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Antifungal activity of *Terminalia chebula* fruit extracts

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ABSTRACT

The present study was aimed to investigate the anticandidal and antifungal potential of dried fruit extracts of *Terminalia chebula* against *Candida albicans*, *C. tropicalis C. glabrata*, *C. krusei*, *C. parapsilosis*, and *Aspergillus flavus*, *A. niger*, *A. fumigatus*, *Trichophyton mentagrophytes*, *T. rubrum*, *Microsporum gypseum*. Phytochemical analysis of methanol extracts of *T. chebula* dried fruits showed the presence of flavonoids, alkaloids, glycosides, saponins, tannins, terpenoids and steroids. Among the tested four extracts, the methanol extracts of *T. chebula* dried fruits exhibited the highest antifungal activity and their inhibition zone was ranged between 7.5 to 19.5mm. MIC and MFC values were between 62.5-250µg/ml and 250-500µg/ml respectively. Zone of inhibition (19.5 mm), MIC (62.5µg/ml) and MFC (125µg/ml) values observed in methanolic extracts of *T. chebula* dried fruits against *A. fumigates* and *T. mentagrophytes*. Our findings proved that methanolic extracts of *T. chebula* dried fruits were possessed substantial anticandidal and antifungal properties.

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INTRODUCTION

In tribal community, dermal and mucous infections are the most common type of infections occurs due their poor personal hygiene including food habits, sanitation and lacking of clean drinking water. The most common dermal fungal pathogens are belongs to dermatophytes and *Candida* sp. [1, 2]. Among the recent fungal infection case studies, >90% infections are due to *Candida albicans*, which leads to *Candidiasis* [3]. Due to the affiliations between domesticated animals and humans often end with either ringworm or tinea infections. Dermatophytic species generally grows on the outer surface such as nails, hair and skin on humans as well as domestic animals appendages also. Dermatophytes specifically infest keratin protein present in hair, hooves, beaks and skin. Dermatophyte microbes belong to three genuses such as *Trichophyton*, *Microsporum* and *Epidermophyton* [4].

Another pathogen, *Aspergillus fumigates* also cause deleterious fungal infections especially in less immunocompetent humans patients which eventually end with Aspergillosis [5,6]. Similarly, immunocompetent individuals are more susceptible towards *Cryptococcus neoformans*, *Candida albicans*, *Asperillgus fumigatus* and *Histoplasma capsulatum* [7]. Recently, long term antifungal therapies prone to microbial evolution and became a multi drug resistant strain against broad spectrum of antibiotics which currently used in drug therapies against fungal infection [8]. Indeed, conventional antifungal agents from medicinal plants were used as an effective alternative source.

Various human and plant microbes such as *Corynebacterium accolans*, *C. albicans*, *Staphylococcus aureus* and *Erwinia carotovora* were treated with polyphenolic ellagic acid, gallic acid and corilagin [9]. *Terminalia chebula* Retzius belongs to the Family Combretaceae which is native to India and Southeast Asia. It is commonly called as black myroblans [10]. This plant popularly used in folk medicine either alone or with other herbs and also used as cardiotonic, denrifrice, stomache tonic, laxative and purgative. *T. chebula* fruits are used to treat skin infections, diabetes, digestive disorders, burns, and kidney dysfunction and eye diseases [11,10]. Thus, the current study was undertaken to analyze the antifungal activity of various solvent extracts of *T. chebula* dried fruits.

MATERIALS AND METHODS

Collection and Preparation of Crude Extracts

The experimental plant *Terminalia chebula* Retzius (Combretaceae) were collected from Kovilur Panchyath at Javadhu hills of Eastern Ghats (Figure 1) and the fruits were utilized in this study. *T. chebula* fruits washed with tap water, 10% of Sodium hypochlorite solution and finally rinsed with distilled water. The dried fruits were grounded finely into powder. In Soxhlet apparatus (>78°C), 500gms of fine powder was mixed

Figure 1: Morphology of *Terminalia chebula* fruits

with non-polar to polar solvents *viz*., hexane, ethyl acetate, chloroform and methanol for 72hours of each solvent. In a rotary evaporator, (Heidolph, Germany) the solvents were evaporated under vacuum and the dried extracts were stored (4 °C).

