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Anticandidal and synergistic anticandidal activity and chemical composition of *Citrus reticulata* and *Azadirachta indica* essential oils

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Abstract: *The aim of the present work was to evaluate anticandidal activity of Azadirachta indica A. Juss. and Citrus reticulate Blanco (neem and orange) essential oils alone and their synergistic activity i.e. essential oils in combination with some two polyene antibiotics viz. Amphotericin B, Nystatin and four azole antibiotics viz. Fluconazole, Ketoconazole, Clotrimazole and Itraconazole against multidrug resistant clinical isolates of Candida. The synergistic antifungal activity of both essential oils with six commercial antibiotics was evaluated against clinical isolates by disc diffusion assay. C. reticulata fruit peel essential oil showed better synergistic antifungal activity than A. indica seed essential oil. C. reticulata fruit peel essential oil showed better synergistic activity with all the four azole antibiotics; best activity was with fluconazole. It showed less activity with polyene antibiotics. The essential oil of C. reticulata fruit peel in combination with Fluconazole antibiotic can be successively used as a natural source of antifungal agent and can be effectively used to treat infections caused by Candida species*
Keywords:*, Azadirachta indica, Citrus reticulate, Candida albicans, Fluconazole, essential oils, Synergistic activity*

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I. INTRODUCTION

Candida albicans is an opportunistic pathogen that causes severe infections in immunocompromised patients. Candidiasis caused by *Candida* species has increased substantially in the past 20 years, and they stand as fourth rank among microbes. *Candida* species are opportunistic pathogens that can cause local and systemic infections in predisposed persons, commonly affecting immunologically compromised patients and those undergoing prolonged antibiotic treatment [1]. Candidiasis is a common infection of the skin, nails, oral cavity, esophagus, and vagina, caused by *Candida* genus. Generally, *Candida albicans* causes vulvovaginal candidiasis in women world wide affecting women of all ages at least once in life time and in some cases the causative agent is *C. glabrata,* a non albican species [2]. The effective treatment of *Candida* infections is either with imidazole antibiotics (imidazoles clotrimazole, miconazole and econazole) or polyene antibiotics (Nystatin and Amphotericin B).

There has been a dramatic increase in systemic infections by opportunistic fungi mainly because of their resistance to normally available antifungal agents which is because of their wide spread use; incidence of resistance to traditional antifungal therapies is also increasing [3]. Additionally, the antifungal agents commonly used in patients cause side effects, including hypersensitivity, allergic reactions, and immunosuppression [4]. Also fungi contamination in stored products is responsible for modifications in the flavour, appearance and reduction of nutritional value of these products, as well as occurrence of allergies and mycotoxin intoxications [5].

The increased incidence of fungal infections and inflammatory-related diseases, difficulties encountered in their treatment, increase in drug resistance, side effects of conventional medication, and treatment costs justify the search for effective alternatives and development of new effective, less toxic and cheaper antifungal drugs especially from natural sources. Natural products have played an important role worldwide in the treatment and prevention of fungal infections and many other diseases and disorders. Screening by using *in vitro* evaluation is a useful tool for the discovery of new potential antifungal agents from natural products such as essential oils and plant extracts [6].

Essential oils are volatile compounds synthesized from glucose through the secondary metabolism of plants. They are characterized by strong odor and complex composition and can be obtained from different plant structures through hydro distillation or steam distillation [7]. They are soluble in organic solvents and lipids. They show antibacterial, antifungal, antiviral, insecticidal, antiparasitical, anti-inflammatory, anticancer properties and are also used in pharmaceutical and cosmetic industries [8, 9].

Citrus reticulata Blanco belongs to the family Rutaceae. The citrus genus consists of about 1,000 species, all of which produce a characteristic and distinct flavor used in foods, pharmaceutical, perfumery, and cosmetic industry. The genus citrus includes many species of lemons, limes, oranges, grapefruits, tangerines and mandarins [10]. The composition of essential oils of these species is affected by plant organ, climate, soil type, geographical location, age and vegetative stage of the plant, etc [11].

Azadirachta indica A. Juss. (syn. *Melia indica* Brandis; *Melia azadirachta* Linn.) belongs to the family Meliaceae. It grows in tropical and semi-tropical regions. All parts of the plant (root, stem, leaf, seed, bark flower) including neem oil show various pharmacological properties and are traditionally used to cure a number of diseases [12]. It has more than 140 active compounds and shows insecticidal, anti-inflammatory, anti-ulcer, antipyretic, antiarthritic, antifungal, antimalarial, antibacterial, antitumor, immunomodulatory, spermicidal, diuretic, etc activities [13].

Considering the above, in the present work, two essential oils viz. *Azardirachta indica* seed and *Citrus reticulata* fruit peel oil were evaluated alone and in combination with six commercial commonly used antibiotics (Amphotericin B, Nystatin, Ketoconazole, Fluconazole, Clotrimazole and Itraconazole) for their antifungal potential against some clinical isolates of *Candida*.

