Original Article

A Study to Compare the In-vitro Antifungal Activity of Cymbopogon Citratus and Terbinafine By Disc Diffusion Method Against the Selected Clinical Isolates of Dermatophytosis

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Objective: To compare the in-vitro antifungal activity of Cymbopogon citratus and terbinafine by disc diffusion method against the selected clinical isolates of dermatophytosis".

Methods: This randomized controlled study comprised of 20 clinical isolates of superficial mycotic infections. Each of the 20 samples was inoculated in three petri dishes (total 60) on Sabouraud dextrose agar (SDA) medium. The prepared discs containing methanol extract of Cymbopogon citratus and terbinafine were placed on the inoculated plates. The diameter of clear zone around each disc in millimeters was measured after three days.

Results: The antifungal activity of Cymbopogon citratus was 76%, 70%, 36% and 24% against Trichophyton rubrum, Trichophyton mentagrophyte, Microsporum canis and Microsporum audouinii respectively as compared to terbinafine (100%).

Conclusion: The Cymbopogon citratus is the potential candidate to be utilized for the treatment of superficial mycosis alternative to terbinafine.

Keywords: cymbopogon citratus,dermatophytosis, disc diffusion method.

Introduction

BiThe dermatophytosis is produced by dermatophytic fungi in the keratinized tissues. These cutaneous mycoses affect 20 - 25% of the world's population.[1] Superficial mycotic infections do not usually threaten life, but their intolerant itching cause misery and their presence may be a social stigma.[2]

The dermatophytosis is caused by 40 species of fungi which are grouped into three genera; Trichophyton, Microsporum and Epidermophyton.[3] These fungi invade the stratum corneum of epidermis and other keratinized tissues derived from it; such as skin, hair and nails of humans and animals. These dermatophytes utilize keratinous substrates of hair, skin and nails as the carbon, nitrogen and sulphur sources.[4,5]

The dermatophytosis can be classified clinically depending upon the site of the body involved. These include tinea capitis (scalp), tinea corporis (non-hairy skin), tinea ungium (nail), tinea cruris (groin), tinea pedis (athlete's foot), and tinea barbae (bearded areas of the face and neck).The gross appearance of the lesion includes an outer ring of active, progressing infection with central healing within the ring accompanied with itching, redness, scaling, or fissuring of the skin. The dermatophytosis transfers from animal (zoophilic dermatophytes) and soil (geophilic dermatophytes) to man or through direct infection by personal contact (anthropophilic dermatophytes).[1,6]

Dermatophyte infections are usually limited to the epidermis. These organisms cause a range of pathologic clinical presentations, including tinea pedis, tinea corporis, tinea cruris, Majocchi's granuloma (dermatophyte invades the dermis or subcutaneous tissue), tinea capitis, and tinea ungium. The dermatophytes of genus Trichophyton cause infections on skin, hair, and nails, and dermatophytes of genus Microsporum causes infections on skin and hair.[7] Trichophyton rubrum is the most commonly seen pathogen.[8]

Several antifungal agents are available for the treatment of superficial mycoses, and they are administered either topically or systemically. Terbinafine (Lamisil) is the drug of choice for treating dermatophytosis.[9] The commonly used antifungal drugs have many problems regarding toxicity, efficacy, economy, and their repeated use results in emergence of resistant strains.[10]

Now a day, the investigation of the efficacy of plant-based drugs in traditional medicine has attained great attention because these drugs produce few side effects, are low-priced and easily available.[11] Several plants native to Pakistan have been found with remarkable medicinal properties. Natural products, either as pure compounds or as standardized plant extracts, provide indefinite opportunities for new drugs. It is expected that plant extracts demonstrate target sites other than those used by currently available antimicrobials.[12]

Some of the plants with antifungal activities include Aloe vera (Kawar Gandal), Allium sativum (Garlic), Azadirachta indica (Neem), Cuminum cyminum (Jira), Cymbopogon martini (Tikhadi), Cymbopogon citratus (Lemon grass), Cinnamomum zylanicum (Dalchini), Eucalyptus globulus (Nilgri), Eugenia caryophyllata (Clove), Elettaria cardamomum (Cardamom), Mentha spicata (Mint), Ocimum sanctum (Tulsi), Trachyspermum captivum (Ajwain), Zingiber officinale (Zinger), Cassia angustifolia (Seena) and Trigonella foenumgraecum (Mathery)[13,14]

