML-ME and PAS-ME

Tables 3 and 4, respectively, present the findings of the qualitative and quantitative analyses of the AML-ME and PAS-ME, respectively. Both AML-ME and PAS-ME contained flavonoids, phenolics, tannins, alkaloids, steroids, and saponins. However, terpenoids was found only in PAS-ME. The results of quantitative analysis of AML-ME and PAS-ME were similar to that of qualitative analysis. The concentrations of flavonoids, saponins and tannins were high in PAS-ME. Whereas in AML-ME the concentrations of flavonoids, saponins, tannins and alkaloids were high. The compounds identified to be present in AML-ME and PAS-ME through GC-MS analysis is give in Table 5 and 6, respectively. The main compounds identified in AML-ME are N, N,-dimethyl-o-(1-methyl-butyl)-hydroxylamine (41.52%), 13-tetradece-11-yn-1-ol (15.21%), n-hexadecanoic acid (12.53%), Propanal, 2,3-dihydroxy-, (s)- (5.22%) and 1,3-butanediol (4.46%). The main compounds identified in PAS-ME are hexamethylene chloroiodide (41.4%), cyclopentene,3-hexyl (19.23%), E-2-octadecadecen-1-ol (12.02%), n-hexadecanoic acid (10.31%) and 2-cyclopentene-1-undecanoic acid, ethyl ester (8.57%).

In vitro antioxidant activities of AML-ME and PAS-ME

AML-ME and PAS-ME showed *in vitro* antioxidant activities such DDPH radical scavenging activity (Figures 3A and 3B) and ABTS radical scavenging activity (Figures 3C and 3D). The radical scavenging activity by AML-ME and PAS-ME is dose-dependent. The IC_{50} (half maximal inhibitory concentration) value of PAS-ME was determined to be 146.06 µg/ml for DPPH radical scavenging activity and 59.81 µg/ml for ABTS radical scavenging activity, respectively. Further, figure 3E and 3F showed dose-dependent FRAP activity of AML-ME and PAS-ME at the concentrations 100 µg/ml to 500 µg/ml and 50 µg/ml to 250 µg/ml, respectively.

DISCUSSION

The antifungal activity of methanolic extract obtained from leaf or seed of 12 medicinal plants belonging to different families was tested against the dermatophyte *T. rubrum*. Among the plants tested, *A. marmelos* leaf and *P. anisum* seed showed high antifungal activity against *T. rubrum*. *A. marmelos* belongs to family rutaceae and has been used in traditional medicine in India from pre-historic period. All the parts of *A. marmelos* have been studied for various biological activities such as antidiarrheal anticancer, immune system stimulation, antidiabetic,

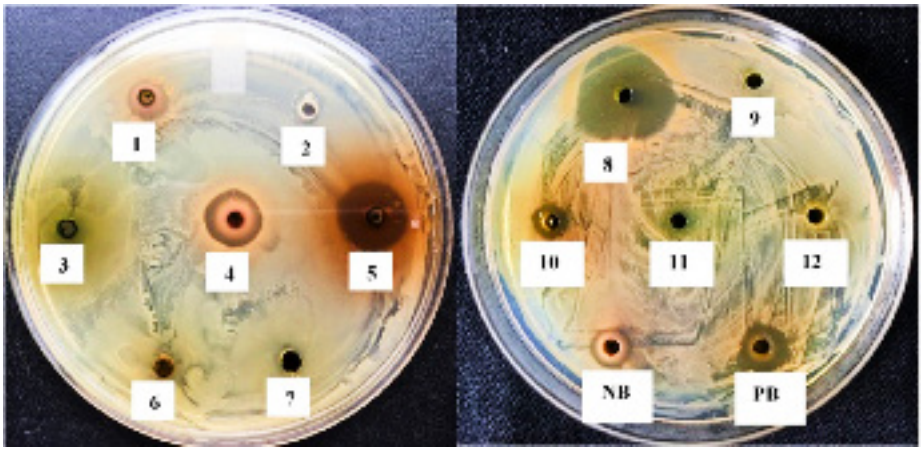

Figure 1: Screening of antifungal activity by different plants. Zone of antifungal inhibition of leaves or seed methanol extract of various plants (numbering as per Table 1) on *T. rubrum*, PB-Positive control, NB-Negative control.

Table 1: Zone of Inhibition (ZOI) values of the plant extracts against *T. rubrum* on PDA medium.