The details of plants selected are shown below:

***Azardirachta indica* A Juss.**
Family: Meliaceae
Vernacular name: Limbdo
Part used: seed essential oil
Reported activity: Antimicrobial activity [14], antitumor and antiviral activity [15], Anticancer activity [16], Immunomodulatory activity [17], Cytotoxic and apoptosis activity [18], Anti-inflammatory, pro-apoptotic, and anti-proliferative [19].

***Citrus reticulata* Blanco**
Family: Rutaceae
Vernacular name: Santra
Part used: fruit peel essential oil
Reported activity: Antioxidant and anticancer activity [20], antimicrobial activity [21], hepatoprotective activity [22], antifungal activity [23], anti-inflammatory, insecticidal and antimicrobial activities [24].

Fig. 1 Photograph of *Azardirachta indica*

Fig. 2 Photograph of *Citrus reticulata*

II.MATERIALS AND METHODS

2.1. Essential oils

The *Azardirachta indica* (neem) seed essential oil and *Citrus reticulata* (orange) fruit peel essential oils were purchased from Yucca Enterprise Mumbai, India.

2.2. Antibiotics used

Six different antibiotics (Hexa Disc) used in the study are Amphotericin B-AMP (100 units/ disc), Nystatin –NYS (100 units/ disc), Ketoconazole-KT (30 mcg/ disc), Fluconazole-FLC (10 mcg/ disc), Clotrimazole-CC (10 mcg/ disc) and Itraconazole –IT (30 mcg/disc).

2.3. 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 urine sample. The isolates were cultured on Sabouraud dextrose agar medium with Chloramphenicol under aerobic conditions within a temperature range of 28º C. The isolates were identified as *Candida* species on the basis of some biochemical tests like Blastospore / Chlamydospore formation, color of colony on HiChrome *Candida* differential agar, carbohydrate assimilation test (sucrose, mannose, lactose, 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.

2.4. Synergistic anticandidial assay

Synergistic anticandidial activity of the Neem essential oil and Orange essential oil with antibiotics (Amphotericin B, Nystatin, Ketoconazole, Fluconazole, Clotrimazole, Itraconazole) were evaluated by using disc diffusion method [25]. The Petri plates were prepared by pouring 20 ml Sabouraud dextrose agar seeded with 200 µl test culture containing 1×10^8cfu/ml as McFarland 0.5 turbidity standard. Plates were allowed to solidify. Standard antibiotics paper discs (6 mm) were impregnated with 20 µl of Neem and Orange essential oils (20%) dissolved in ethanol separately. The sterile paper discs were impregnated with 20 µl of Neem and Orange essential oils (in 20% Ethanol) 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.

2.5. Increase in fold area

Increase in fold area (IFA) was calculated as $\mathbf{(B^2 - A^2) / A^2}$

Where **A**- inhibition zone for Antibiotics and **B** - Inhibition zone for essential oil + antibiotics.

2.6. Gas-chromatography-mass spectrometry (GC-MS) analysis

The chemical composition of the essential oil was analyzed using GC-MS. The essential oil solution was injected into GC-MS (QP-2010 Plus, Shimadzu). The capillary column was BPX-35 (length=30 m, i.d.=0.25mm, thickness = 0.25µm). The GC-MS oven temperature was increased from 40º C to 310º C at rate of 10º C/min with final hold time 5 min. The injector and detector temperature were maintained at 300º C and 290º C while spectra recorded in the 40 -700 m/z range and ion source temperature was 200º C. Other operating condition were as follows: Injection mode: split, Flow control: Liner velocity, Column flow: 0.80ml/min and Split ratio: 0.5 Essential oil components were quantified by relative percent peak area of TIC from the MS signal and identified by comparing their mass fragmentation pattern with those stored in the spectrometer database.

III. RESULTS

3.1. Chemical composition of Neem essential oil

Chromatogram of GC-MS analysis of Neem essential oil is given in Fig. 3. The identified compounds of essential oil are listed in Table- 1, with their respective retention time and percent compositions. The results of GC-MS analysis of Neem essential oil led to identification of 21 compound accounting for the total oil. The principal compound identified were 2-Methylcoumaran-3-one (14.07%), n-Pentatriacontane (12.24%), 10-Octadecenoic acid (8.56%), Palmitin (7.58%), Naphthalenediol (4.78%), 1, 3- Tetracosane (3.67%), Heptadecanoic acid (3.62%), Heptacosane (3.40%). Several other compounds such as D-Limonene (0.36%), Monomethyl phthalate (1.07%), Phthalic acid (1.78%), Methyl hexadecanoate (2.12%), Tetradecanoic acid (1.33%), 13-Docosenoic acid (2.26%), Nonadecane (2.05%), n-Triacontane (0.60%), Triacontane (2.30%), Oleic acid (1.04%), Linoleoyl chloride (2.20%), Hexatriacontane (2.92%), Methanamine (2.85%) were in less amounts.

