International Journal of Current Trends in Pharmaceutical Research
CODEN (USA): IJCTGM | ISSN: 2321-3760
Journal Home Page: www.pharmaresearchlibrary.com/ijctpr

Anti Fungal Activity of Ocimum Sanctum Linn (Lamiaceae) On Clinically Isolated Dermatophytic Fungi

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A B S T R A C T

Fungal diseases represent a critical problem to health and they are one of the main causes of morbidity and mortality worldwide. Human infections, particularly those involving the skin and mucosal surfaces, constitute a serious problem, especially in tropical and subtropical developing countries. Dermatophytoses are one of the most frequent skin diseases of human, pets and livestock. Pathogenic fungi, dermatophytes, have the ability to invade keratinized tissues of animals and humans and cause a disease, dermatophytosis, which is the commonest human contagious fungal disease. The present study aims to explore the antifungal activity of *Ocimum sanctum* on clinically isolated dermatophytic fungi. To know the phytoconstituents of *Ocimum sanctum*, the extracts were subjected to the analysis of macromolecules and secondary metabolites by using thin layer chromatography and high performance thin layer chromatography methods. The present study concluded that *Ocimum sanctum* leaf extracts showed both inhibitory and fungicidal activity against the dermatophytes studied. Secondary metabolites may be the reason for inhibition and fungicidal activity of this plant leaf extracts and fractions.

Keywords: Fungal diseases, dermatophytes, chromatography, fungicidal activity.

A R T I C L E I N F O

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Article History: Received 05 July 2021, **Accepted** 12 September 2021, **Available Online** 19 December 2021

Citation: Dharani Prasad P, *et al.* Anti Fungal Activity of Ocimum Sanctum Linn (Lamiaceae) on Clinically Isolated Dermatophytic Fungi. *Int. J. Curnt. Tren. Pharm, Res.*, 2021, 9(1): 36-45.

C O N T E N T S

1. Introduction

The occurrence of fungal infections is increasing at alarming rates, especially among immune compromised subjects, such as AIDS patients, transplanted patients, and neonates. Among the pathogens, species of Candida are generally associated with these infections, whose incidence is attributed to a variety of factors, either exogenous or endogenous. Fungal diseases represent a critical problem to health and they are one of the main causes of morbidity and mortality worldwide. Dermatophytoses are one of the most frequent skin diseases of human, pets and livestock. Pathogenic fungi, dermatophytes, have the ability to invade keratinized tissues of animals and humans and cause a disease, dermatophytosis, which is the commonest human contagious fungal disease. The disease is widely distributed all over the world with various degrees and more common in men than in women. There are three genera of fungi that cause dermatophytosis. They are Epidermophyton, Trichophyton and Candida spp. Contagiousness among animal communities, high cost of treatment, difficulty of control and the public health consequences explain their great importance. Different treatments have been recommended to control dermatophytes[(2)].

Herbal medicines are in great demand in the developed countries primarily for their cost effectiveness and no side effects. Patients requiring oral antifungal therapy are usually treated with terbinafine, itraconazole, or fluconazole. Griseofulvin can also treat tinea pedis, but may be less effective than other oral antifungals and requires a longer duration of therapy. Herbal therapy is becoming increasingly popular among patients and physicians. Plants contain a spectrum of secondary metabolites such as phenols, alkaloids, flavonoids, quinines, tannins and their glycosides and their essential oils. Traditional medicine practice is wide spread in China, India, Japan, Pakistan, Srilanka and Thailand. In china about 40% of the total medicine consumption is attributed to traditional tribal medicines. Having known the facts like incidence of dermatophytosis, etiological agents of dermatophytosis, the ill effects of antifungal drugs of today and the significance of plant based remedy for fungal infection, the present study was aimed to look for the prevalence pattern of various dermatophytic fungi that cause dermatophytosis. An attempt was also made to look for antifungal activity of three selected Indian medicinal plants. In addition to that the phytochemical constituents of the selected plants were also aimed [(22)].

2. Materials and Methods

Plant collection and Authentication

The plant material *Ocimum sanctum* was collected from Tirupati and its surrounding areas during the month of june 2020. It was identified and authenticated by an expert taxonomist Dr. K. Madhava chetty, Asst. Professor, Department of Botany, Sri Venkateswara University, Tirupati.

Collection of plant material

The fresh leaves were collected and dried. The dried leaves were ground to powder and stored in an air tight container until further use. The known quantity of *Ocimum sanctum* leaf powder was subjected for cold extraction with water and 100% ethyl alcohol and the aqueous extracts were collected. The extracts were dried in vacuum desicator and were stored in a sterile container for further use. The known quantity of *Ocimum sanctum* coarse powder was also successively extracted with various solvents like hexane, benzene, chloroform, ethyl acetate, methanol and water. Different fractions collected were filtered and evaporated in vacuum. Coding was given to various extracts and fractions and was stored till use. The dried extracts and fractions were weighed and dissolved in 5% dimethyl sulfoxide (DMSO) and was used for *in-vitro* testing of its activity against dermatophytes.