Cymbopogon citratus is an herb worldwide recognized as citronella grass or lemon grass and contains aldehyde, esters, saponin, terpenes, alcohols, ketone, flavonoids, chlorogenic acid, caffeic acid, p-coumaric acid and sugars.[15] It's reported phytoconstituents are essential oils that contain citral a, citral b, nerol, geraniol, citronellal, terpinolene, geranyl acetate, myrecene and terpinol methylheptenone.[16] A number of investigations have demonstrated antimicrobial and antifungal activities of Cymbopogon citratus leaves.[17,18]

The study conducted by Bokhari (2009),[18] revealed that methanol extract of lemon grass suppressed the growth of Trichophyton rubrum (95% inhibition) and Microsporum canis (75% inhibition) compared to griseofulvin (100% inhibition). It was noted by Ganjewala et al. (2012),[19] that >0.1 mg/ml dose of citral (one of the active principle of Cymbopogon citratus) was very effective against the hyphal growth of Trichophyton mentagrophyte while 0.2 mg/ml dose of citral caused irreversible damage to the cell membrane and organelles.

The antifungal tests are classified into three major groups, consisting of diffusion, dilution and bio-autographic methods.[20] The disc diffusion method is a qualitative test which could provide the information whether the crude extract possess antifungal properties.[21] Absorbent discs or spherical reservoirs having different amount of the substances to be examined are kept in contact with an inoculated solid medium and the area of the clear zone around the disc or reservoir is measured at the end of the incubation period and compared with standard drugs.[22]

Although many studies have been carried out on effects of Cymbopogon citratus extracts on some fungal species but no study is available on comparison of this herb to terbinafine. Keeping in view that, this study has been proposed "to estimate the possibility of using Cymbopogon citratus as alternative antifungal agents for dermatophytosis".

Methods

This randomized controlled study was conducted in the department of Pharmacology, Postgraduate Medical Institute in collaboration with department of Dermatology, Mayo Hospital and department of Pathology, King Edward Medical University, Lahore.

45 samples of skin scraping and 9 samples from infected nail were selected. The sample was mounted on a slide in 20% potassium hydroxide solution for 60-90 minutes for microscopic examination. The selected dermatophytes of superficial mycoses (Trichophyton rubrum, Trichophyton mentagrophyte, Microsporum canis, and Microsporum audouinii) were identified microscopically by their typical macroconidia pattern,[23] and then these samples were taken to the Department of Pathology, King Edward Medical University, Lahore for culture and sensitivity. The samples were excluded from the study if there was the growth of other non-selected pathogens. The specimens from clinically diagnosed cases by the dermatologist were taken by convenience sampling.

The plants were taken from local plant nursery and identified by the Department of Botany, University of Punjab, Lahore as "Cymbopogon citratus". The identified plants were thoroughly washed in running tap water and then rinsed in distilled water. The extract was prepared by solid liquid extraction principle. The Cymbopogon citratus extract was prepared with 90% methanol (1:10 w/v). Extract was preserved in airtight glass bottle and stored at 4ºC until further use. Subsequently, the working solution was prepared by dissolving the extract in distilled water in a concentration of 100 µg/ml18. The working solution of terbinafine was prepared by dissolving the terbinafine powder in distilled water to obtain a solution of terbinafine 0.25 µg/ml24. Terbinafine powder was taken from manufacturing company (Novartis).

Five samples of each selected four pathogens were included in the study. Each of the 20 samples was inoculated in three petri dishes (total 60) on the surface of the Sabouraud dextrose agar (SDA) medium with the sterile inoculating loop. Following inoculation, the culture plates were incubated in aerobic environment at 30-37oC for seven days and inspected daily for growth23.

