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Isolation, identification and anti-fungal susceptibility pattern of dermatophytes from clinical samples

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ABSTRACT

Aims: Dermatophytosis is an emerging health problem in all age group people. The treatment is very challenging due to the high side effects of oral anti-fungal drugs and the increased resistance pattern against drugs and ointments. The aim of this study was to evaluate the sensitivity and resistance pattern of isolated and characterized dermatophytes to find the sensitive essential oil as a natural source of medicine to inhibit dermatophytosis.
Methodology and results: A total of 50 dermatophytes were isolated from different age groups and further sensitivity testing was performed against anti-fungal drugs, ointments, and essential oils. The best sensitivity was observed against anti-fungal cream and three essential oils.
Conclusion, significance and impact of study: From 50 dermatophytes, the highest percentage of dermatophytes were obtained by the male patient under the age group of 19-64. In all, *Trichophyton* were the highly isolated species and showed high sensitivity against essential oil. Anti-fungal susceptibility testing is also vital for resistance surveillance and for comparing the *in vitro* activity of new and existing agents. The present study shows that natural essential oil can be a good alternative source of medication against these dermatophytes with no side effects.

Keywords: Dermatophytes, antimicrobial susceptibility, resistance, sensitive

INTRODUCTION

Dermatophytes are considered under a group of keratinophilic and keratinolytic fungi having the capacity to infect keratinized tissue (skin, nails and hair) of humans and animals. Dermatophyte infections are generally divided into three categories, *viz*., cutaneous (infection in an upper nonliving layer), subcutaneous (under the skin) and systemic (deeper tissue infection). The severity of dermatophyte infection depends on various factors like the secreted products of fungus and the consequence of the host's immune reactions, the virulency of the infecting strain or species, the site of infection and local environmental factors (Grumbt *et al.*, 2013).

Epidermophyton, *Microsporum* and *Trichophyton* are the three main genera that belong to dermatophytes. Dermatophytes are transmitted through direct contact (anthropophilic organism), animals (zoophilic organism) and soil (geophilic organism) or indirect contact (Venketsan *et al*., 2007).

In healthy individuals, most dermatophytic infections are not considered severe, mild infections can be easily treated with topical anti-fungal ointment preparation, but severe and systemic infections always require oral anti-fungal therapy in combination with topical treatment. The diagnosis of disease is mainly done clinically, but confusion may persist due to the topical application of steroid ointment and cream, lead misdiagnosis and efficient laboratory diagnosis and identification is needed. The laboratory identification is based on macroscopic observations like culture characteristics, pigmentation, growth rate, texture, etc., and microscopic examination of conidia (Frías-De-León *et al*., 2020).

In the past decades, the consumption of the anti-fungal drug has been raised due to low immunity and humans easily acquire environmental fungal infections. So there is an urgent need to screen out the common available dermatophytic strain against different tropical ointment and oral anti-fungal drugs for their susceptibility and resistance. The increased resistance indicates the possibility of inventing new anti-fungal preparation that may be synthetic, semi-synthetic or natural (Chaudhary and Kumar, 2016).

This study primarily focuses on isolation, identification of common dermatophytes in a community, their susceptibility pattern against tropical ointment, anti-fungal drugs and naturally occurring essential oils with high sensitivity and resistance patterns (Lassella *et al*., 2002).

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MATERIALS AND METHODS

Study design

The present study was performed in the Department of Microbiology, Sri Guru Ram Rai University, Dehradun, India, between November 2020 and July 2021. Clinically suspected patients of mild and chronic dermatophytosis from OPDs of different clinics in the Dehradun District were included in this study.

Patients who are under topical or systemic anti-fungal treatment were excluded from this study. Patient's personal information was always noted during the collection of samples like history, age, gender, occupation and economic profile. Samples were collected after obtaining informed oral and written consent from the patients.