The different clinical isolates of dermatophytic fungi like *Trichophyton mentagrophytes*, *Trichophyton Rubrum*, *Microsporum nanum*, *Microsporum canis*, *Microsporum gypseum* and *Epidermophyton floccosum* were taken for antifungal activity of *Ocimum sanctum*. The selected isolates were grown on sabouraud dextrose agar (SDA). Twenty one day old culture of dermatophytic fungi was scraped with a sterile scalpel and macerated with sterile distilled water. The suspension was adjusted spectrophotometrically to an absorbance of 0.600 at 450 nm. By this way the fungal inoculum was prepared. For further study known quantity of this inoculum was used. The susceptibility testing was performed by the reference broth micro dilution method. Minimum fungicidal concentration (MFC) and minimum inhibitory concentration (MIC) were determined after 21 days of incubation at 35℃ by NCCLS (National Committee for Clinical Laboratory Standards) method. Medicinal plants are the good source of macromolecules and secondary metabolites. To know the phytoconstituents of *Ocimum sanctum*, the extracts were subjected to the analysis of macromolecules and secondary metabolites by using thin layer chromatography and high performance thin layer chromatography methods.

Preparation of plant extracts

The collected plant materials of *Ocimum sanctum* were dried with active ventilation at ambient temperature (25±1ºC) and chopped into small pieces, shade dried and coarsely powdered in by using suitable milling equipment. The coarsely powdered material weighed and extracted with Methanol using a soxhlet apparatus for five to six hours at temperature not exceeding the boiling point of the solvent. For each 100 grams of dry material 2 liters of solvent was used. The extracted solvents were concentrated under reduced pressure (in vacuum at 40°c) using a rotary evaporator. The residue obtained were

designated as crude extracts and stored in a freezer at -20°c until assayed [23].

Fungal isolation and maintenance

The swab samples from patients were taken by gently rubbing a sterile cotton swab over the infected area and were incubated in saboured dextrose agar for primary isolation of the pathogens. These plates were then aerobically incubated for 24-72 h at 30°C in B.O.D. (Biological Oxygen Demand) incubator for fungal species isolation. When growth was appeared on cultured plates, fungal pathogens were identified on the basis of carbohydrate assimilation or fermentation, their macroscopic and microscopic morphologic features after growth on specialized media. Moulds are identified primarily on the basis of their macroscopic and microscopic morphologic features [24]. The newer chromogenic method that couple for the presumptive identification of yeasts and moulds from clinical specimens. All isolated pathogens were compared with MTCC standard strains like *Trichophyton mentagrophytes*, *Trichophyton Rubrum*, *Microsporum gypseum*, *Microsporum canis*, *Epidermophyton floccosum* and *Candida albicans* respectively [25]. The different clinical isolates of dermatophytic fungi like *Trichophyton mentagrophytes*, *Trichophyton Rubrum*, *Candida albicans*, *Microsporum canis*, *Microsporum gypseum* and *Epidermophyton floccosum* were taken for antifungal activity of *Ocimum sanctum*. The selected isolates were grown on sabouraud dextrose agar (SDA). Twenty-one-day old culture of dermatophytic fungi was scraped with a sterile scalpel and macerated with sterile distilled water. The suspension was adjusted spectrophotometrically to an absorbance of 0.600 at 450 nm. The strains were maintained on sabouraud dextrose agar slants and the cultures were kept under refrigerated conditions and were sub cultured at every 15 days [26].