Sl. No.	Plant name	Diameter of ZOI (mm) against *Trichophyton rubrum*
1	*Hypericum scabum*	4
2	*Terminalia chebula*	2
3	*Zanthoxylum americanum*	1
4	*Anagalis arvensis*	7
5	*Aegle marmelos*	16
6	*Xanthium strumarium*	1
7	*Mentha piperita*	1
8	*Pimpinella anisum*	22
9	*Juglans regia*	2
10	*Syzygium jambolanum*	6
11	*Achillea millefolium*	3
12	*Rhoicissus digitata*	2
PB	Positive Control	6
NB	Negative Control	3

radio-protective, antioxidant, antipyretic, and antigenotoxic.[17] *Staphylococcus aureus, Bacillus cereus, Proteus mirabilis, S. epidermidis, Streptococcus haemolyticus* group A, *Pseudomonas aeruginosa, Klebsiella pneumoniae, Salmonella typhi* and *Escherichia coli*, have all been reported to be sensitive to the leaf extract of *A. marmelos*.[18,19] Further, antifungal activity against the fungus such as *Candida albicans, Candida tropicalis* and *Aspergillus flavus* has been shown by *A. marmelos* leaf extract.[18] In a previous study, the cold extract prepared from *A. marmelos* leaf has shown antifungal activity against the dermatophytes *Microsporum canis, T. rubrum, Epidermophyton floccosum, M. gypseum* and *T. mentagrophytes*.[20] However, the MIC of cold methanol extract was found to be 200 µg/ml concentration, which is high in comparison to MIC obtained in the present study (100 µg/ml) against *T. rubrum* by hot extraction of *A. marmelos* leaf. This result shows that hot extraction of *A. marmelos* leaf is advantageous in obtaining the phytochemicals required for antifungal activity. *P. anisum* L. (Anise), belongs to *Umbelliferae* family and traditionally used against seizure and epilepsy.[21] *P. anisum* has broad-spectrum pharmacological effects such as analgesic, antidiabetic, antioxidant, anticonvulsant and muscle relaxant.[22] Numerous investigations have demonstrated the antibacterial activity of Proteus vulgaris essential oil against *B. cereus, E. coli, S. aureus, K. pneumoniae, P. aeruginosa* and

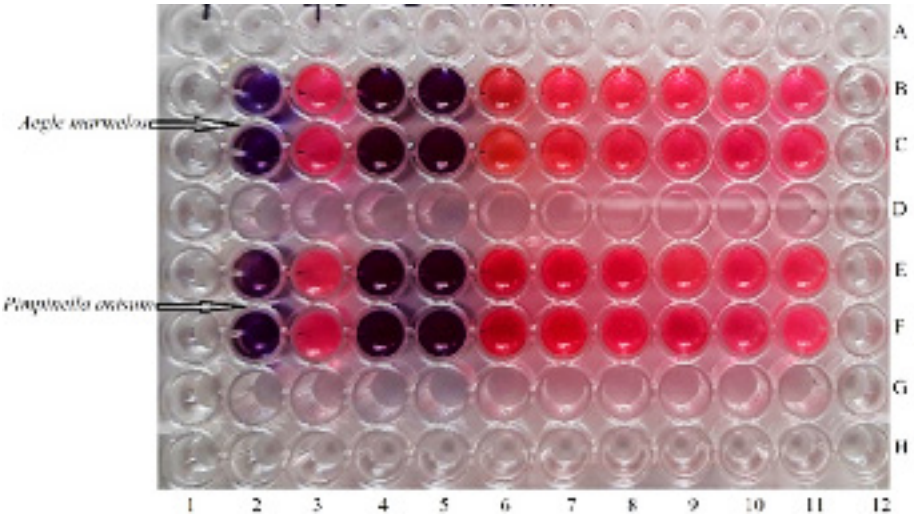

Figure 2: Determination of MIC values of AML-ME and PAS-ME. Visual color development in micro titer plate of growth inhibition by AML-ME and PAS-ME on *T. rubrum.*

Table 2: Inhibition effect of PAS-ME and AML-ME at different concentrations against *T. rubrum*.

Plant name	MIC (µg/ml) against *Trichophyton rubrum*							
	2	5	10	25	50	75	100	125
AML-ME	-	-	-	-	-	-	+	++
PAS-ME	-	-	-	-	-	-	+	++

+ indicates appearance of blue color (inhibition)- indicates appearance of pink color (no inhibition)

Table 3: Qualitative determination of the various phytochemicals in PAS-ME and AML-ME.

Phytochemicals	Sample	
	AML-ME	PAS-ME
Alkaloids	+	++
Phenols	++	++
Flavonoids	+	++
Terpenoids	-	+
Tannins	++	+++
Saponins	++	+++
Steroids	++	+++

+++ Highly Present; ++ Slightly present; + Present; - Absent

Table 4: Quantitative determination of the various phytochemicals in PAS-ME and AML-ME.