Sample collection

All dermatophytes patients were scrutinized by taking all the aseptic precautions and hygenicity. The infected area was wiped correctly with 70% ethyl alcohol, and skin scales and nail cutting were collected by swabbing and gentle scrapping methods around the inflamed margin of the lesions (Chaya and Pande, 2007). Hairs were collected aseptically with sterilized tweezers, which contain hair follicles with shafts (Figure 1A, 1B and 1C).

Preliminary examination of sample

For initial identification, 10% KOH with 40% DMSO solution was used, and a processed sample was examined directly under a microscope and noticed the presence of unstained refractile fungal elements (Shalaby *et al*., 2016).

Screening of dermatophytes on selective media

Sabouraud's dextrose agar (SDA) and cycloheximide are the most commonly used media for the primary isolation of dermatophytes. In the present study, all the dermatophytic isolates were again screened and identified with the help of a suitable selective medium composed of milk, honey and bromothymol blue (MHB media). It is a new medium consisting of 10 g glucose, 10 g skimmed milk, 10 mL honey and 1 mL 1.6% bromothymol blue per 500 mL distilled water (honey: dissolved in hot distilled water and skimmed milk: dissolved in cold distilled water). After mixing the ingredients the medium was then autoclaved for 15 min at 121 °C (Taha *et al*., 2013).

All the specimens were cultured on MHB for a 15 days incubation period at 25 °C and the growth/colour change was observed every third day. The identification of dermatophytes on this medium depends upon the casein hydrolysis and colour change i.e. if media changed their colour from straw yellow to green and later followed blue, indicating the growth of dermatophytes (Figure 1D, 1E and 1F). Further, all the isolated samples were recultivated on Sabourad's dextrose agar (SDA) medium as working and stock culture.

Microscopic examination

All preliminary isolated identified, and screened samples were further microscopically examined through lactophenol cotton blue staining and all the confirmed samples were preserved in glycerol stock for further use (Figure 1G, 1H and 1I) (Havlickova *et al*., 2008).

Anti-fungal sensitivity

Anti-fungal susceptibility tests were performed against pathogenic dermatophytes as per NCCLS guidelines to define the percentage sensitivity and resistance of dermatophytes against oral anti-fungal drugs (fluconazole, terbinafine, ketoconazole, griseofulvin and itraconazole), anti-fungal ointment (fusidic acid, luliconazole, ketoconazole, clobestol, clotrimazole, terbinafine and miconazole) and especially against those essential oil which were commonly used by society from ancient time like clove oil, eucalyptus oil, garlic oil, teatree oil, neem oil, rosemary oil, onion oil, lemon oil and almond oil (Figure 2) (Bansod and Rai, 2008). Essential oil which are commonly used in Indian traditional system of treatment from the past two decades as an alternative conventional therapy for dermis infections known as Ayurveda and Unani treatment methods (Raut and Karuppayil, 2014; Lopes *et al*., 2017).

For anti-fungal susceptibility a spore suspension were prepared by using 2 mL of sterile water, vortex the suspension for 1 min and further viable count were determined by plating 100 µL of 10-fold dilutions to MHA plates. The lawn prepared by suspension on medium were used for further sensitivity analysis, suspension of anti-fungal drugs, anti-fungal ointment and essential oil were added to each disk (10 µL/disk) and kept aside for 10 min for better diffusion. All the plates were incubated at 27-30 °C for 21 days (Dogra *et al*., 2019).

All the incubated plates were further examined on a daily basis to check out the proper growth. Zones of inhibition were observed, then ZOI was measured in mm and all changes in zone diameter were noted over time. Interpretation of the zone was recorded as sensitive, intermediate, and resistant as per the size of the inhibition zone diameter.

RESULTS

A total of 50 dermatophytes were isolated from different clinics in Dehradun District, India and all were identified and confirmed through macroscopic and microscopic examination (Figure 1). It was recorded that a high percentage of dermatophytic samples were isolated from a male patient (56%) instead of a female (44%). Among all positive cases, a high percentage of *Trichophyton* spp. (82%) were recorded and considered a predominant etiological agent as a significant health concern in both genders (Table 1).