Assay for antifungal activity

Fungal inoculums were prepared from overnight grown cultures (24 h) in peptone water (HiMedia, Mumbai, India), and the turbidity was adjusted equivalent to 0.5 McFarland units (equivalent to 1.5 X 105 or 106 CFU/ml). Flucanozole (10µg/ml) was taken as the positive control for antifungal activity. The DMSO was taken as negative control to determine possible inhibitory activity of the *Ocimum sanctum* dilutant extract. The susceptibilities of the isolated pathogens were determined by using agar well diffusion method. 20ml of sabouraud dextrose agar (SDA) was taken into sterile universal bottles and these were then inoculated with 0.2ml of overnight culture of the test organism, mixed gently and poured into sterile petridishes. After setting wells of 6mm was punctured with the help of a sterilized cork borer into the pre-solidified sabouraud dextrose agar plates containing the test organism. Using the micropipette, 40 µl of each extract was poured into the different wells of the inoculated plates. Standard antifungal was used as positive control and fungal plates was incubated at 37^{o}C for 72 h. The diameter of zone of inhibition was measured. Each experiment was done in triplicate and mean values were taken. Antifungal activity was measured by the diameter (mm) of the clear inhibitory zone formed around the well [27]. Determination of minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC). The minimum inhibitory concentration (MIC) of different extracts was determined by micro dilution method using serially diluted (2 folds) plant extract according to the National Committee for Clinical Laboratory Standards (NCCLS). Equal volume of each extract and peptone broth were mixed in test tubes. The specifically, 0.1ml of standardized inoculums (1-2 ×105 cfu/ml) was added in each tube. The tube was incubated aerobically at 37ºC for 24 to 48hours. Two control tubes were maintained for each test batch, these included antifungal control (tube containing extract and growth media without inoculums) and organism control (tube containing the growth medium, saline and the inoculums). The lowest concentration (highest dilution) of the extract that produced no visible fungal growth (no turbidity) when compared with the control tube was regarded as MIC. However, the MFC was determined by sub culturing the test dilution on to SDA medium and incubated further for 72 h. The highest dilution that yielded no fungal colony on the solid medium was taken as MFC [28]. To know the phytoconstituents of *Ocimum sanctum*, the extracts were subjected to the analysis of macromolecules and secondary metabolites by using thin layer chromatography and high performance thin layer chromatography methods [29]

Preliminary Phytochemical Screening of Ocimum sanctum Extracts and fractions

A. Detection of Flavonoids

Magnesium and HCL Reduction Test:

The crude ethanolic extract was dissolved in a few ml of alcohol and few pieces of Magnesium ribbons and concentrated Hydro Chloric acid was added drop by drop. Pink or crimson red colour developed indicating the presence of flavonoids. The extract and fractions showed positive response for the above test.

B. Test for Phenolic compounds:

Ferric Chloride Test: To the ethanolic extract Ferric chloride solution was added. The appearance of blue colour indicates the presence of Phenolic compounds.

C. Test for Steroids/Terpenoids:

Liebermann-Burchard Test: The extract was dissolved in acetic anhydride by heating the mixture to boiling, cooled and then 1ml of cold concentrated sulfuric acid was added along the sides of the test tube. Color change at the junction was observed. Steroids/Triterpenoids and their glycosides give red, pink or blue color.

D. Test for Alkaloids: About 50mg of the extract was stirred with few ml of dilute Hydrochloric acid and filtered. The filtrate was subjected to various alkaloid reagents. The details of the tests are given hereunder.

Mayer's test: To a few ml of filtrate, 2-3 drops of Mayer's reagent was added along the sides of the test tube. A creamy precipitate indicates presence of alkaloids.

Wagner's test: To a few ml of filtrate, 2-3 drops of Wagner's reagent was added along the sides of the test tube. A reddish-brown precipitate indicates presence of alkaloids.

Hager's test: To a few ml of filtrate, 2-3 drops of Hager's reagent was added along the sides of the test tube. A yellow precipitate indicates presence of alkaloids.

Dragendroff's test: To a few ml of filtrate, 2-3 drops of Dragendroff's reagent was added along the sides of the test tube. A reddish-brown precipitate indicates presence of alkaloids.

E. Test for Carbohydrates:

Molisch Test: To the extract taken in a test tube, 1ml of water and 1ml of 5% (w/v) alcoholic α-naphtol solution were added and mixed well. The mixture was cooled and 1ml of concentrated sulfuric acid was added along the sides of the test tube. No color developed at the junction was recorded. Carbohydrates give a violet color at the junction.

Test for Saponins:

Froth Test: A small quantity of the extract was taken into a test tube. To it, was added 15ml of water and shaken vigorously and set aside. The froth produced was observed after 15 minutes. Saponins, if present, produce a stable froth [30]. The extract and fractions shows negative result for the above tests.

Identification of pathogenic fungi by microscopic examination

A. Lacto phenol cotton blue mount [LCB mount]

Lacto phenol cotton blue was used as a mounting medium. Lactic acid preserves the fungal structure and phenol acted as a disinfectant. A drop of lacto phenol cotton blue was placed on a clean slide. A small amount of fungal specimen was placed on it and the specimen was teased. Cover slip was placed on it. Then it was examined under the low power (l0x) of light microscope. For closer examination it was observed under high power objective (40x). Appearance of fungal hyphae and the arthroconidia were looked. They appear pale to dark blue due to its staining. The results were recorded.

B. Micro slide cidture technique

The slide culture technique was used when the lacto phenol cotton blue wet mount fails. Slide culture technique gives a clear picture about hyphae and conidia. Conidia are formed and attached to the conidiophores. The pattern of conidia formation on the conidiophores was essential for the identification of dermatophytic fungi.