The discs were prepared from Whatmann filter paper No. 1, 5.0 mm in diameter & 1mm in thickness. These discs were labeled as "C" (Cymbopogon citratus) and "T" (terbinafine). After sterilization, the discs labeled

and the discs labeled as "T" were impregnated with 20 µl (0.005µg) of terbinafine working solution (0.25 µg/ml).The antifungal activities of methanol extract of Cymbopogon citratus and aqueous solution of terbinafine were determined by disc diffusion method24. The discs were placed with equal distance on the pathogen inoculated plate. All plates were incubated at 30-37oC for three days. The inhibition zone was determined by measuring the diameter of clear zone around each disc in millimeters25. The readings from three plates for each sample were taken and the mean was calculated.

Statistical Analysis:

The data was entered and analyzed by Statistical Package for Social Sciences software (SPSS, version 18; SPSS Inc., Chicago, IL). The antifungal activity (zone of inhibition in mm) was expressed as mean ± standard deviation in table and bar diagram. One way ANOVA was applied to compare antifungal activity against individual species and against all species. Post hoc Tukey's test was applied to observe difference among treatments and difference among species. The p-value was calculated to note the significance of the results. A p-value of ≤ 0.05 was considered as statistically significant.

Results

Antifungal activity of Terbinafine against selected fungal pathogens. Mean zone of inhibition by terbinafine was 12.46±1.70 mm for Trichophyton rubrum,12.73±1.09 mm for Trichophyton mentagrophyte, 12.66±0.62 mm for Microsporum canis and 12.73±1.06 mm for Microsporum audouinii. Effect of terbinafine among all fungal pathogens was not statistically different as shown int **Table-1.** Antifungal activity of Cymbopogon citratus against selected fungal pathogens

Mean zone of inhibition by Cymbopogon citratus was 9.53±1.12 mm for Trichophyton rubrum, 8.93±0.98 mm for Trichophyton mentagrophyte, 4.53±0.64 mm for Microsporum canis and 3.06±1.68 mm for Microsporum audouinii as shown in **Table-2.** Comparison of antifungal activity of terbinafine, and Cymbopogon citratus against selected fungal pathogens

The antifungal activity of methanol extract of Cymbopogon citratus against Trichophyton rubrum, Trichophyton mentagrophyte, Microsporum canis and Microsporum audouinii was 76%, 70%, 36% and 24% respectively as compared to terbinafine (100%) as shown in **Fig-1.**

Table-1: Antifungal activity of terbinafine against selected fungal pathogens.

		Mean	SD	Minimum	Maximum	P-value
Terbinatine [control]	***Trichophyton rubrun***	12.46	1.70	11.00	15.33	0.087 (insignificant)
	Trichophyton mentagrophyte	12.73	1.09	11.00	14.00	
	Microsporum canis	12.66	0.62	12.00	13.33	
	Microsporum audouinii	12.73	1.06	11.33	14.00	

Table-2: Antifungal activity of Cymbopogoncitratus against selected fungal pathogens.

		Mean	SD	Minimum	Maximum	P-value
Cymbopogon [control]	***Trichophyton rubrun***	9.53	1.12	8.33	11.33	0.0000*
	Trichophyton mentagrophyte	8.93	0.98	7.33	9.67	
	Microsporum canis	4.53	0.64	3.67	5.33	
	Microsporum audouinii	3.06	1.68	1.33	5.33	

Table-3: Multiple comparison of antifungal effects of Cymbopogon citratus.

(I) Study Grpuops	(J) Study Groups)	P-value
	Triphophyton mentagrophyte	0.91
Trichophyton rubrum	Microsporum canis	0.00***
	Microsporum audouinii	0.00***
	Microsporum canis	0.00***
Trichophyton mentagrophte	Microsporum audouinii	0.00***
Microsporum canis	Microsporum audouinii	0.26

** ≤ 0.05 - Statistically significant*

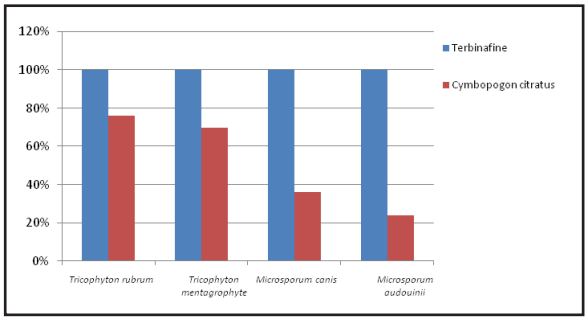

Fig-1: Comparison of antifungal activity of terbinafine to Cymbopogon citratus in percentage.