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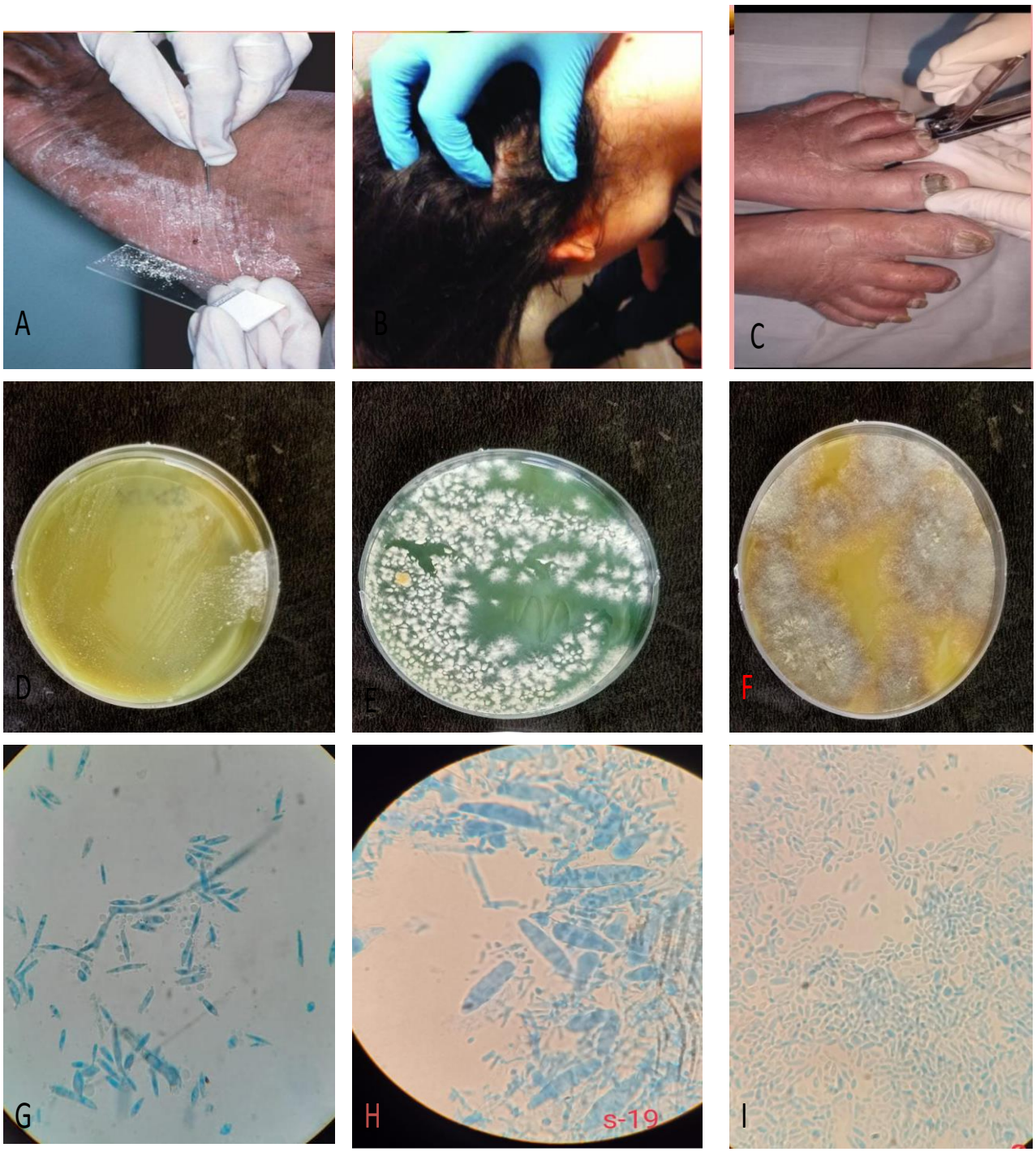

Figure 1: (A, B and C) Collection of dermatophyte samples from skin, hair and nails; (D) Isolation of *Trichophyton* on MHB media, showing colour change of media slight yellow to greenish; (E) Isolation of *Microsporum* on MHB media, colour change of media slight yellow to bluish-green; (F) Isolation of *Epidermophyton* on MHB media, colour change of media slight yellow to dark yellow colour; (G, H and I) Microscopic view of *Trichophyton*, *Microsporum* and *Epidermophyton*.

