

SYNERGISTIC ANTICANDIDAL ACTIVITY OF TWO *TERMINALIA* SPECIES WITH POLYENE AND AZOLE GROUP OF ANTIBIOTICS AGAINST MULTIDRUG-RESISTANT CLINICAL ISOLATES OF *CANDIDA*

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ABSTRACT

Objectives: The aim of the present study was to evaluate synergistic anticandidal activity of two *Terminalia* species with polyene and azole group of antibiotics against multidrug-resistant clinical isolates of *Candida*.

Methods: The synergistic effect of ethanol extract of leaf of *Terminalia cattapa* and *Terminalia chebula* with six standard antibiotics, namely, amphotericin B, nystatin, fluconazole (FLC), ketoconazole (KT), clotrimazole (CC), and itraconazole (IT) was evaluated against the clinical isolates by disk diffusion assay.

Results: The synergistic activity of the antibiotics with ethanol extract of *T. chebula* was better than with that of *T. cattapa*. *T. chebula* ethanol extract increased the anticandidal effect of azole antibiotics FLC and KT, while it had a less synergistic effect on CC and IT.

Conclusion: Therefore, FLC/KT plus ethanol extract of *T. chebula* can be an interesting and alternative source of anticandidal agent against *Candida* species.

Keywords: *Terminalia chebula*, *Terminalia catappa*, Antibiotics, Fluconazole, Synergistic activity, Anticandidal agent.

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INTRODUCTION

Infections due to opportunistic fungi are occurring at an alarming rate. The incidence of fungal infection is rising not only in hospital environment but also in normal population. Fungal infections are the 4th leading cause of hematogenous infections, and the most common fungi are *Candida* species [1], namely, *Candida albicans*, *Candida tropicalis*, *Candida glabrata*, *Candida parapsilosis*, *Candida stellatoidea*, *Candida krusei*, and *Candida kyfer*; *C. albicans* being the most important one [2]. The range of fungal infections varies from colonization of mucosa to invasive deadly infections. *Candida* infections are treated with antifungal drugs such as amphotericin B (AMP), nystatin (NYS), itraconazole (IT), fluconazole (FLC), ketoconazole (KT), econazole, miconazole, clotrimazole (CC), and tioconazole. However, overuse of these drugs causes many side effects, including hypersensitivity, allergic reactions, immune suppression, and results in resistant strains [3,4]. These problems are also positively impacted by diseases such as cancer, diabetes, and AIDS [5,6]. The high cost, toxic effects, and increase in resistant strains compel the researchers to search for herbal drugs with fewer side effects or new approaches such as combination therapy or synergistic approach which results in additive or synergistic effect. The synergistic approach increases the spectrum of activity, decreases the risk of emergence of resistant strains, and deletes the harmful effects of immunotherapy with chemical agents [7]. The increasing recognition and importance of fungal infections with respect to resistance to antifungal drugs have stimulated the search for safe, natural therapeutic alternatives which are more effective, eco-friendly, and less toxic. It is essential to search for antifungals belonging to a wide range of structural classes selectively acting on different targets with less lateral effects. The best opportunity is natural plant extracts which can be tried individually or in combination. Plant extracts showed promising antibacterial and antifungal activities [8,9]. The concept of using single drugs to treat infections is changing. When drugs are used

in combination, the phytochemicals present in them exert different mode of action which increases the effectiveness of their antimicrobial therapy [10,11]. Thus, synergistic therapy may be the answer to the increase in fungal resistance to the existing antifungal drugs.