Phytochemical Screening

The hexane, ethyl acetate, chloroform and methanolic crude extracts were used for the preparatory phytochemical examination of flavonoids, alkaloids, saponins, glycosides, tannins, steroids and terpenoids [11].

Microorganisms

The fungal strains *viz*., *Candida albicans*, *C. glabrata*, *C. tropicalis*, *C. parapsilosis*, *C. krusei*, *Aspergillus niger*, *A. flavus*, *A. fumigatus*, *Trichophyton rubrum*, *T. mentagrophytes* and *Microsporum gypseum* were obtained from Christian Medical College (CMC) Vellore, India. Sabouraud Dextrose Agar (SDA), Sabouraud Dextrose Broth (SDB) from Himedia Ltd., (Mumbai) used to study the antifungal activity for yeast and mould fungi.

Antifungal Assays

Disc diffusion method

T. chebula dried fruits crude extracts antifungal properties were studied by disc diffusion method [12]. For susceptibility test, 20ml SDA medium poured into Petri plates for yeast and filamentous fungi. 100µl of fungal suspension containing 10^6 CFU/ml and 10^4 spore/mL for yeast and mould fungi respectively were inoculated into the media. Under aseptic conditions, sterile (HiMedia) paper disc (6mm) were impregnated with 20µl of disparate extract concentrations (1000, 500 and 250µg/disc) dissolved in 10% DMSO and placed on the agar plates.

For positive control, Amphotericin - B (100units/disc) and Ketoconazole (10µg/disc) for yeast and *Aspergillus*, dermatophytes were used and 10% DMSO used as negative control. Finally, the inoculated plates were incubated for 24 - 48h at 28 °C for yeast, for 72-96 h for *Aspergillus* sp. and 4-7 days with dermatophytes at 30°C. Inhibition zone was measured in mms.

Determination of Minimum Inhibitory Concentration (MIC)

T. chebula dried fruit extracts MIC was determined by broth micro dilution technique as recommended by CLSI M27-A3 [13] and M38-A2 [14] for yeasts and filamentous fungi, respectively. The MIC values were analyzed in RPMI-1640 (pH 7.0, Himedia, Mumbai) which is composed of L-glutamine with morpholine propane sulfonic acid (MOPS) without $NaHCO_3$. 50mg/ml (20µL stock) of crude extracts mixed with 10% DMSO and dissolved in 980µl of RPMI-1640 medium solution. From this, solutions were two fold serial diluted in the ranges from 500 to 15.7µg/mL. In 96 well microtitre plates, 200µL of solution was poured into first well and then 100µL transferred to the next well containing 100µL of RPMI-1640, and the serially diluted to the other wells.

100µL of inoculum suspension was transmitted to each well to achieve a concentration of approximately $0.5\text{-}2.5\times10^3$ CFU/mL and $0.4\text{-}5\times10^4$ CFU/mL for yeasts and filamentous fungi. Without inoculum only sterile water was added for control well. The microtitre plates were incubated for 24-48 hrs at 28 °C for yeast and for 72-96 hrs for *Aspergillus* sp. and 4-7 days with dermatophytes at 30°C. Growth inhibition of different concentrations of crude extracts on *Candida*, *Aspergillus* and dermatophytic strains were compared with control results and the lowest concentration was recorded as MIC.

Determination of Minimum Fungicidal Concentration (MFC)

From each MIC titre well, SDA plates were inoculated with a loop of samples and then incubated for 24-48hrs at 28°C for yeast and for 72-96hrs for *Aspergillus* and 4-7 days for dermatophytes at 30°C. The lowest concentration of the extract which inhibits visible fungal growth after incubation was recorded as MFC.

Statistical Analysis

A result of statistical analysis was performed by using SPSS software 16.0 version (SPSS Inc., Chicago, IL, USA). The antifungal activity between the crude extracts and their significance were studied by student's t-test and their mean comparison was performed by one-way analysis of variance (ANOVA) and Duncan post hoc test. P value <0.05 was considered as statistically significant and the results were expressed as mean ± SD.