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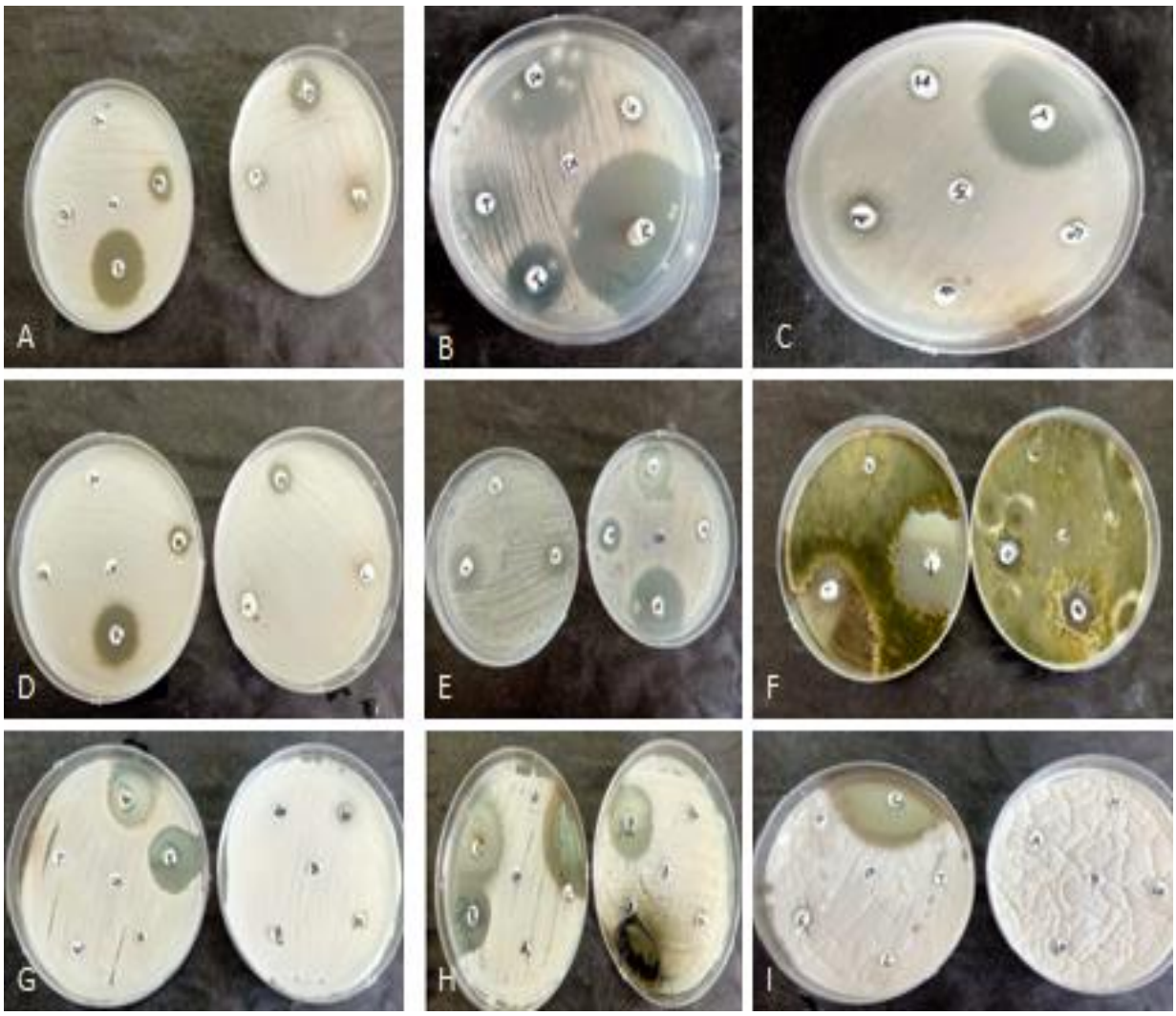