Terminalia chebula Retz. and *Terminalia catappa* L. belong to the family Combretaceae. They are traditionally used to treat many diseases and disorders. There are many reported activities for both these plants. Some of the reported activities of *T. chebula* are analgesic activity, antifertility activity, anti-inflammatory activity, antimicrobial activity, antioxidant activity, nephroprotective activity, and plasmid curing activity while that of *T. catappa* are allelopathic activity, antibacterial activity, anticlastogenic activity, antioxidant and antimicrobial activity, antiulcer activity, and molluscicidal activity. [12]. However, there are no reports till date on the anticandidal activity of these plants against clinical isolates of *Candida*. In the present work, we evaluated the anticandidal activity of ethanol extract of leaf of *T. chebula* and *T. catappa* against 19 clinical isolates of *Candida*. We also evaluated the synergistic effect of ethanol extract of leaf of these plants with six standards antibiotics, namely, AMP, NYS, FLC, KT, CC, and IT against the clinical isolates.

METHODS

Plant materials

The leaves of *T. chebula* Retz and *T. catappa* L. were collected from Jamnagar, Gujarat, India. The plant was compared with voucher specimen (voucher specimen No. PSN 291 and PSN292) deposited at the Department of Biosciences, Saurashtra University, Rajkot, Gujarat, India. The leaves were thoroughly washed with tap water and air-dried under shade. The dried leaves parts were homogenized to a fine powder and stored in airtight bottles which were later used for solvent extraction.

Extraction

The dried powder of leaf was extracted by the cold percolation method [13]. Ten grams of dried powder was added to 100 ml of hexane in a conical flask, which was plugged with cotton wool and kept on a rotary shaker at 120 rpm for 24 h. After 24 h, the extract was filtered with 8 layers of muslin cloth and centrifuged at 5000 rpm for 10 min. Supernatant was collected, and the solvent was evaporated. The residue was then added to 100 ml of ethanol in a conical flask, which was plugged with cotton wool and kept on a rotary shaker at 120 rpm for 24 h. After 24 h, the extract was filtered with 8 layers of muslin cloth and centrifuged at 5000 rpm for 10 min. The supernatant was collected, and the solvents were evaporated; the dry extract was stored at 4°C in airtight bottles.

Antibiotics used in the study

The six different antibiotics (Hexa Disc): AMP (100 units/disc), NYS (100 units/disc), KT (30 mcg/disc), FLC (10 mcg/disc), CC (10 mcg/disc), and IT (30 mcg/disc) were used in the study.

Fungal strains

Fifty clinical strains of *Candida* spp. were collected from various microbiological laboratories of Rajkot, Gujarat, India. Most of the strains were isolated from patients with skin infections and some from a urine sample. The isolates were cultured on Sabouraud dextrose agar medium with chloramphenicol under aerobic conditions within a temperature range of 28°C. The isolates were identified as *Candida* species on the basis of some biochemical tests such as blastospore/chlamyospore formation, color of colony on HiChrome *Candida* differential agar, carbohydrate assimilation test (sucrose, mannose, lactose, and malate), and negative absorption (urea and nitrate). The susceptibility test revealed 19 isolates as multidrug-resistant (MDR), and hence, these 19 isolates were used for further study. The 19 isolates are named as C1, C2, C3, C5, C6, C12, C13, C14, C15, C18, C21, C22, C23, C26, C30, C41, C42, C43, and C44.

Synergistic anticandidial assay

Synergistic anticandidial activity of the *T. chebula* and *T. catappa* leaf extracts with antibiotics (AMP, NYS, KT, FLC, CC, and IT) were evaluated using disc diffusion method [14]. The Petri plates were prepared by pouring 20 ml Sabouraud dextrose agar seeded with 200 µl test culture containing 1×10^8 cfu/ml as McFarland 0.5 turbidity standard. Plates were allowed to solidify. Standard antibiotics paper discs (6 mm) were impregnated with 20 µl of *T. chebula* and *T. catappa* leaf extracts (20 mg/ml) dissolved in dimethyl sulfoxide (DMSO) separately. The sterile paper discs were impregnated with 20 µl of *T. chebula* and *T. catappa* leaf extracts (in DMSO) separately. All the discs were allowed to saturate for 30 min and were placed on the surface of the agar plates which had previously been inoculated with *Candida* isolates, respectively. All plates were incubated for 48 h at 28°C. Results were recorded by measuring the zone of inhibition appearing around the discs. All the tests were performed in triplicate, and the mean values are presented.