C. Hair perforation test

The hair was observed at regular intervals by placing them in a drop of water on a microscopic slide. Microscopic examination was made for the presence of conicaJ perforation of the hair shaft and the results were recorded.

D. Urease test

The results of fungal stock culture the identified dermatophytes es were stored in a refrigerator at 40°C and were maintained for 3-4 months without sub culture. For long term preservation, agar slant cultures were covered with sterile mineral oil and were maintained without sub culture for up to 12 months.

Antifungal susceptibility test

In-vitro susceptibility testing was done for all fungal isolates to look for their antifungal susceptibility. This was done by standard broth dilution technique (NCCLS M-38A, 2002).

Preparation of fungal inoculum

Six different clinical isolates of dermatophytic fungi were taken for this study. Dermatophytic fungi taken were *Trichophyton mentagrophytes*, *Trichophyton rubrum*, *Microsporum nanum*, *Microsporum canis*, *Microsporum gypseum* and *Epidermophyton floccosum* are recorded and used for identification of the isolates. The selected clinical isolates were grown on sabouraud dextrose agar (SDA). Twenty one day old culture of dermatophytic fungi was scraped with sterile scalpel and macerated with 10ml of sterile distilled water. The suspension was adjusted spectrophotometrically to an absorbance of 0.600 at 450mm. This suspension was used as inoculums for MIC and MFC. Each tube witJi media was inoculated with 20µgm/ml of fungal suspension.

Minimal inhibitory concentration (MIC)

The susceptibility testing was determined by broth micro dilution method. MIC was analyzed by incorporating various concentrations of medicinal plant extracts and fractions of the plants chosen. The SD broth was prepared in a tube. The plant leaf extracts or fractions were diluted serially with dimethyl sulfoxide (DMSO). The diluted extracts were added to the SD broth and made u p to 2 ml volume. 20µgm/LI fungal inoculum was added to the extract containing broth. Positive controls were also maintained with known antifungal agents. Negative control was maintained with DMSO. All the tubes were incubated at 30° C and were read at every two days up to 14 days of incubation. The MICs were determined by visual observation for the inhibition of growth and were compared with that of the control (positive and negative) tubes.

Minimal fungicidal concentration (MFC)

The MFC was determined using the method of Rotimi et al, 1988. The MIC tubes, which showed no visible growth after 21 days incubation, were sub cultured on antibiotic free SDA plates and incubated at room temperature for 7 days. The MFC was recorded as the lowest concentration of the extract that prevented the growth of any fungal colony on the solid medium.

Assessment of fungi from environment

Most of the fungal infections spread through wind (fungal spores) hence airborne microbes were assessed by open plate method. The plates with media were exposed in the area like sampling area, hospital environment, consulting

room of the dermatologist's and laboratory atmosphere. SDA was used for fungus isolation.

Analysis of macromolecule

Macro and micro molecules are available in plants. These compounds are generally considered as a major elements utilized as nutrition. Secondary metabolites of plants are considered to be the active constituents which are used as a medicine. To know the active principle and nutritive value of *Aegle marmelos*, *Lawsonia inetinis* and *Ocimum sanctum*, the following simple methods were done to look for primary and secondary metabolites of the medicinal plants. Qualitative analysis of macromolecules was done for carbohydrates, proteins and lipids by the following methods.

Qualitative analysis of secondary metabolites by TLC (WHO, 1998)

TLC was performed by making use of readymade silica coated aluminium plate supplied by M/s Qualigens chemicals Pvt Ltd, Mumbai. The thickness of the TLC plate is about 0.25mm. TLC plate was activated by heating at 110°C for 10 miinutes in a hot Eiir oven and allowed to cool.

Application of sample 20x20cm size

TLC plate was taken. Starting line was drawn 15 mm above the lower edge using marking pencil. Plant extract was applied on the starting line as spot by making use of capillary tube. All the extracts and fractions of the single plant were appUed in a single plate with 15 mm space in between. Spot was made up to 4mm diameter and was allowed to cool at room temperature.

Development of chromatogram

TLC chamber was saturated with solvent mixture which contains chloroform: ethyl acetate: formic acid in the ratio of 5:4:1. The TLC plate was placed in the saturated chamber and the chromatogram was allowed to run. The chamber was closed and the chromatogram was developed at room temperature by allowing the solvent to absent the specified distance. TLC plate was removed from the chamber and position of the solvent front was marked. Solvent available in the plate was allowed to evaporate at room temperature.

Observation

TLC plate was observed in daylight initially. Sulphuric acid reagent was sprayed on the plate. Plate was placed in a hot air oven and heated at 60°C for 10 minutes, the coloured spot developed was observed. The distance of each spot to the point of application was recorded. Rf value was calculated by making use the formula,

Rf= Distance of the spot/Distance of the solvent travelled

Statistical analysis

Statistical analysis was done by students "t" test and one-way ANOVA using Origin 6.0 software for epidemiological studies. Anti-microbial activity results were assessed by calculating mean ± SD. p values were noted and the significance level was made.