Phytochemicals	Sample	
	AML-ME	PAS-ME
Alkaloids (AE/g of extract)	215 ± 1.84	2.5 ± 0.82
Phenols (GAE/g of extract)	16.5 ± 1.21	25 ± 2.4
Flavonoids (QE/g of extract)	65 ± 2.6	100 ± 4.6
Saponins (DE/g of extract)	97.4 ± 3.2	167.2 ± 6.5
Tannins (TAE/g of extract)	182.5 ± 4.6	433 ± 4.3

Proteus typhi.[21,23] It has been previously documented that oral cavity-derived *C. parapsilosis, C. albicans, C. krusei*, and *C. glabrata* exhibit antifungal activity.[24] Encapsulation of essential oil from *P. anisum* and *Coriandrum sativum* using chitosan biopolymer has been found to act as food preservative through antifungal (against *Aspergillus flavus*) and antiaflatoxigenic activity.[25] Also, Antifungal effect of *P. anisum* essential oil against *Penicillium brevicompactum, P. polonicum, P. funiculosum, P. crustosum, P. glabrum, P. expansum, P. oxalicum*, and *P. chrysogenum* has been reported.[26]

The presence of secondary metabolites such flavonoids, phenolics, tannins and saponins in the extract may be the cause of AML-ME and PAS-ME antifungal activity. Different levels of interactions between phytoconstituents and fungus are involved in the antifungal activity such as interaction with the outer cellular components, or cytoplasmic membrane or cytoplasmic constituents. In most cases, interaction with all three levels is involved in the antifungal activity by plant-derived extracts or compounds.[8] The antifungal activity of secondary metabolites found in plants involves a variety of mechanisms of action, including ergosterol biosynthesis, apoptosis induction through activation of metacaspase and promoting cytochrome c release, antifolate activity, inhibition of fatty acid synthesis, inhibition of the synthesis of virulent factors, inhibition of (1,3)-D-glucan synthase activity, and fungal cell wall disruption.[27,28] Alkaloids

Table 5: List of phytochemicals identified by GC-MS analysis in AML-ME.

Retention Time	Compounds	Area (%)	Molecular formula	Molecular weight
2.518	2-Aminononadecane	1.719	$C_{19}H_{41}N$	283
4.449	1,3-Butanediol	4.465	$C_4H_{10}O_2$	90
6.055	3-Amino-2-Oxazolidinone	1.610	$C_3H_6O_2N_2$	102
7.085	N, N,-Dimethyl-o-(1-methyl-butyl)-hydroxylamine	41.522	$C_7H_{17}ON$	131
8.491	Hydroperoxide, 1-Methylbutyl	2.927	$C_4H_8O_2$	88
8.901	M-Dioxan-4-ol, 2,6-dimethyl	1.428	$C_5H_{12}O_2$	104
9.046	Propanal, 2,3-dihydroxy-, (s)-	5.221	$C_3H_6O_3$	90
12.352	5-(aminooxy) pentanoic acid	1.336	$C_8H_{18}O_2$	146
16.654	Hexane, 1-(ethenyloxy)-	1.550	$C_8H_{16}O$	128
18.375	n-Hexadecanoic acid	12.531	$C_{16}H_{32}O_2$	256
19.005	1,6;3,4-Dianhydro-2-deoxy-beta-d-lyxo-h exopyranose	1.437	$C_6H_8O_3$	128
20.070	13-Tetradece-11-yn-1-ol	15.218	$C_{14}H_{24}O$	208
20.365	1-Dodecyne	3.427	$C_{12}H_{22}$	166
20.886	2,7-Octadiene-1,6-diol, 2,6-dimethyl-, (z)-	1.330	$C_{10}H_{18}O_2$	170
21.211	cis-3-Hexenyl iso-butyrate	2.056	$C_{10}H_{18}O_2$	170
22.666	Trimethylamine, 1-cyclohexyl	2.223	$C_9H_{19}N$	141