Discussion

In-vitro study of the antifungal activity enables the comparison between different antimycotics, which in turn may explain the reason for the lack of clinical response and help out clinicians in choosing an effective therapy for their patients.[26] Therefore, this study was planned to evaluate in-vitro antifungal activity of Cymbopogon citratus and terbinafine on 20 clinical isolates of Trichophyton rubrum, Trichophyton mentagrophyte, Microsporum canis and Microsporum audouinii.

In the present study, the mean zone of inhibition around Trichophyton rubrum growth was 9.53±1.12 mm with Cymbopogon citratus discs. A higher value of zone of inhibition of 15.5 mm was observed by Owoseni et al., (2010)[27] in their study. This difference in the observation relates possibly with the use of fungal pathogen from collection center rather than clinical isolates, use of a different growth medium (potato dextrose agar), different solvent (ethanol) for extraction preparation and different antifungal method (agar well diffusion method). All the above mentioned factors might have led to a higher value of zone of inhibition.

In the present study, the mean zone of inhibition around discs impregnated with methanol extract of Cymbopogon citratus for Trichophyton mentagrophyte growth was 8.93±0.98 mm. The higher value of diameter of clear zone i.e. 90 mm was observed in the study conducted by Wannissorn et. al, (1996).[28] This difference in the observation may be due to the use of Cymbopogon citratus oil cream rather than its methanol extract that may possibly have different phytoconstituents. There is also difference in the application of method to note antifungal activity (hole diffusion assay instead of disc diffusion method) and incubation period was 10 days which is quite different from 03 days in our study.

Microsporum canis is one of the fungal pathogens responsible for common infections in humans, which are difficult to treat. In this study, the in-vitro antifungal activity of methanol extract of Cymbopogon citratus was 4.53 ± 0.64 mm for Microsporum canis. A very high value of inhibition zone (30 ± 1.5) was recorded by Bokhari, (2009).[18]. The pathogen used in that study was obtained from culture collection laboratory of Nancy, France. The stock solution was diluted with distilled water but the dilution factor has not been mentioned. The method used to note the antifungal activity was agar well diffusion assay and the diameter of zone of inhibition was measured after seven days of incubation. The use of pure strain of pathogen, method of antifungal activity and duration of incubation; all are different from this study that might be the reason of a higher value of zone of inhibition in that study.

In order to predict the ability of a given antimycotic agent to eradicate the fungal isolate, in-vitro susceptibility test is helpful. Hence, in-vitro antifungal activity of methanol extract of Cymbopogon citratus on Microsporum audouinii growth was noted in this study. The mean zone of inhibition was 3.07±1.68 mm.

According to this study, the discs of terbinafine showed mean zone of inhibition of 12.47±1.70, 12.73±1.09, 12.67±0.62 and 12.73±1.06 mm around Trichophyton rubrum, Trichophyton mentagrophyte, Microsporum canis and Microsporum audouinii respectively. All these values are higher as compared to their counterpart observations of Cymbopogon citratus. This study corroborated previous findings made by Diogo et al., (2010)[29] and Nweze et al., (2010),[30] in which the higher values of zones of inhibition was observed for in-vitro use of terbinafine disc around the above said fungal pathogens.

Possible Mechanism: The antifungal activities of Cymbopogon citratus are linked to the presence of bioactive secondary metabolites like alkaloids, tannins, saponins, flavonoids, phenols, glycosides and diterpenes. Their mechanism of action appears to be predominantly on the fungal cell membrane, disrupting its structure causing leakage and cell death; blocking the membrane synthesis; inhibition of spore germination, fungal proliferation and cellular respiration.[27]

Conclusion

This work has indicated that methanol extract of Cymbopogon citratus possessed intermediate antifungal activity (7670%) against Trichophyton

Rubrum and Trichophyton mentagrophyte. The Microsporum canis and Microsporum audouinii were markedly resistant (36-24%) to Cymbopogon citratus as compared to terbinafine (100%). The ultimate conclusion of this study is that Cymbopogon citratus is the potential candidate to be utilized for the treatment of superficial mycosis alternative to terbinafine.

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