3. Results and Discussion

Phytochemical Investigations of *Ocimum sanctum*:

The *Ocimum sanctum* plant's leaves are used for their medicinal value or for their nutritive value, analysis was done to look for their primary metabolites and secondary metabolites. The extracts were looked for the presence of primary metabolites like carbohydrates, proteins and lipids. *Ocimum sanctum* fractions like benzene, chloroform and ethyl acetate showed the presence of proteins only. Lipid was absent in all the fractions and extracts of *Ocimum sanctum.* The secondary metabolites of *Ocimum sanctum* showed in the presence of steroids in all extracts and fractions and all the extracts and fractions showed tannins except OSF 2 and OSF 4, given in table 5.1 and table 5.2.

Table 1: Phytochemical studies of primary metabolites in *Ocimum sanctum*

S.No	Extracts and fractions of Ocimim sanctum codes	Carbohydrates	Proteins	Lipids
1	OSF-1	-	+	-
2	OSF-2	-	-	-
3	OSF-3	-	+	-
4	OSF-4	-	+	-
5	OSF-5	-	+	-
6	OSF-6	-	+	-
7	OSE-7	-	+	-
8	OSE-8	-	+	-

(+) indicates presence of primary metabolites

(-) indicates absence of primary metabolites

OSF=*Ocimun sanctum* fraction, OSE=*Ocimum sanctum* extract

Table 2: Phytochemical studies of Secondary metabolites in *Ocimum sanctum*

S.No	Extracts and fractions of OS codes	Alkaloids	Terpenoids	Flavonoids	Phenolic compounds	Tannins	Steroids
1	OSF-1	-	-	-	-	+	+

2	OSF-2	-	-	-	-	-	+
3	OSF-3	-	-	-	-	+	+
4	OSF-4	-	-	-	-	-	+
5	OSF-5	-	-	-	-	+	+
6	OSF-6	-	-	-	-	+	+
7	OSE-7	-	-	-	-	+	+
8	OSE-8	-	-	-	-	+	+

(+) = Indicates the presence of metabolites, (-) = Indicates the absence of metabolites

The results of preliminary quantitative phytochemical screening of aqueous and alcoholic extracts of *Ocimum sanctum* revealed the presence of multiple polar and nonpolar chemical constituents. Steroids, terpenoids, flavonoids, phenolic compounds, lignin, proteins, carbohydrates were present in both extracts. Hexane fraction showed the availability of steroid, terpenoids, benzene extract also indicated the availability of steroid.

Alkaloid was also available in benzene fraction along with steroid. Chloroform and ethylacetate fractions yielded alkaloid, terpenoids and flavonoids. Methanol and water fractions showed the presence of terpenoids, flavonoids, phenolic compounds. Though charbohydrates, reducing sugars, proteins and amimoacids were available in all the extracts and fractions, they were not effectively involved in any of the inhibitory activities of fungus as they are the primary metabolites. *Ocimum sanctum* showed the presence of proteins in all the fractions and extracts. The Rf was between 0.02 and 0.98. Tannins and steroids were the two major secondary metabolites seen in *Ocimum sanctum*.

Antifungal activity of *Ocimum sanctum*

The use of medicinal plants as cure for dermatophytosis is an age old practice. Based on Siddha and Ayurveda medicines and natural system of medicines practiced in India, the Indian medicinal plant was chosen to look for their antidermatophytic activity. The plant chosen was *Ocimum sanctum*. The leaves of the plant was taken, cleaned, dried and subjected for cold extraction and fractionation with various solvents. The solvents used were hexane, benzene, chloroform, ethyl acetate, methanol, water and ethyl alcohol. Accordingly, three different crude extracts and five different fractions were collected.

Extracts were dried in vacuum and stored in -20°C till use. Coding was given to the extracts and fractions. The soluble nature of extracts and fractions were assessed and it was observed that crude water extract was soluble in PBS and crude ethyl alcohol extract was soluble in PBS: methanol in the ratio of 2:1. Whereas the fractions taken with various organic solvents and water were soluble in PBS: methanol: DMSO in the ratio of 2:1:1

Table 3: Solubility nature of extracts and fractions of *Ocimum sanctum*

S.No	Solvents	Extracts and fractions of Ocimum sanctum codes	Solubility
1	Hexane	OSF-1	PBS:Methanol:DMSO (2:1:1)
2	Benzene	OSF-2	PBS:Methanol:DMSO (2:1:1)
3	Chloroform	OSF-3	PBS:Methanol:DMSO (2:1:1)
4	Methanol	OSF-4	PBS:Methanol:DMSO (2:1:1)
5	Ethyl acetate	OSF-5	PBS:Methanol:DMSO (2:1:1)
6	Water	OSF-6	PBS:Methanol:DMSO (2:1:1)
7	Ethyl alcohol	OSE-7	PBS (100%)
8	Crude water	OSE-8	PBS:Methanol (1:1)