3.2. Chemical composition of Orange essential oil

Chromatogram of GC-MS analysis of Orange essential oil is given in Fig. 4. The identified compounds of essential oil are listed in Table- 2, with their respective retention time and percent compositions. The results of GC-MS analysis of Orange essential oil led to identification of 13 compound accounting for the total oil. The principal compound identified were Limonene (37.44%), Linalool L (7.66%), Decanal (6.42%), Octacosane (1.84%), Pentatriacontane (4.40%), Heptacosane (4.34%). Several other compounds such as Octanal (3.07%), cis-p-Mentha-

2, 8-dien-1-ol (2.06%), Cyclohexanol (2.47%), trans-Carveol (2.90%), Dodecanal (2.36%), 2, 6 Di-Tert-Butyl Phenol (3.36%), 1, 2 Benzenedi-carboxylic acid (2.32%) were in less amounts.

3.3. Antifungal activity

3.3.1. Amphotericin B

The antifungal activity of essential oil of Neem and Orange, antibiotic Amphotericin B (AMP) and their synergistic activity i.e. essential oils plus Amphotericin B against all 19 clinical *Candida* isolates is given in Fig. 5A and their mean zone of inhibition is given in Fig. 5B. Increase in fold area (IFA) values are given in Table 3. The antibiotic AMP alone showed antifungal activity with mean zone of inhibition 14.26 mm (Fig. 5B). The inhibition zone of AMP ranged from 12 mm to19 mm and AMP alone exhibited maximum zone of inhibition against isolate C42 (19 mm) (Fig. 5A). Both essential oils alone did not show antifungal activity against any of the 19 clinical *Candida* isolates (Fig. 5A).

AMP plus Neem essential oil showed antifungal activity against 8 isolates i.e. C1, C3, C6, C12, C18, C21, C23 and C26 (Fig. 5A). The inhibition zone ranged from 9.5 mm to 10.5 mm. The synergistic antifungal activity was against isolate C12 (Fig 5A) and its IFA value was 1.78 (Table 3). AMP plus Orange essential oil showed antifungal activity against 16 isolates (Fig. 5A). The inhibition zone ranged from 9.5 mm to 12.5 mm and maximum zone of inhibition was against isolates C22 and C42. The synergistic antifungal activity was against only isolate C12 and its IFA value was 2.06 (Table 3).

3.3.2. Nystatin

The antifungal activity of essential oils of Neem and Orange, antibiotic Nystatin (NYS) and their synergistic activity i.e. essential oils plus Nystatin against all 19 clinical *Candida* isolates is given in Fig. 6A and their mean zone of inhibition is given in Fig. 6B. Increase in fold area (IFA) values are given in Table 3. The antibiotic NYS alone showed antifungal activity with mean zone of inhibition 18.56 mm (Fig. 6B). The inhibition zone of NYS alone ranged from 16 mm to 23 mm and NYS alone exhibited maximum zone of inhibition against isolates C21, C41 and C42 (23 mm) (Fig.6A). Both essential oils alone did not show antifungal activity against any of the clinical *Candida* isolates (Fig. 6A).

NYS plus Neem oil showed antifungal activity against all the 19 isolates (Fig. 6A). The inhibition zone ranged from 11.5 mm to 19 mm and maximum zone of inhibition was against isolates C6, C12 and C18 (19 mm). The synergistic antifungal activity was against only 2 isolates i.e. C12 and C15 (Fig. 6A). Their IFA values were 0.2 and 7.51 respectively (Table 3). NYS plus Orange essential oil showed antifungal activity against all the isolates except C44 (Fig. 6A). The inhibition zone ranged from 13.5 mm to 20.5 mm and maximum zone of inhibition was against isolate C42 (20.5 mm) The synergistic antifungal activity was against 3 isolates i.e. C12, C15 and C26 (Fig 6A). Maximum IFA value was against isolate C12 (6.56) (Table 3).

3.3.3. Ketoconazole

The antifungal activity of essential oils of Neem and Orange, antibiotic Ketoconazole (KT) and their synergistic activity i.e. essential oils plus Ketoconazole against all 19 clinical *Candida* isolates is given in Fig. 7A and their mean zone of inhibition is given in Fig. 7B. Increase in fold area (IFA) values are given in Table 3. The antibiotic KT alone showed antifungal activity with mean zone of inhibition 7.58 mm (Fig. 7B). The inhibition zone of KT ranged from 16 mm to 26 mm and KT alone exhibited maximum zone of inhibition against isolate C41 (26 mm) (Fig. 7A). Both essential oils alone did not show antifungal activity against any of the 19 clinical *Candida* isolates (Fig. 7A).