Increase in fold area (IFA)

IFA was calculated as $(B^2 - A^2)/A^2$

Where A - inhibition zone for Antibiotics and B - inhibition zone for plant extract + antibiotics.

RESULTS

In disc diffusion method, the ethanol extract of *T. chebula* and *T. catappa*, two polyene antibiotics, namely, AMP and NYS, and four azole antibiotics, namely, KT, FLC, CC, and IT were evaluated for their antifungal activity against 19 *Candida* spp. The ethanol extract of both *Terminalia* species did not show any antifungal activity against any of the 19 clinical *Candida* isolates. Both polyene antibiotics showed a greater zone of inhibition as compared to azole antibiotics. Maximum zone of inhibition was shown by NYS (18.89) and minimum by IT (6.74). However, all the six antibiotics showed a greater zone of inhibition than ethanol extract of *T. catappa* and *T. chebula* (Figs. 1b-6b).

The synergistic antifungal activity of *T. chebula* and *T. catappa* ethanol extract with 6 antibiotics (AMP, NYS, KT, FLC, CC, and IT) against 19 clinical isolates of *Candida* is given in Figs. 1a-6a. IFA values are given in Table 1. The antifungal activity of ethanol extract of both plants, antibiotics AMP, NYS, and their synergistic activity, i.e. ethanol extract of plant plus antibiotic AMP and NYS against all 19 clinical *Candida* isolates are given in Figs. 1a and 2a, and their mean zone of inhibition is given in Figs. 1b and 2b, respectively. Both these antibiotics did not exhibit any significant synergistic activity. *T. chebula* ethanol extract with both the antibiotics exhibited synergistic activity against only 3 isolates while *T. catappa* exhibited synergistic activity against 3 and 7 isolates with AMP and NYS, respectively. Maximum IFA value was 5.67 and 3.34 against C12 by ethanol extract of *T. catappa* and *T. chebula*, respectively, with AMP (Fig. 1 and Table 1); while it was 5.67 and 8.51 with NYS (Fig. 2 and Table 1).

The antifungal activity of *T. chebula* and *T. catappa* ethanol extract, 4 azole antibiotics KT, FLC, CC and IT, and their synergistic activity against all 19 clinical isolates are given in Figs. 3a-6a, and their mean zone of inhibition is given in Figs. 3b-6b, respectively. The mean zone of inhibition of antibiotic KT against 19 clinical isolates was 7.58. Both the plant extracts showed significant synergistic activity. The mean synergistic activity of *T. chebula* ethanol extract with KT was 12.21 while that of *T. catappa* was 13.16 (Fig. 3b). *T. chebula* ethanol extract showed synergistic activity against 17 isolates while *T. catappa* ethanol extract showed synergistic activity against 13 isolates. Maximum IFA value was 8.5 against isolate C42 by *T. catappa* extract and 4.44 against isolate C3 by *T. chebula* extract (Table 1). The IFA value was more with *T. catappa* extract than *T. chebula* extract (Table 1).

The antifungal activity with antibiotic FLC is given in Fig. 4. The ethanol extract of *T. chebula* with FLC showed synergistic activity against all the 19 isolates while *T. catappa* showed activity only against 8 isolates. The IFA values of *T. chebula* ranged from 0.94 to 4.84 (Table 1). Maximum IFA value was 4.84 against isolate C42. Maximum IFA value was 5.25 against isolate C1 and C26 by *T. catappa* extract.

The trend of antifungal activity with azole antibiotic CC was similar to that of FLC (Fig. 5). Mean synergistic activity (i.e. mean zone of inhibition) of *T. chebula* and *T. catappa* was 11.36 and 9.66, respectively.