Figure 2: (A, D and G) Antimicrobial susceptibility of *Trichophyton* (dermatophytes) against anti-fungal drugs, anti-fungal ointment and essential oil, respectively; (B, E and H) Antimicrobial susceptibility of *Microsporum* (dermatophytes) against anti-fungal drugs, anti-fungal ointment and essential oil respectively; (C, F and I) Antimicrobial susceptibility of *Epidermatophytes* (dermatophytes) against anti-fungal drugs, anti-fungal ointment and essential oil, respectively.

Table 1: Analysis of dermatophytes (Gender-wise presentation).

Etiological agent	Clinical isolates		Male patient isolates		Female patient isolates	
	Number	Percentage	Number	Percentage	Number	Percentage
Trichophyton spp.	41	82	23	46	18	36
Microsporum spp.	4	8	2	4	2	4
Epidermophyton spp.	5	10	3	6	2	4
Total (n=50)	50	100	28	56	22	44

It was noted that the maximum infection rate was prevalent among the age group of 19 to 64 and it was also reported that a maximum of patients carried their infection for more than 6 months. The maximum number of samples were collected and isolated from the dermis layer (skin - 71.4%) (Table 2). It was also noted that all the strains of *Trichophyton*, *Microsporum* and *Epidermophyton* are less susceptible against anti-fungal drugs, whereas shows a good susceptibility against anti-fungal ointment and essential oil (Table 3, 4 and 5). It was observed that *Trichophyton* is highly resistant to drugs and ointment but shows a good sensitivity against two essential oils i.e., clove and eucalyptus oil (82.9% sensitivity each) whereas *Epidermatophyton* and

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Table 2: Characteristics of patients with dermatophytes by age group (2020-2021).

Variable	All ages (n=50)	0-18 years (n=8)	19-64 years (n=28)	≥65 years (n=14)
Age	16.66 ± 8.96	2.66 ± 1.45	9.33 ± 5.36	4.66 ± 1.764
Source of specimen				
Skin	34 (68%)	5 (62.5%)	20 (71.4%)	2 (14.2%)
Nail	12 (24%)	3 (37%)	5 (17.9%)	8 (57.14%)
Hair	4 (8%)	0 (0%)	3 (10.7%)	4 (28.6%)

Table 3: Anti-fungal susceptibility pattern of *Trichophyton* spp. against anti-fungal ointment, drug and essential oil.

Anti-fungal agent	Potency used	Susceptibility pattern; n=41			
		Susceptible (S)	Intermediate (I)	Resistant (R)	MTCC 296
Anti-fungal ointment					
Fusidic acid	10 g	1 (2.4%)	5 (12.1%)	35 (85.36%)	R
Luliconazole	10 g	15 (36.6%)	15 (36.6%)	11 (26.8%)	S
Ketoconazole	30 g	25 (60.9%)	9 (21.9%)	7 (17.1%)	S
Clobestol	20 g	0 (0%)	6 (14.6%)	35 (85.36%)	R
Clotrimazole	15 g	19 (46.3%)	13 (31.7%)	9 (21.9%)	S
Terbinafine	15 g	8 (19.5%)	12(29.2%)	21(51.2%)	S
Miconazole	15 g	14 (34.1%)	17 (41.5%)	10 (24.4%)	S
Anti-fungal drug					
Fluconazole	150 mg	30 (73.2%)	3 (7.3%)	8 (19.5%)	S
Terbinafine	250 mg	20 (48.8%)	10 (24.4%)	11 (26.8%)	S
Ketoconazole	200 mg	32 (78%)	2 (4.9%)	7 (17.1%)	S
Griseofulvin	250 mg	1 (2.4%)	14 (34.1%)	26 (63.4%)	R
Itraconazole	100 mg	16 (39%)	16 (39%)	9 (21.9%)	S
Essential oil					
Clove oil	Crude oil	34 (82.9%)	0 (0%)	7(17.1%)	S
Eucalyptus oil	Crude oil	34(82.9%)	0 (0%)	7(17.1%)	S
Garlic oil	Crude oil	8 (19.5%)	0 (0%)	33 (80.5%)	R

Table 4: Anti-fungal susceptibility pattern of *Microsporum* spp. against anti-fungal ointment, drug and essential oil.