RESULTS

Hexane, ethyl acetate, chloroform and methanol extracts of *T. chebula* dried fruits were used to study the phytochemicals such as flavonoids, saponins, alkaloids, glycosides, terpenoids, steroids and tannins. Among the various extracts, methanolic extract of *T. chebula* dried fruits showed the strong presence of phytochemicals. Ethyl acetate extract contains all the phytochemicals tested except glycosides and steroids. Whereas, the chloroform extract contains only alkaloids, flavonoids, glycosides and tannins other tested phytochemicals are absent. The presence of alkaloids, flavonoids and glycosides was observed in the hexane extract (Table 1).

The antifungal activity of ethyl acetate, hexane, chloroform and methanol crude extracts of *T. chebula* dried fruits was possessed different degrees of activity against five *Candidal* strains, three *Aspergillus* species and three dermatophytic fungal strains. Methanol extract showed the highest activity (19.5mm) against *T. mentagrophytes*, followed by ethyl acetate, chloroform and hexane. The mean zone of inhibition, MIC and MFC values are presented in (Tables 2 and 3). The least MIC and MFC values (62.5 and 125 µg/ml) were observed against A. *fumigatus* and *T. mentagrophytes*. Among the four solvents, methanolic extracts showed significant antifungal activities. The positive control Amphotericin - B (100units/disc) for Yeast and Ketoconazole (10µg/disc) for filamentous fungi showed inhibition zone ranges from 13.5 to 16.8mm and 30.5 to 33.8mm respectively. The negative control (10 % DMSO) showed no inhibition zone against the tested fungal strains.

Table 1: Phytochemical analysis of the different extracts of *T. chebula* dried fruits

S. No	Phytochemicals	Hexane	Chloroform	Ethyl acetate	Methanol
1	Alkaloids	++	++	+++	+++
2	Flavonoids	+	++	++	+++
3	Saponins	-	-	++	+++
4	Glycosides	+	+	-	+++
5	Tannins	-	++	++	+++
6	Terpenoids	-	-	++	+++
7	Steroids	-	-	-	+++

- = Absence, + = weak, ++ = medium, +++ = strong

DISCUSSION

Based on the presence of secondary metabolites, the medicinal plants were used to treat human ailments. Recently, traditional pharmacological and ethnomedical approaches were received great attention and recognition in modern medicine due to the prospective medicinal plants [15].

In this studies, antifungal activity of hexane, ethyl acetate, chloroform and methanol extracts of *T. chebula* dried fruit were tested. All the tested microbial strains showed varied degree of inhibitions. *T. chebula* dried fruit methanol extract exhibited good activity against *T. mentagrophytes* [Mean inhibition zone (19.5mm), MIC (62.5µg/ml) and MFC (125µg/ml)] followed by ethyl acetate, chloroform and hexane extracts.

Aqueous extracts of *T. chebula* showed high antifungal activities whereas MIC and MFC values were found as 3.125µg/ml and 12.5µg/ml respectively.

T. chebula leaf extracts possessed antibacterial activities against four Gram positive bacterial strains such as *Enterococcus faedalis*, *Bacillus subtillis*, *Corynebacterium*, *Staphylococcus aureus* and three Gram-negative bacterial strains such as *Klebsiella pneumonia*, *Shigella boydii* and *Salmonella typhi* [17]. The aqueous extract of *T. chebula* showed antifungal activity against various dermatophytes and yeast (*Floccosum*, *Tricophyton rubrum*, *Microsporum gypseum*, *Epidermophyton* and *C. albicans*) [18,19,20].