Antifungal activity of various extracts and fractions of the *Ocimum sanctum* plant was looked by tube dilution method. Both MIC and MFC were looked. Both extracts and all the fractions of *Ocimum sanctum* showed activity against dermatophytes starting from 300µg/ml concentration. Except ethyl alcohol extracts and water fractions all others were active against *T. rubrum*, *T. mentagrophytes* and *M. canis*. The water fraction act against *T. rubrum*, *M. nanum*, *M. canis*, *M. gypseum* and *E. floccosum*. Surprisingly, water fraction of *Ocimum sanctum*, did not show activity against *E. floccosum* even at 400 pg/ml.

Antifungal susceptibility

Azole compounds being given to cure fungal infections. In general for dermatophytosis fluconazole is the drug of choice. To have an understanding about the effect of these antifungal compounds on the clinical isolates sensitivity assay was performed by tube dilution method. The fungal isolates taken for sensitivity assay were *Trichophyton mentagrophytes*, *Trichophyton rubrum*, *M. canis*, *M. gypseum*, *M. nanum* and *Epidermophyton floccosum*. The results revealed that all the six predominant fungal isolates were sensitive to fluconazole at 400 µg/ml levels, except Microsporum nanum against flucanozole, which was sensitive even at 300 µg/ml level.

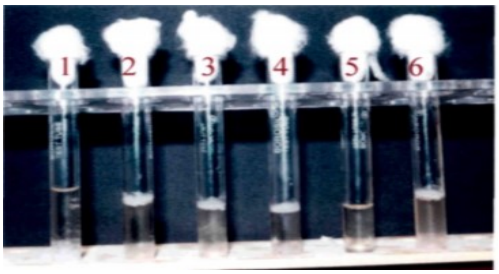
Table 4: Effect of flucanazole on various dermatophytic fungi

S.No	Dermatophytic fungi	Concentration of fluconazole (µgm/ml) and activity				
		50	100	200	300	400
1	Trichophyton mentagrophytes	-	-	-	-	+
2	Trichophyton rubrum	-	-	-	-	+
3	Microsporum canis	-	-	-	+	+
4	Microsporum gypseum	-	-	-	-	+
5	Microsporum nanum	-	-	-	-	+
6	Epidermophyton floccosum	-	-	-	-	+

(+) = Inhibition, (-) = No inhibition

In our study the percent activity i.e the total antifungal potency of studied *Ocimum sanctum* extracts was evaluated with varying concentrations ranging from 50µg/ml to 400µg/ml. The potency of the extract based on the zone of inhibition, was compared with standard commercial available antibiotic such as flucanzole (400µgm/ml) observed that the antifungal activity of crude extracts was less than those of the standard drug.

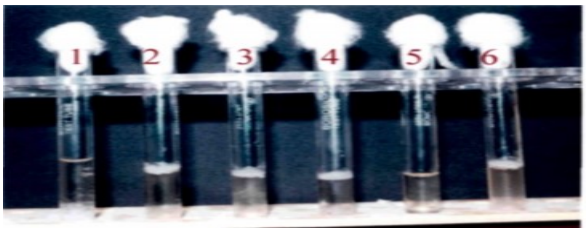
Trichophyton mentagrophytes



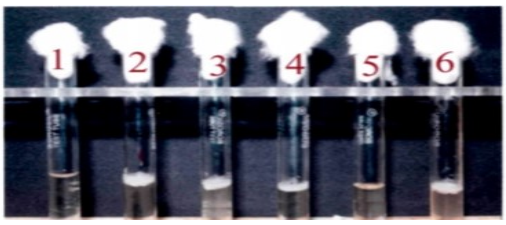
2) Trichophyton rubrum

3) Microsporum canis

4) Microsporum gypseum



5) Microsporum nanum



6) Epidermophyton floccosum

1=50 µgm/ml concentration, 2=100 µgm/ml concentration, 3=200 µgm/ml concentration
4= 300 µgm/ml concentration, 5=400 µgm/ml concentration, 6=Positive control

Figure 5.1: Effect of flucanazole on dermatophytic fungi

Table 5: Effect of *Ocimum sanctum* leaves extracts and fractions on Dermatophytic fungi