Anti-fungal agent	Potency used	Susceptibility pattern; n=4			
		Susceptible (S)	Intermediate (I)	Resistant (R)	MTCC 2819
Anti-fungal ointment					
Fusidic acid	10 g	0 (0%)	1 (25%)	3 (75%)	R
Luliconazole	10 g	3 (75%)	0 (0%)	1 (25%)	S
Ketoconazole	30 g	0 (0%)	0(0%)	4 (100%)	R
Clobestol	20 g	4 (100%)	0(0%)	0 (0%)	S
Clotrimazole	15 g	2 (50%)	0(0%)	2 (50%)	S
Terbinafine	15 g	4 (100%)	0(0%)	0 (0%)	S
Miconazole	15 g	2 (50%)	0(0%)	2 (50%)	S
Anti-fungal drug					
Fluconazole	150 mg	4(100%)	0(0%)	0(0%)	S
Terbinafine	250 mg	1(25%)	2(50%)	1(25%)	S
Ketoconazole	200 mg	4(100%)	0(0%)	0(0%)	S
Griseofulvin	250 mg	0(0%)	1(25%)	3(75%)	R
Itraconazole	100 mg	4(100%)	0(0%)	0(0%)	S
Essential oil					
Clove oil	Crude oil	4(100%)	0(0%)	0(0%)	S
Eucalyptus oil	Crude oil	4(100%)	0(0%)	0(0%)	S
Garlic oil	Crude oil	0(0%)	0(0%)	4(100%)	R

Microsporum show a good sensitivity against all the antimicrobial agents with minimum resistance. So, this revealed that *Trichophyton* is a major concern and developed as a resistant strain in society and an urgent need to develop some natural therapeutic alternatives including those from natural sources such as essential oi-

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Table 5: Anti-fungal susceptibility pattern of *Epidermophyton* spp. against anti-fungal ointment, drug and essential oil.

Anti-fungal agent	Potency used	Susceptibility pattern; n=5			
		Susceptible (S)	Intermediate (I)	Resistant (R)	MTCC 7880
Anti-fungal ointment					
Fusidic acid	10 g	0 (0%)	0 (0%)	5 (100%)	R
Luliconazole	10 g	5 (100%)	0(0%)	0 (0%)	S
Ketoconazole	30 g	3 (60%)	0(0%)	2(40%)	S
Clobestol	20 g	0 (0%)	0(0%)	5 (100%)	S
Clotrimazole	15 g	3(60%)	1(20%)	1(20%)	S
Terbinafine	15 g	5 (100%)	0 (0%)	0 (0%)	S
Miconazole	15 g	3(60%)	0(0%)	2 (40%)	S
Anti-fungal drug					
Fluconazole	150 mg	3(60%)	0(0%)	2(40%)	S
Terbinafine	250 mg	5 (100%)	0(0%)	0(0%)	S
Ketoconazole	200 mg	4(80%)	1(20%)	0(0%)	S
Griseofulvin	250 mg	0(0%)	2(40%)	3(60%)	S
Itraconazole	100 mg	1(20%)	3(60%)	1 (20%)	S
Essential oil					
Clove oil	Crude oil	5(100%)	0(0%)	0(0%)	S
Eucalyptus oil	Crude oil	3(60%)	0(0%)	2(40%)	S
Garlic oil	Crude oil	0(0%)	0(0%)	5(100%)	R

-ls, secondary plant metabolites or its components for disease management with no side effect and cost effective (Figure 2).

CONCLUSION

Results suggest that the natural essential oil can be used as a therapeutic agent for the treatment of dermatophytes with no side effects. Further research is required for the preparation of herbal medicines through the use of natural products.

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