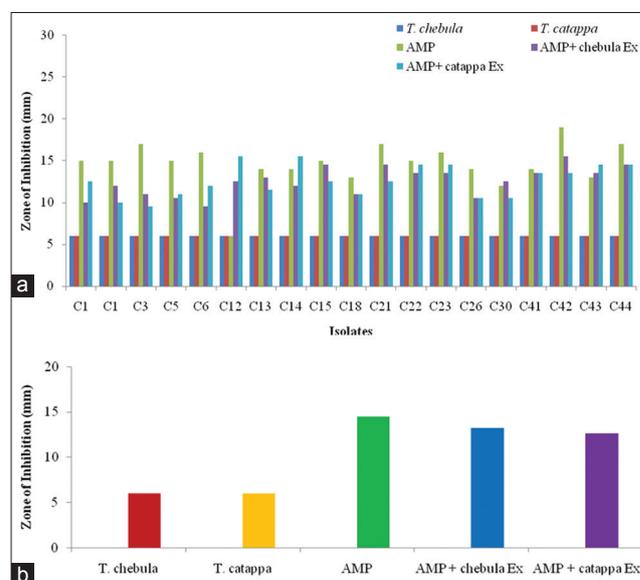


Fig. 1: (a) Synergistic anticandidial activity of *Terminalia chebula* and *Terminalia catappa* with amphotericin B (AMP) against *Candida* isolates. (b) Mean values of zone of inhibition of plant extracts and antibiotic alone and in combination against *Candida* isolates

Table 1: IFA of six different antibiotics with *T. chebula* and *T. catappa* leaf extracts

Isolate No.	AMP		NYS		KT		FLC		CC		IT	
	<i>T. chebula</i>	<i>T. catappa</i>										
C1	-	-	-	0.06	2.06	5.67	3.34	5.25	2.67	3.69	2.67	3.69
C2	-	-	-	-	2.06	3.69	3	3.69	2.67	-	2.06	2.67
C3	-	-	-	-	4.44	3.69	2.67	2.67	-	3.69	-	3.69
C5	-	-	-	0.30	1.78	3.69	2.06	-	2.06	-	2.06	2.67
C6	-	-	-	0.05	0.08	4.44	3.34	3.34	-	4.84	-	6.56
C12	3.34	5.67	8.51	5.67	4.06	-	2.67	-	2.67	-	3.34	-
C13	-	-	-	2.67	3	5.67	3	-	3.34	-	3	-
C14	-	0.23	-	0.06	2.67	6.11	2.06	-	2.67	-	3.34	-
C15	-	-	0.34	0.06	3	4.84	4.06	5.25	3.69	5.25	4.84	-
C18	-	-	-	-	3	3	2.67	3.34	2.67	3.34	3	3.69
C21	-	-	-	-	3.34	5.67	2.67	-	3	-	3.34	-
C22	-	-	-	-	3.34	-	3.69	-	3.34	-	4.06	-
C23	-	-	-	-	3.34	-	2.67	5.25	4.06	3.69	3.69	-
C26	-	-	-	0.26	2.36	8	3	3	2.67	2.67	2.67	4.84
C30	0.08	-	-	-	-	-	3	3	2.67	3	0.08	0.26
C41	-	-	0.04	-	-	-	0.94	-	3.34	-	0.23	1.14
C42	-	-	-	-	3.34	8.50	4.84	3	4.06	4.06	3.69	4.84
C43	-	0.24	-	-	3.34	-	3	-	2.67	-	2.36	-
C44	-	-	-	3.34	2.67	3.34	3.34	-	3	-	4.06	-

IFA: Increase in fold area, AMP: Amphotericin B, NYS: Nystatin, KT: Ketoconazole, IT: Itraconazole, CC: Clotrimazole, FLC: Fluconazole, T. chebula: *Terminalia chebula*, T. catappa: *Terminalia catappa*