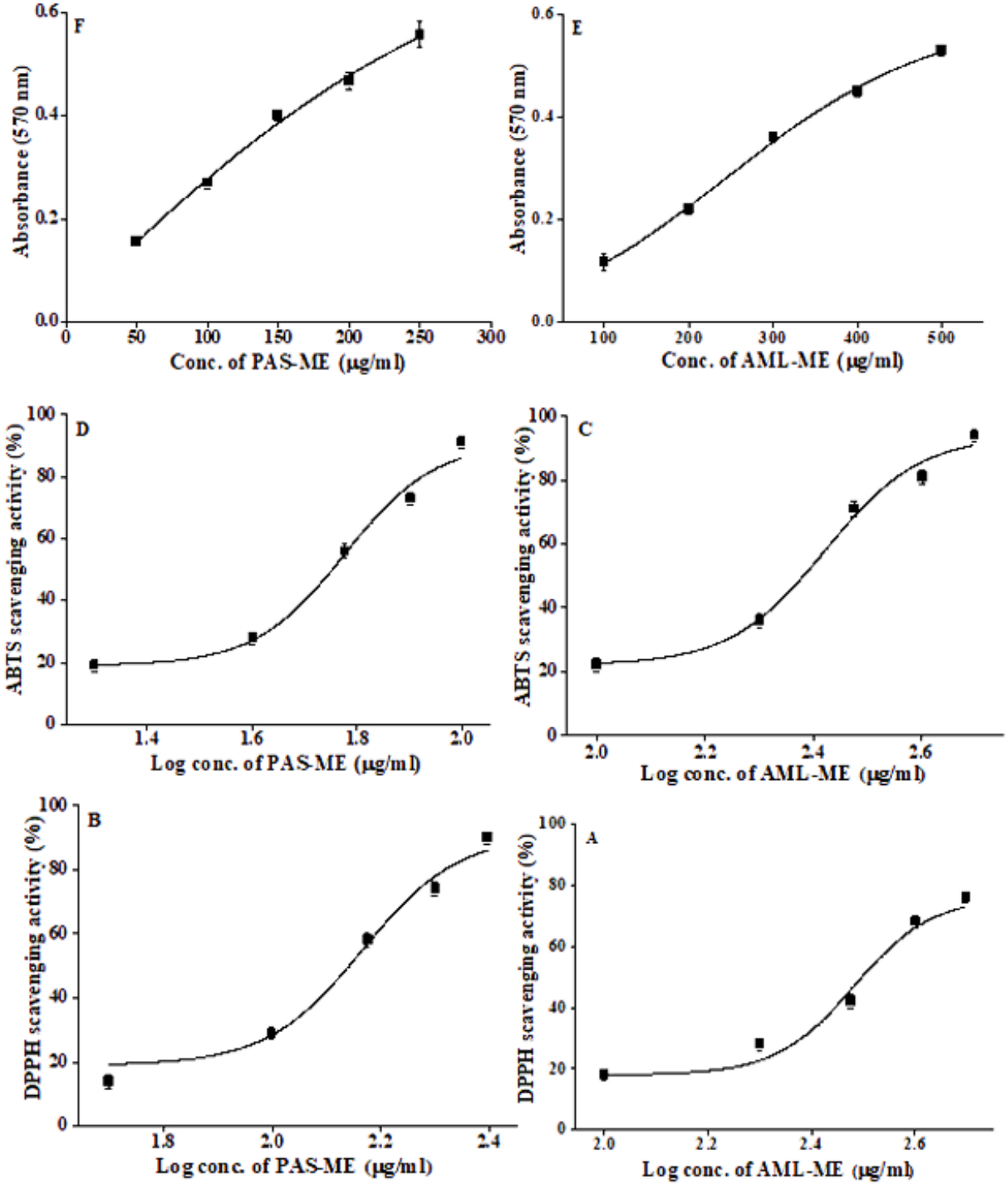

Figure 3: *In vitro* antioxidant activity of AML-ME and PAS-ME. DPPH radical scavenging activity (A and B), ABTS radical scavenging activity (C and D) and FRAP activity (E and F) of various concentrations of AML-ME and PAS-ME are shown.

Table 6: List of phytochemicals identified by GC-MS analysis in PAS-ME.

Retention Time	Compounds	Area (%)	Molecular formula	Molecular weight
18.39	n-Hexadecanoic acid	10.314	$C_{16}H_{32}O_2$	256
18.945	Cyclopentene,3-hexyl	19.235	$C_{11}H_{20}$	152
19.995	9-Octadecynoic acid	4.936	$C_{18}H_{32}O_2$	280
20.060	E-2-Octadecadecen-1-ol	12.029	$C_{18}H_{36}O$	268
20.786	11-(2-Cyclopentenyl) undecanoic acid, ethyl ester	8.577	$C_{18}H_{32}O_2$	280
21.326	Hexamethylene chloroiodide	41.417	$C_6H_{12}ClI$	246
24.707	cis-3-Hexenylpyruvate	1.345	$C_9H_{14}O_3$	170
26.523	4-Pentadecyne, 15-chlorocl	2.147	$C_{15}H_{27}Cl$	242

found in AML-ME might have upregulated oxidative stress, disrupted the cytoplasmic membrane or cross-linked with DNA or protein in the fungal cells thereby induced the fungal death.[29,30]