KT plus Neem essential oil showed antifungal activity against 16 isolates out of the 19 isolates evaluated. They did not show activity against C13, C22 and C30 (Fig. 7A). The inhibition zone ranged from 10 mm to 15 mm. Maximum zone of inhibition was against isolate C12 (Fig. 7A). The synergistic antifungal activity was against 15 isolates (Fig 7A). The IFA values ranged from 1.78 to 5.25; Maximum IFA value was against isolate C12 (5.25) (Table 3). KT plus Orange essential oil showed antifungal activity against 17 isolates; they did not show activity against C13 and C 44 (Fig. 7A). The inhibition zone ranged from 11.5 mm to 28 mm and maximum zone of inhibition was against C41. The synergistic antifungal activity was against 17 isolates (Fig 7A). The IFA values ranged from 0.16 to 13.06; maximum IFA value was against isolate C6 (13.06) (Table 3).

3.3.4. Fluconazole

The antifungal activity of essential oils of Neem and Orange, antibiotic Fluconazole (FLC) and their synergistic activity i.e. essential oils plus Fluconazole against all 19 clinical *Candida* isolates is given in Fig. 8A and their mean zone of inhibition is given in Fig. 8B. Increase in fold area (IFA) values are given in Table 3. The antibiotic FLC alone showed antifungal activity with mean zone of inhibition 7.10 mm (Fig. 8B) while both the essential oils alone did not show antifungal activity against any of the 19 clinical *Candida* isolates (Fig. 8A). FLC alone exhibited zone of inhibition against only 1 isolate C41 (27 mm) (Fig. 8A).

FLC plus Neem essential oil showed antifungal activity against 8 isolates C1, C5, C6, C15, C21, C23, C41 and C42 (Fig. 8A). The inhibition zone ranged from 10.5 mm to 19.5 mm. Maximum zone of inhibition was against isolate C41 (19.5 mm) (Fig. 8A). The synergistic antifungal activity was against 6 isolates i.e. C1, C5, C6, C15, C23, C26 and C42 (Fig 8A). The IFA values ranged from 2.06 to 3.34; Maximum IFA value was against isolate C15 (3.34) (Table 3). FLC plus Orange essential oil showed antifungal activity against the 7 isolates C1, C3, C5, C15, C41, C42 and C43 (Fig. 8A). The inhibition zone ranged from 12 mm to 20.5 mm and maximum zone of inhibition was against isolate C41 (20.5 mm). The synergistic antifungal activity was against 6 isolates i.e. C1, C3, C5, C15, C42 and C43 (Fig 8A). The IFA values ranged from 3 to 6.56; Maximum IFA value was against isolate C42 (6.56) (Table 3).

3.3.5. Clotrimazole

The antifungal activity of essential oils of Neem and Orange and antibiotic Clotrimazole (CC) and their synergistic activity i.e. essential oils plus Clotrimazole against all 19 clinical *Candida* isolates is given in Fig. 9A and their mean zone of inhibition is given in Fig. 9B. Increase in fold area (IFA) values are given in Table 3.

The antibiotic CC alone showed antifungal activity with mean zone of inhibition 7.58 mm (Fig. 9B). The inhibition zone of CC alone ranged from 12 mm to 22 mm and CC alone exhibited maximum zone of inhibition against isolate C41 (22 mm) (Fig. 9A), while both the essential oils alone did not show antifungal activity against any of the clinical *Candida* isolates (Fig. 9A). CC plus Neem essential oil did not show any antifungal activity or any synergistic antifungal against any of the 19 isolates (Fig. 9A). On the other hand, CC plus Orange essential oil showed antifungal activity against 10 isolates C3, C5, C6, C15, C23, C42 and C43 (Fig. 9A). The inhibition zone ranged from 11.0 mm to 17.0 mm and maximum zone of inhibition against isolate C15 (17 mm). The synergistic antifungal activity was against 7 isolates i.e. C3, C5, C6, C15 C23 C42 and C43 (Fig 9A). Maximum IFA value was against isolate C15 (7.03) (Table 3).

3.3.6. Itraconazole

The antifungal activity of essential oils of Neem and Orange, antibiotic Itraconazole (IT) and their synergistic activity i.e. essential oils plus Itraconazole against all 19 clinical *Candida* isolates is given in Fig. 10A and their mean zone of inhibition is given in Fig. 10B. Increase in fold area (IFA) values are given in Table 3. The antibiotic IT alone showed antifungal activity with mean zone of inhibition 6.74 mm (Fig. 10B) The inhibition zone of IT ranged from 12 mm to 14 mm and IT alone exhibited maximum zone of inhibition against isolate C41 (14 mm) (Fig. 10A). Both essential oils alone did not show antifungal activity against any of the clinical *Candida* isolates (Fig. 10A).