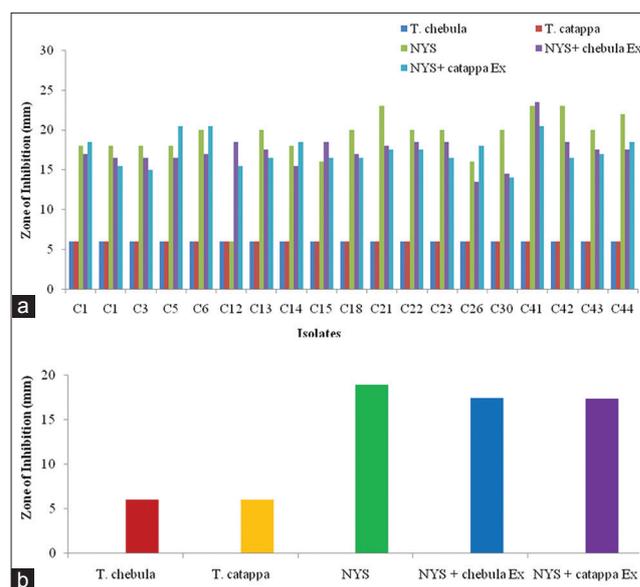


Fig. 2: (a) Synergistic anticandidal activity of *Terminalia chebula* and *Terminalia catappa* with nystatin (NYS) against *Candida* isolates. (b) Mean values of zone of inhibition of plant extracts and antibiotic NYS alone and in combination against *Candida* isolates

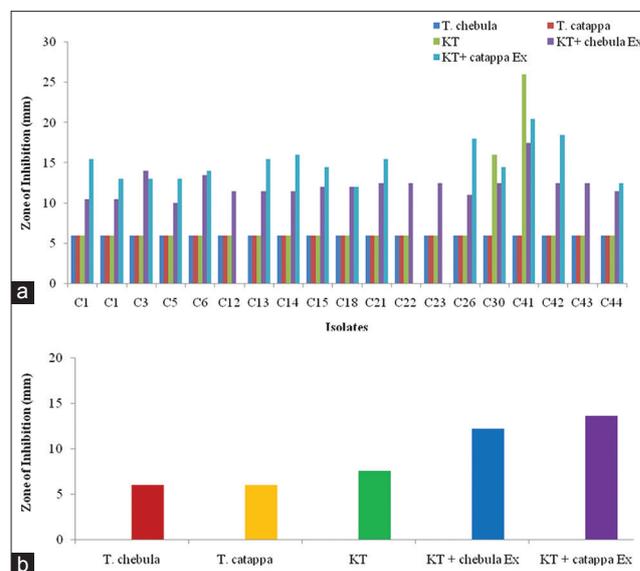


Fig. 3: (a) Synergistic anticandidal activity of *Terminalia chebula* and *Terminalia catappa* with ketoconazole (KT) against *Candida* isolates. (b) Mean values of zone of inhibition of plant extracts and antibiotic KT alone and in combination against *Candida* isolates

The former showed inhibitory activity against 17 isolates while the later showed activity against 7 isolates. IFA value of 4.06 was shown by *T. chebula* against isolates C23 and C42; IFA value of 5.25 was shown by *T. catappa* extract against isolate C15.

The antifungal activity of antibiotic IT was almost same as that of other azole antibiotics. The mean synergistic activity and IFA values were also similar to other azole antibiotics. The mean synergistic activity of *T. chebula* was 11.79 while that of *T. catappa* was 10.29 while that of antibiotic IT alone was only 6.74. Maximum IFA value was 4.84 and 6.56 against isolate C15 and C6 by *T. chebula* and *T. catappa*, respectively (Fig. 6 and Table 1).