S.No	Dermatophytic fungi	Extraction and fraction code	Concentration of extracts and fractions (µgm) and activity of *Ocimum sanctum* (OS)				
			50	100	200	300	400
1	*Trichophyton*	OSF-1	-	-	-	+	+
		OSF-2	-	-	-	+	+
		OSF-3	-	-	-	+	+
		OSF-4	-	-	-	+	+
		OSF-5	-	-	-	+	+

	mentagrophytes	OSF-6	-	-	+	+	+
		OSE-7	-	-	+	+	+
		OSE-8	-	-	-	-	+
2	*Trichophyton rubrum*	OSF-1	-	-	-	-	+
		OSF-2	-	-	-	+	+
		OSF-3	-	-	-	+	+
		OSF-4	-	-	-	+	+
		OSF-5	-	-	-	+	+
		OSF-6	-	-	+	-	+
		OSE-7	-	-	-	+	+
		OSE-8	-	-	-	-	+
3	*Microsporum canis*	OSF-1	-	-	-	+	+
		OSF-2	-	-	-	+	+
		OSF-3	-	-	-	+	+
		OSF-4	-	-	-	+	+
		OSF-5	-	-	-	+	+
		OSF-6	-	-	-	-	+
		OSE-7	-	-	-	+	+
		OSE-8	-	-	-	-	+
4	*Microsporum gypseum*	OSF-1	-	-	-	+	+
		OSF-2	-	-	-	+	+
		OSF-3	-	-	-	+	+
		OSF-4	-	-	-	+	+
		OSF-5	-	-	-	+	+
		OSF-6	-	-	-	-	+
		OSE-7	-	-	-	+	+
		OSE-8	-	-	-	+	+
5	*Microsporum nanum*	OSF-1	-	-	-	+	+
		OSF-2	-	-	-	+	+
		OSF-3	-	-	-	+	+
		OSF-4	-	-	-	+	+
		OSF-5	-	-	-	+	+
		OSF-6	-	-	-	-+	+
		OSE-7	-	-	-	+	+
		OSE-8	-	-	-	-	+
6	*Epidermophyton floccosum*	OSF-1	-	-	-	+	+
		OSF-2	-	-	-	+	+
		OSF-3	-	-	-	+	+
		OSF-4	-	-	-	+	+
		OSF-5	-	-	-	+	+
		OSF-6	-	-	-	-	-+
		OSE-7	-	-	-	+	+
		OSE-8	-	-	-	+	+

(+) = Inhibition, (-) = No inhibition

MIC value is used to evaluate antimicrobial nature of plant extracts and minimum quantity of antimicrobial compound required to kill or arrest multiplication of all microorganisms present in the medium or body fluid. Extracts and fractions of *Ocimum sanctum* leaves showed effective antifungal activity against all dermatophytic fungus tested and the MIC ranged from (129.10±29.12) µg/mL to (428.26±34.16) µg/mL. Methanol fraction yielded effective MIC against *Trichophyton mentagrophytes* with MIC value of about (129.10±29.12) µg/mL concentrations. All the extracts and fractions effectively inhibited the growth of *Trichophyton mentagrophytes* at lower concentration from (129.10±29.12) µg/mL to (252.18±10.35) µg/mL, whereas other fungal growth inhibition needed at least (297.56±18.85) µg/mL of extracts and fractions. Among extracts alcoholic extract showed best activity. MIC required inhibiting the growth of *Trichophyton mentagrophytes* was (171.66±28.41) µg/mL

and (389.53±48.54) µg/mL against *Trichophyton rubrum*, (375.59±39.18) µg/mL against *Microsporum gypseum*, (389.13±15.42) µg/mL against *Microsporum nanum*, (381.48±39.15)µg/mL against *Microsporum canis* and (382.39±47.13) µg/mL against *Epidermophyton floccosum* (Table 5.6). Among fractions methanol fraction exhibited best activity against all dermatophytic fungus those are, (129.10±29.12) µg/mL against *Trichophyton mentagrophytes*, (368.28±56.15) µg/mL against *Trichophyton rubrum*, (375.36±35.80) µg/mL against *Microsporum gypseum*, (428.26±34.16) µg/mL against *Microsporum nanum*, (389.25±45.70) µg/mL against *Microsporum canis* and (392.45±15.33) µg/ mL against *Epidermophyton floccosum*.