IT plus Neem essential oil showed antifungal activity against only isolate C41 (Fig. 10A) and its zone of inhibition was 9.5 mm. IT plus Neem essential oil did not show any synergistic antifungal activity against any of the 19 clinical *Candida* isolates (Fig. 10A). IT plus Orange essential oil showed antifungal activity against 11 isolates C3, C5, C6, C15, C21, C22, C23, C26, C30, C41 and C42 (Fig. 10A). The inhibition zone ranged from 11 mm to 15 mm and maximum zone of inhibition was against isolate C15 (15 mm). The synergistic antifungal activity was against 11 isolates i.e. C3, C5, C6, C15, C21, C22, C23, C26, C30, C41 and C42 (Fig 10A). The IFA values ranged from 2.36 to 5.25; Maximum IFA value was against isolate C15 (5.25) (Table 3).

IV. DISCUSSION

Antibiotic misuse has been considered as a major cause of antibiotic resistant fungi. As a result, fungi became resistant to antibiotics, which are in turn less effective after long use. Therefore search and research for new antifungal agents has become a very important and urgent task, especially in recent times, considering the increasing levels of antibiotic resistance among pathogenic microorganisms. One of the best and promising approach has been the use of medicinal plants as extracts or essential oils alone or in combination which are widely available resources, less expensive, with less or no side effects and have shown antimicrobial properties.

The therapeutic properties of medicinal plants and essential oils from various parts of plants belonging to different families is very well documented. Antifungal activity of *Lavandula angustifolia* essential oil against *Candida albicans* is reported by D'auria *et al.,* [26]. *Citrus reshni* leaf and fruit peel essential oil showed antifungal activities especially on *A. niger* which reached to 82.6% activity of Amphotericin B as an antifungal standard [27]. Essential oils from *Cinnamodendron dinisii* Schwacke and *Siparuna guianensis* Aublet showed antifungal activity [28]. The essential oil of *Aegle marmelos* (L.) Correa leaves showed better antifungal activity against *Candida albicans* and *Aspergillus niger* than the standard antifungal antibiotics used erythromycin, methicillin, oxacillin, bacitracin and nystatin check the antibiotics [29].

Synergistic approach is more beneficial than single application of essential oils. Combination may be between two or more than 2 compounds [30]. This combination approach may give synergistic effect or antagonistic effect but more often the combination is between compounds showing strong and weak activity and they end up being complementary and the net result is synergistic effect [31]. The synergistic approach or combination may be between two plant extracts, two essential oils or plant extract plus antibiotic or essential oil plus antibiotic, etc. [32, 33, 34, 35]. Synergy may be between antibiotics and some chemical compounds like sulfated sterols, tetracyclic indoles, allicin, amiodarone and piperazinyl quinolines [36]. Essential oils in combination with other substances exhibit different effects (indifferent, additive, antagonistic and synergistic) against bacteria and fungi. This is probably due to different mechanisms of action involved in this case.

In the present work, the GC-MS analysis of Neem essential oil revealed the presence of various compounds like D-Limonene, Monomethyl phthalate, Phthalic acid, Methyl hexadecanoate, Tetradecanoic acid, 10-Octadecenoic acid, Heptadecanoic acid, 13-Docosenoic acid, Nonadecane, Palmitin, n-Triacontane, Triacontane, Oleic acid, Linoleoyl chloride, n-Pentatriacontane, Hexatriacontane, Methanamine, Heptacosane, 1,3-Naphthalenediol, Tetracosane, 2-Methylcoumaran-3-one. The GC-MS analysis of Orange essential oil revealed the presence of various compounds like Limonene, Octanal, Linalool L, cis-p-Mentha-2,8-dien-1-ol, Cyclohexanol, Decanal, trans-Carveol, Dodecanal, 2,6 Di-Tert-Butyl Phenol, 1,2 Benzenedi-carboxylic acid, Octacosane, Pentatriacontane, Heptacosane.

The chemical components of the essential oils of Neem and Orange are responsible for the observed synergistic antifungal activity. Amongst both the oils, Orange essential oil showed better anticandidal activity than Neem essential oil. Orange essential oil contains compounds like Limonene and linalool which are well known for antimicrobial activity [37]. Obidi *et al.,* [38] also reported antimicrobial properties of orange oil against Gram positive bacteria (*S. aureus, E. fecalis*), Gram negative bacteria (*E. coli, P. aeruginosa*) and fungi (*C. albicans*).