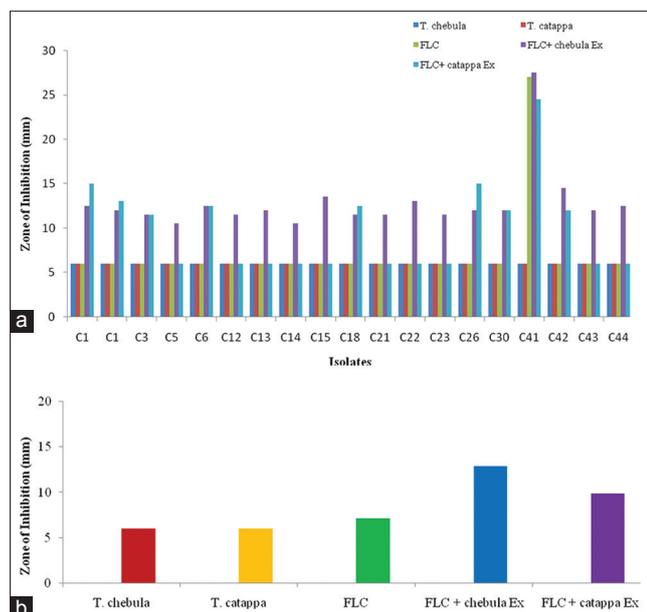


Fig. 4: (a): Synergistic anticandidal activity of *Terminalia chebula* and *Terminalia catappa* with fluconazole (FLC) against *Candida* isolates. (b) Mean values of zone of inhibition of plant extracts and antibiotic FLC alone and in combination against *Candida* isolates

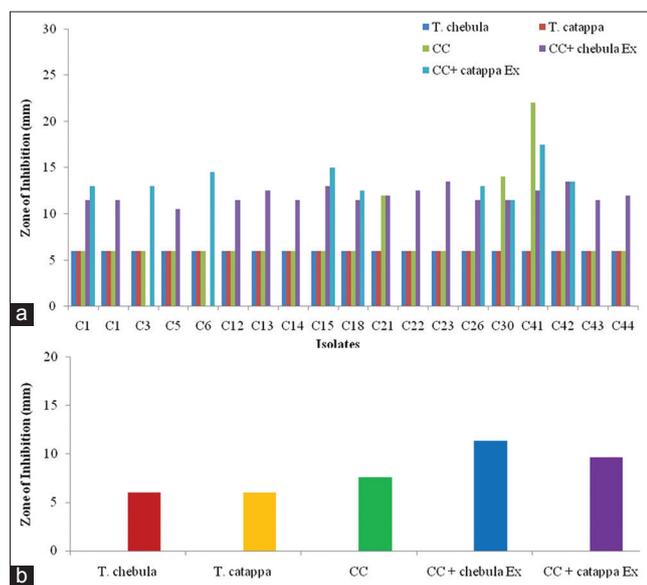


Fig. 5: (a) Synergistic anticandidal activity of *Terminalia chebula* and *Terminalia catappa* with clotrimazole (CC) against *Candida* isolates. (b) Mean values of zone of inhibition of plant extracts and antibiotic clotrimazole alone and in combination against *Candida* isolates

DISCUSSION

There is a dramatic increase in resistant antifungal strains, which is the result of widespread and repeated use of antibiotics and antifungal agents for a longer duration. Several *Candida* species, namely, *C. albicans*, *C. tropicalis*, *C. glabrata*, *C. parapsilosis*, *C. stellatoidea*, *C. krusei*, and *C. kyferare* becoming resistant to AMP, a polyene antibiotic [15], and azole resistant [16] which is worrisome and threatening. Thus, gradually the antibiotic therapy failed which prompts for new line of treatment. Great efforts were made to identify natural agents to combat these opportunistic infections. Medicinal plant extracts are