Table 2: Anticandidal activities of different crude extracts of *Terminalia chebula* dried fruits

S. No	*Candida* sp. /Name of the crude extracts	Mean zone of inhibition(mm)[a]				MIC (µg/ml)	MFC (µg/ml)
		1000µg/disc	500µg/disc	250µg/disc	Positive control		
1	*Candida albicans*						
	Hexane	-	-	-	14.8±0.76	ND	ND
	Chloroform	7.48±0.46	-	-	14.8±0.76	ND	ND
	Ethyl acetate	8.47±0.50	-	-	14.8±0.76	ND	ND
	Methanol	10.98±0.49	8.49± 0.48	-	14.8±0.76	250	500
2	*Candida glabrata*						
	Hexane	-	-	-	14.81±0.76	ND	ND
	Chloroform	-	-	-	14.79±0.76	ND	ND
	Ethyl acetate	8.00±0.50	-	-	13.51±0.50	ND	ND
	Methanol	10.49±0.50	7.51±0.50	-	14.8±0.76	250	500
3	*Candida tropicalis*						
	Hexane	-	-	-	14.79±0.76	ND	ND
	Chloroform	-	-	-	14.78±0.76	ND	ND
	Ethyl acetate	-	-	-	14.79±0.76	ND	ND
	Methanol	11.49±0.50*	9.48±0.50	7.49±0.50	14.79±0.76	250	500
4	*Candida parapsilosis*						
	Hexane	-	-	-	14.49±0.50	ND	ND
	Chloroform	-	-	-	14.81±0.76	ND	ND
	Ethyl acetate	8.53± 0.50	-	-	14.79±0.76	ND	ND
	Methanol	10.50± 0.50	7.51±0.50	-	14.52±0.50	250	500
5	*Candida krusei*						
	Hexane	-	-	-	15.79±0.76	ND	ND
	Chloroform	7.81±0.76	-	-	14.83±0.76	ND	ND
	Ethyl acetate	9.51±0.50	7.53±0.50	-	16.81±0.76	ND	ND
	Methanol	12.49±0.50*	9.54±0.50	7.51±0.50	16.80±0.76	250	500

a-Mean of three replicates; ± - Standard deviation; ND: not determined.
Positive control: Amphotericin-B (100 units/disc), * $p<0.05$ levels considered as significant

Table 3: Antifungal activities of Terminalia chebula dried fruits different crude extracts

S. No	Fungal sp./ Name of the crude extracts	Mean zone of inhibition(mm)[a]				MIC (μg/ml)	MFC (μg/ml)
		1000μg/disc	500μg/disc	250μg/disc	Positive control		
6	*Aspergillus niger*						
	Hexane	10.49±0.50	7.51±0.50	-	30.5±0.50	250	500
	Chloroform	11.50±0.50	9.5±0.50	-	32.5±0.50	250	500
	Ethyl acetate	14.71±0.64	11.50±0.50	9.51±0.50	30.81±0.75	250	500
	Methanol	16.81±0.76	14.49±0.50	11.03±0.50	30.53±0.50	125	250
7	*Aspergillus flavus*						
	Hexane	12.51±0.50	10.49±0.50	8.80±0.76	31.51±0.50	250	500
	Chloroform	14.49±0.50	11.01±0.50	9.03±0.50	31.80± 0.76	250	500
	Ethyl acetate	15.79±0.76	13.52±0.50	10.49±0.50	30.51±0.50	250	500
	Methanol	18.50±0.50*	15.81±0.76	13.48±0.50	31.50±0.50	62.5	125
8	*Aspergillus fumigates*						
	Hexane	12.99±0.50	10.99±0.50	9.51±0.50	30.51±0.50	250	500
	Chloroform	14.83±0.76	12.51±0.50	10.52±0.50	31.52±0.50	250	500
	Ethyl acetate	16.52±0.50	13.53±0.50	11.83±0.76	30.53±0.50	125	250
	Methanol	18.50±0.50*	15.50±0.50	13.51±0.50	30.81±0.76	62.5	125
9	*Trichophyton rubrum*						
	Hexane	11.99±0.50	9.53±0.50	7.54± 0.50	32.81± 0.76	250	500
	Chloroform	13.51±0.50	11.03± 0.50	9.55± 0.50	32.52± 0.50	250	500
	Ethyl acetate	16.83±0.76	14.51± 0.50	11.03± 0.50	30.53± 0.50	125	250
	Methanol	17.71±0.64	15.53± 0.50	12.54± 0.50	30.84± 0.76	125	250
10	*Trichophyton mentagrophytes*						
	Hexane	13.54±0.50	11.01±0.50	9.54±0.50	33.51±0.50	250	500
	Chloroform	15.04±0.50	13.52±0.50	11.01±0.50	33.81±0.76	250	500
	Ethyl acetate	17.73±0.64	14.51±0.50	11.02±0.50	32.51±0.50	125	250
	Methanol	19.51±0.50*	16.52±0.50	14.54±0.76	32.81±0.76	62.5	125
11	*Microsporum gypseum*						
	Hexane	12.03±0.50	9.01±0.50	7.52±0.50	32.81±0.76	250	500
	Chloroform	13.74±0.64	11.51±0.50	9.81±0.76	32.53±0.50	250	500
	Ethyl acetate	15.52±0.50	13.52±0.50	11.02±0.50	32.51±0.50	250	500
	Methanol	17.71±0.64	14.51±0.51	11.51±0.50	31.83±0.76	125	250