Both essential oils showed different level of anticandidal activity. The activity was also different with different antibiotics. The polyene antibiotics, Nystatin and Amphotericin B showed antifungal activity when evaluated alone but in combination i.e. when antibiotics plus essential oils was evaluated, it resulted in antagonistic activity; out of 19 clinical isolates of *Candida*, only 2-3 isolates showed antifungal activity as envisaged by their IFA values. All the four azole antibiotics (Ketoconazole, Fluconazole, Clotrimazole and Itraconazole), on the other hand, showed an entirely different trend. The best combination was shown by FLC with both the essential oils but the best activity was shown by FLC plus Orange essential oil. FLC plus Neem essential oil showed synergistic activity against 15 isolates, maximum IFA value was 5.25 against C12 followed by KT plus Neem essential oil; the synergistic activity was against only 6 isolates; while CC and IT did not show anticandidal activity either alone or in combination with Neem essential oil. Orange essential oil showed an entirely different trend with all the four azole antibiotics. FLC plus Orange essential oil showed synergistic activity against 17 isolates, maximum IFA value was 13.06 against C6 followed by IT plus Orange essential oil; the synergistic activity was against 11 isolates. CC and KT also showed synergistic activity against 8 and 5 isolates respectively. The IFA values were in the order FLC > IT > CC > KT. In our earlier work, *P. mentha* essential oil also showed synergistic anticandidal activity with azole antibiotics but best Activity was with Ketoconazole [39]. Antifungal potential of citrus essential oils is well documented [40, 41]. Garlic clove oil and tangerine fruit volatile oil showed better antimicrobial activity when mixed together rather than when used alone [42].

The antimicrobial activity of essential oils is associated with their disturbances of many vital processes of cell, increased membrane permeability and lipid depolymerization [43]. The components of essential oils induce structural changes in cell membrane of micro organisms making them more permeable to ions and other cell components which leads to cell death [44]. Essential oils can interact with the microbial membrane and cause drastic physiological changes leading to loss in membrane permeability, ultimately resulting in cell death [45]. However, due to the large number of components and synergistic or antagonistic interactions among them, it is possible that essential oils have cellular targets other than cell membranes. They do not have any specific cellular target.

V. CONCLUSION

The present study concludes that *C. reticulata* fruit peel essential oil showed better synergistic antifungal activity than *A. indica* seed essential oil. The best synergistic activity was shown with antibiotic Fluconazole. This suggested that combination of essential oil with antibiotics is most effective approach to treat *candida* infection. The combination of *C. reticulata* fruit peel oil and antibiotics could be used as promising source of antifungal agents.

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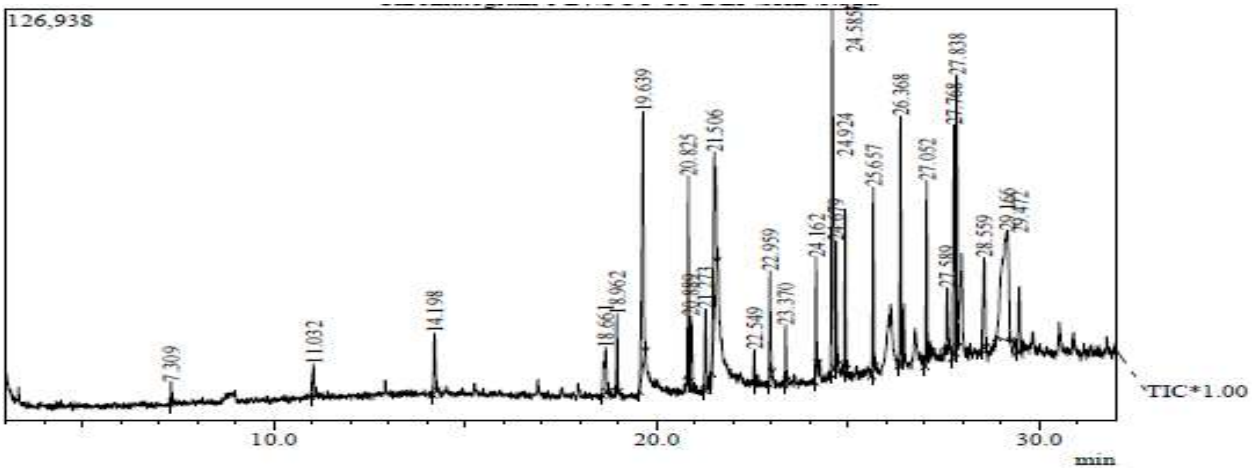

Fig. 3 Chromatogram of GC-MS analysis of *Azardirachta indica* seed essential oil.