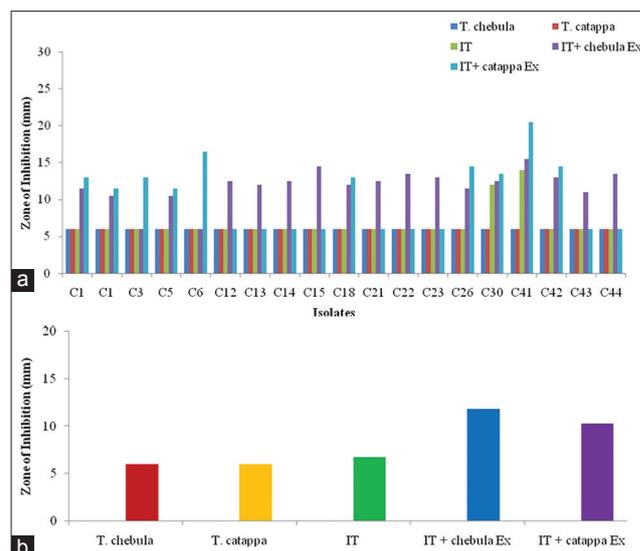


Fig. 6: (a) Synergistic anticandidal activity of *Terminalia chebula* and *Terminalia catappa* with itraconazole (IT) against *Candida* isolates. (b) Mean values of zone of inhibition of plant extracts and antibiotic IT alone and in combination against *Candida* isolates

well known for their antibacterial and antifungal activities [17-19]. The antifungal activity of some plant extracts against *Candida* spp. is also reported [20,21]. The antimicrobial activities of the plants are attributed to their secondary metabolites [22,23]. However, the most promising approach is combination therapy or synergistic approach.

In the present work, the ethanol extract of *T. chebula* and *T. catappa* leaf extract and 6 antibiotics alone and in combination, i.e., plant extract plus antibiotic was evaluated against 19 clinical isolates of *Candida*. Both the plant ethanol extracts did not show any antifungal activity. The zone of inhibition of both AMP and NYS and both polyene antibiotics alone was greater than all the four azoles (KT, FLC, CC, and IT) antibiotics. This differential inhibition might be due to different mechanism of action of both classes of antifungals. Ergosterol is an integral component of fungal cell membrane. Azole antibiotics inhibit the biosynthesis of ergosterol by inhibiting cytochrome P-450-dependent enzyme lanosterol 14-alpha-demethylase which results in blocking of proliferation of *Candida* species. On the other hand, polyene antibiotics interact with ergosterol to form channels which result in leakage of vital cytoplasmic components from inside the fungal cells to outside leading to cell death.

In combination therapy also, both classes of antifungal depicted different results. AMP and NYS did not show any synergistic activity while all the four azole antibiotics showed good synergistic activity. The synergistic activity of the antibiotics with ethanol extract of *T. chebula* was better than with that of *T. catappa*. Among the four azole antibiotics, KT and FLC showed better synergistic activity than CC and IT. Maximum mean zone of inhibition was with FLC in combination with *T. chebula* extract (12.86 mm) followed by KT (12.21 mm); this combination showed synergistic activity against all the 19 and 17 clinical isolates of *Candida*, respectively. The synergistic activity of *S. khuzistanica* ethanol extract with antibiotic KT was reported by Mahboubi and Kazempour [24] while Avijgan *et al.* [25] reported synergistic activity of *E. platyloba* ethanol extract with IT and FLC. Antagonistic effect was reported against CC and miconazole by the same authors, while we report no synergistic effect against AMP and NYS.

The synergistic activity of FLC and KT may be attributed to the high phenolic content present in *T. chebula*. The synergistic activity of plant extracts and antibiotics is reported by many other researchers [26-29]. The combination of azole antibiotics, especially FLC and KT, with

ethanol extract of *T. chebula* can be an alternative way of minimizing side effects of the antibiotics, since it leads to significant synergistic effect, reducing the dose which is necessary for therapeutic use. Endo et al. [30] also reported similar results from pomegranate peels. The plant extracts alone had no antifungal efficiency, but in combination with antibiotics, they could enhance or rather show synergistic effect, similar to the results of present work. The stem bark and leaf extracts of *L. paniculata* had no antifungal activity, but in combination with antibiotic, a significant synergistic effect was observed [31]. Therefore, FLC/KT plus ethanol extract of *T. chebula* can be an interesting and alternative source of treatment for *Candida* caused infections.

CONCLUSION

This study suggests that plant extracts alone did not show antifungal activity, but in combination with antibiotics, enhanced antifungal activity was demonstrated. The combination of *T. chebula* leaf extract and FLC/KT can be used as a new source of an antifungal agent against MDR *Candida* species.

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