a-Mean of three replicates; ND: not determined; Positive control: Ketaconazole (10μg/disc); * $p<0.05$ level

In this study, methanol extract of *T. chebula* showed highest mean of inhibition zone (19.5mm), lowest MIC and MFC (62.5 and 125.0μg/ml) values against *A. fumigatus* and *T. mentagrophytes*. Similarly, Fyhrquist *et al.* [21] reported the methanol extract of the leaf and roots of *T. sericea* and *T. sambesiaca* contains strong antifungal properties against *C. albicans*. Moreover, the methanol extract of *Diospyros virginiana* reported the presence of phenol compound which showed strong antibacterial and antifungal activities against *Enterobacter cloacae*, *E. coli*, *P. aeruginosa*, *S. typhimurium*, *B. cereus*, *Listeria monocytogenes*, *Micrococcus flavus and S. aureus* and *A. fumigatus*, *A. versicolor*, *A. ochraceus*, *A. niger*, *Trichoderma viride*, *Penicillium funiculosum*, *P. ochrochloron* and *P. var. cyclopium* [22]. Rathinamoorthy and Thilagavathi described the methanol extract of *T. chebula* fruits showed antibacterial activity against *Bacillus licheniformis* MTCC 429), and *Corynebacterium acnes* (MTCC 151), *Micrococcus luteus* (ATCC 49732) and *Corynebacterium sp.* (MTCC 8730).

In the present investigation, the methanol extract of *T. chebula* fruit possessed antifungal activity against few *Aspergillus* species, *Candida* species and Dermatophytic strains than other solvent extracts like hexane, chloroform and ethyl acetate which was similar to the previous report [23]. The methanol extract of *Gnaphalium polycaulon* leaf exhibited high antifungal activity against *Aspergillus flavus*, *A. fumigates*, *A. oryzae*, *Candida albicans* and *Penicilium notatum*.

Methanol extract of *T. chebula* showed potent inhibitory zone on tested fungal strains. Methanol solvent is known for its ability to isolate more antimicrobial compounds from plants including tannins, quassinoids, polyphenols, saponins, xanthoxyllines, lactones, terpenoids, totarol, phenones and flavones whereas water extracts showed terpenoids, starches, polypeptides, saponins, anthocyanins, tannins and lectins [24]. Various results confirmed that among the tested four solvents, methanol showed better results on antimicrobial substance extraction from medicinal plants [25,26,27].

In this study, methanol estract of *T. chebula* dried fruits showed highest antifungal activity due to their alkaloids, glycosides, saponins, tannins, flavonoids, terpenoids and steroids. In plants, alkaloids, tannins, flavonoids and many aromatic compounds or secondary metabolites which is involved in defense mechanism against invading microorganisms and various predators like herbivores and insects [28]. Similar results reported on 600μg/ml of ripened *T.chebula* fruits extracts against *T. mentagrophytes* [29]. The results of the present study evident the importance of *T. chebula* dried fruit extracts against various fungal pathogens. Further study on the isolation antifungal molecule and it's characterization from the methanol extract of *T. chebula* dried fruits are under process.

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