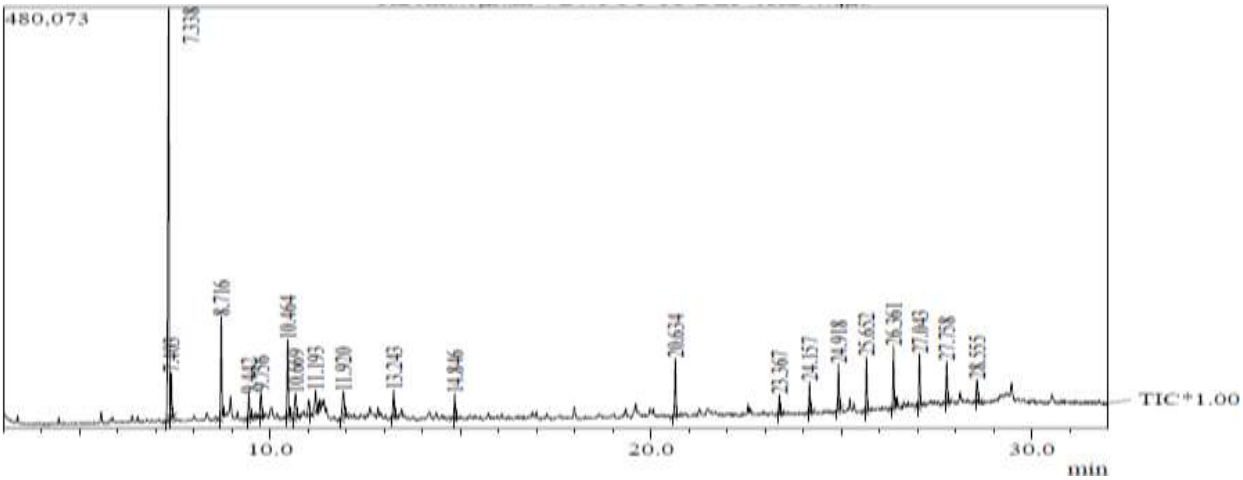

Fig. 4 Chromatogram of GC-MS analysis of *Citrus reticulata* fruit peel essential oil.

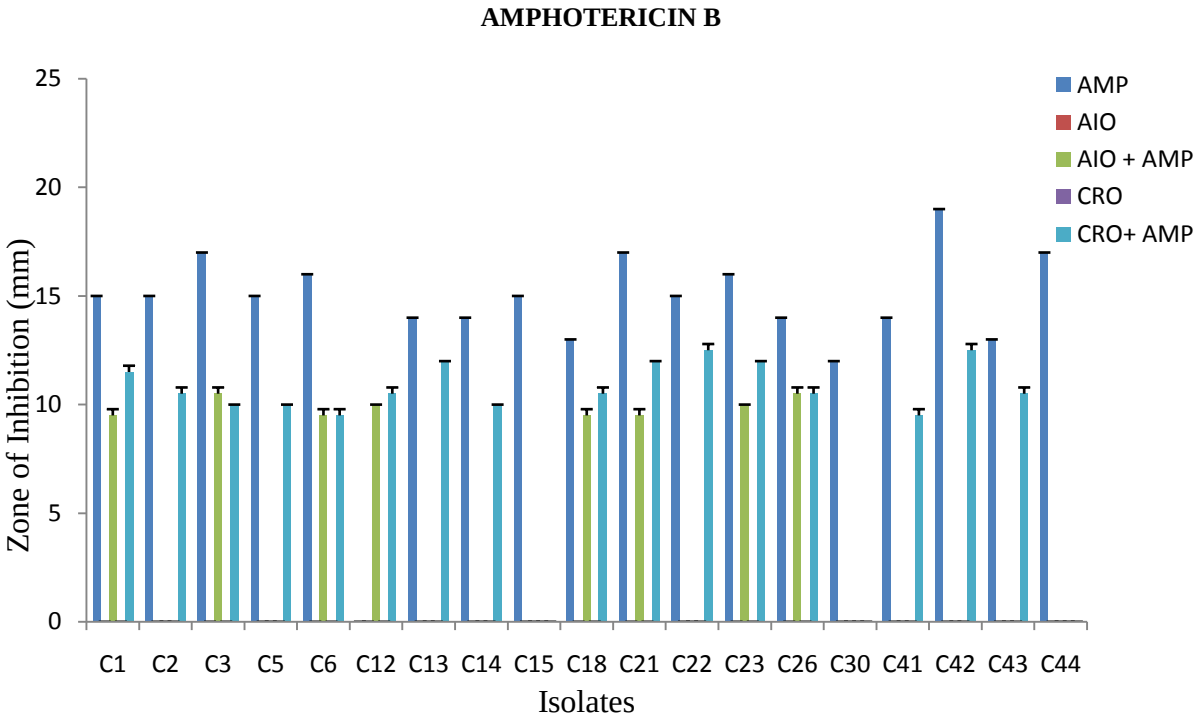

Fig 5A Synergistic antifungal activity of *Azardirachta indica* oil (AIO) and *Citrus reticulata* oil (CRO) with Amphotericin B (AMP) antibiotic against 19 multidrug resistant *Candida* isolates