Table 6: MIC of *Ocimum sanctum* extracts and fractions (µg/mL) (Mean± SD) (n=3)

Extract/ Fraction	Minimum Inhibitory Concentration (MIC)					
	Trichophyton mentagrophyte	*Trichophyton rubrum*	*Microsporum gypseum*	*Microsporum nanum*	*Microsporum canis*	*Epidermophyton flocossum*
Hexane Fraction	161.05±29.15	410.08±05.10	417.30±35.16	401.20±01.20	398.25±15.12	387.53±42.14
Benzene Fraction	210.15±15.01	375.65±18.25	402.53±48.12	411.26±19.82	410.25±28.10	419.15±26.00
Chloroform Fraction	148.35±31.15	402.25±50.20	362.32±52.21	421.12±25.10	378.42±42.20	418.35±48.28
Methanol Fraction	129.10±29.12	368.28±56.15	375.36±35.80	428.26±34.16	389.25±45.70	392.45±15.33
Ethyl acetate Fraction	189.12±23.55	398.52±45.15	387.25±24.85	368.23±25.10	392.35±34.82	408.66±35.26
Water Fraction	218.51±25.17	325.23±28.12	403.07±28.10	371.43±48.10	413.12±25.10	421.15±55.19
Alcoholic Extract	171.66±28.41	389.53±48.54	375.59±39.18	389.13±15.42	381.48±39.15	382.39±47.13
Aqueous Extract	252.18±10.35	365.20±76.65	297.56±18.85	204.26±24.72	310.56±25.18	419.45±18.33

Table 7: Analysis of phytoconstituents of *Ocimum sanctum* by TLC

S.No	Ocimum sanctum codes	Spot/ Rfd	
		1	2
1	OSF-1	-	0.98
2	OSF-2	-	0.98
3	OSF-3	0.52	0.98
4	OSF-4	0.52	0.98
5	OSF-5	-	0.97
6	OSF-6	0.52	0.98
7	OSE-7	-	0.96
8	OSE-8	0.52	0.89

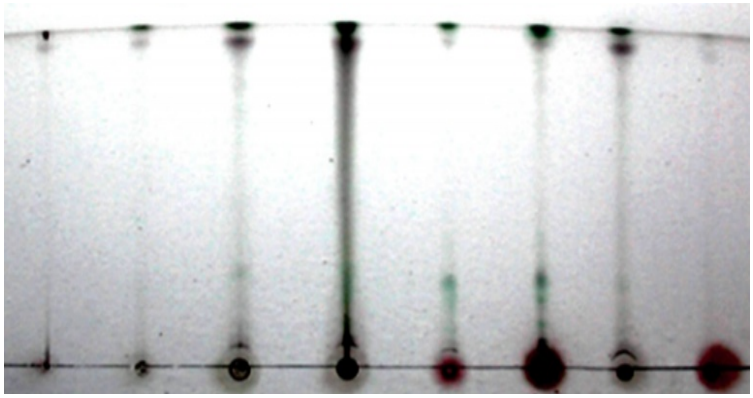


Figure 2: TLC of *Ocimum sanctum* of extracts and fractions

DISCUSSION

The incidence of dermatophytosis has been high since 1980s. It is still a serious problem in today. This is because of the antifungal drug resistance of the pathogen and the side effect exhibited by the drugs used to cure fungal diseases. Hence, there is a great demand for an efficient and safer treatment. Medicinal herbs could be an alternate source for the treatment of dermatophytosis. Herbal medicines are generally safer and free from side effects. Another important reason for this revival is that the effectiveness of many traditional medicines is now an accepted fact. This study showed that the leaf extracts of *Ocimum sanctum* has fungicidal activity. Few previous studies have comprehensively investigated the activity of medicinal plant leaves, bark extracts and oil against dermatophytes and other filamentous fungi. The MICs reported previously are based on broth micro dilution assay and NCCLS methods. Very few works were done on the fungicidal action of the plants *Ocimum sanctum*. The antifungal activity of other plant parts were also been reported earlier. The present study revealed that *Ocimum sanctum* leaf extracts showed both inhibitory and fungicidal activity against the dermatophytes studied. Secondary metabolites may be the reason for inhibition and fungicidal activity of this plant leaf extracts and fractions. The TLC confirmed the presence of these compounds. It is evident that the antidermatophytic activity of this plant may be due to secondary metabolites present in it. *Ocimum sanctum* plant leaf contains alkaloids, glycosides, saponins, tannins, volatile oil and ascorbic acid. Antidermatophytic activity of *Ocimum sanctum* may be due to these metabolites.

4. Conclusion

The present investigation reported that the medicinal plants used to study possess antimicrobial activity against the three clinical fungi which were used. These findings can form the basis for further studies of toxicity testing, isolate active compounds, elucidate the structures, and also evaluate them against wider range of resistant fungal strains with the goal to find new therapeutic principles. Keeping in view the tremendous pharmacological activities, wealth of available literature and the scientific validation of these three plants makes them as a source for a novel drug to alleviate the clinical conditions of dermatophytesis. The present study revealed that *Ocimum sanctum* leaf extracts showed both inhibitory and fungicidal activity against the dermatophytes studied. Secondary metabolites may be the reason for inhibition and fungicidal activity of this plant leaf extracts and fractions. TLC was confirmed the presence of these compounds. It is evident that the antidermatophytic activity of this plant may be due to secondary metabolites present in it.

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