Some of the compounds identified in AML-ME and PAS-ME were reported to be present in extracts prepared from other plants which showed antimicrobial activity. The flavonoid compound 2,4-dimethyl-1,3-dioxane isolated from ayurvedic medicine Arjunarishta similar to the structure of compound m-dioxane-4-ol, 2,6-dimethyl identified in AML-ME is known to possess antifungal activity.[31,32] The ethanol stem and leaf extracts of *Saccharum munja* containing 5-(aminooxy) pentanoic acid has shown antibacterial activity.[33] The cyanobacterial extract that contained 1,6;2,3-dianhydro-4-deoxy-β-D-lyxo-h exopyranose has shown antifungal activity. There is antifungal action in the hydroxy fatty acids. The length of the fatty acid and the position of the hydroxyl group are two factors that generally affect the hydroxy fatty acid's antifungal action.[34,35] Hence, lengthy hydroxy fatty acid such as 13-Tetradece-11-yn-1-ol found in AML-ME would have contributed for antifungal activity. The ginseng extract that contained 1-dodecyne has previously shown antifungal activity against different species of fungus. The compound, hydroxylinalool (2,7-Octadiene-1,6-diol, 2,6-dimethyl-, (z)-), which is the derivative of linalool is identified in AML-ME. Linalool is well-known for its antifungal activity through disrupting the integrity of plasma membrane of fungus.[36] The macroalgae fraction prepared that contained abundant n-hexadecanoic acid has shown antifungal activity.[37] Antifungal activity of the acetylenic acids, 9-octadecynoic acid from the plant *Sommera sabiceoides* has been reported.[38] Similarly, 11-(2-Cyclopentenyl)undecanoic acid, ethyl ester (hydnocarpic acid) is a cyclopentenyl fatty acid present in plant genus *Hydnocarpus* and through inhibition of biotin synthesis has shown antimicrobial activity against *Mycobacterium.*[39] In addition to the compounds identified in GC-MS analysis, other compounds that might have present in AML-ME and PAS-ME would have altogether shown the antifungal activity against *T. rubrum*. The AML-ME and PAS-ME showed potent *in vitro* antioxidant activity in addition to antifungal activity which might be advantageous in curbing inflammatory activity as well as side-effects such as oxidative stress in mammalian cells when applied.[8] Hence, the presence of antifungal activity as well as antioxidant property by AML-ME and PAS-ME would give a therapeutic option without much side-effects. Also, if these extracts are used as complementary medicine along with known conventional antifungal agents, these extracts might not only enhance the antifungal potential but might also curb the side-effects caused due to oxidative stress in mammalian cells by the application of these conventional antifungal agents.

CONCLUSION

Among the 12 methanol extracts prepared from leaves or seeds of plants belonging to different family studied, the leaves extract of *A. marmelos* leaf and seed extract *P. anisum* showed high antifungal activity against dermatophyte *T. rubrum*. The extract's MIC was determined to be 100 µg/ml. We identified the phytochemicals in these extracts that may have combined forces to combat the fungus and restrict its growth. Interestingly, antioxidant activity has been found to be present in the extract which would have application to reduce the side-effects when used along with other conventional antifungal agents. Altogether, the therapeutic use of these extracts against dermatophytes is worth exploring further.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

GC-MS: Gas Chromatography Mass spectrophotometer; **MDR:** Multi drug resistance; **TPC:** Total phenolic concentration; **TFC:** Total flavonoid concentration.

SUMMARY

The antifungal efficacy of methanolic extracts of medicinal herbs against *T. rubrum* was tested.

To identify the phytochemicals included in the extracts, a minimum inhibitory concentration (MIC) of the extract was performed along with qualitative and quantitative analyses. The extracts of *Aegle marmelos* leaves (AML-ME) and *Pimpinella anisum* seed (PMS-ME) showed high zone of inhibition of 16 and 22 mm, respectively. MIC of both the extracts was found to be 100 µg/ml. Phytochemical analysis revealed the presence of phenolic compounds, flavonoids, tannins and saponins in AML-ME and PAS-ME. The existence of antifungal and antioxidant activities in the AML-ME and PAS-ME may be helpful in further investigating these extracts as an alternative or supplemental therapy to the currently known antifungal drugs.

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