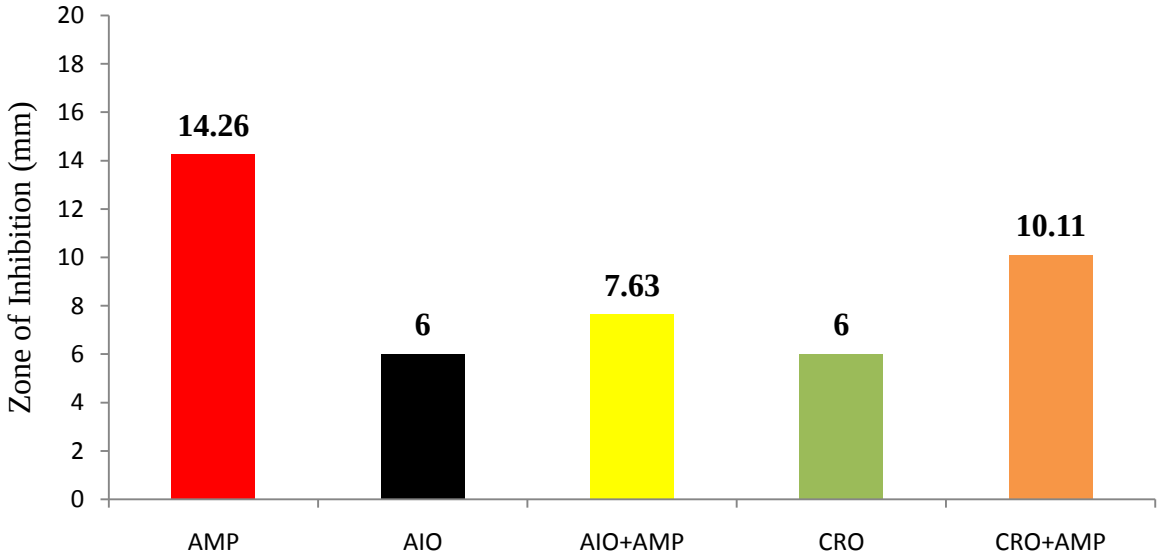

Fig 5B Mean values of zone of inhibition of Amphotericin B (AMP) alone and *Azardirachta indica* oil (AIO) and *Citrus reticulata* oil (CRO) and antibiotic in combination against 19 multidrug resistant *Candida* isolates

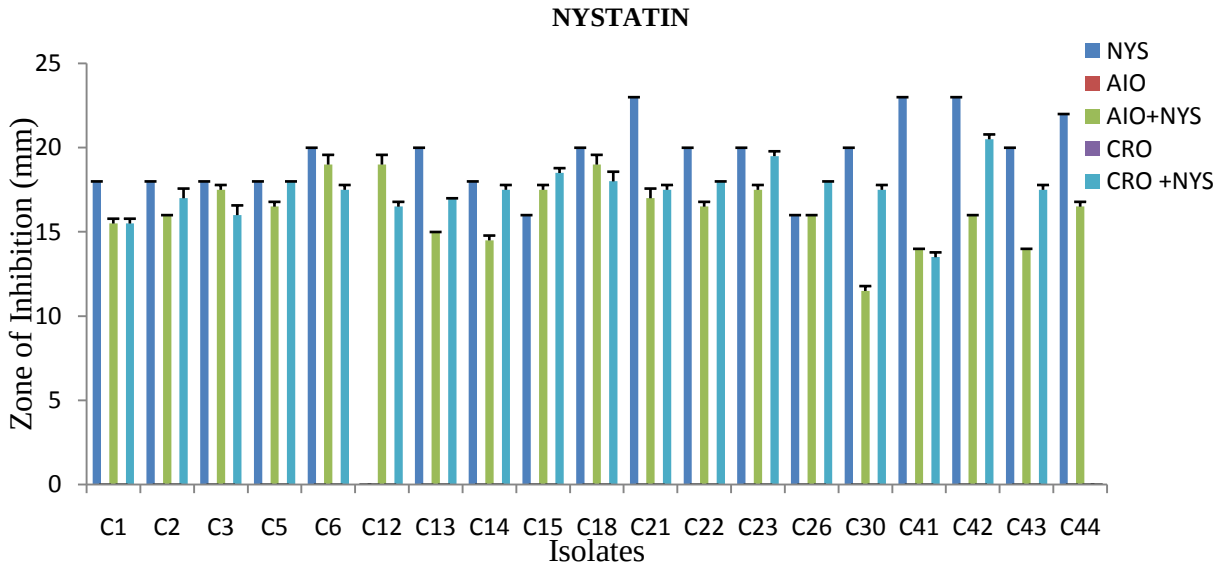

Fig 6A Synergistic antifungal activity of *Azardirachta indica* oil (AIO) and *Citrus reticulata* oil (CRO) with Nystatin (NYS) antibiotic against 19 multidrug resistant *Candida* isolates

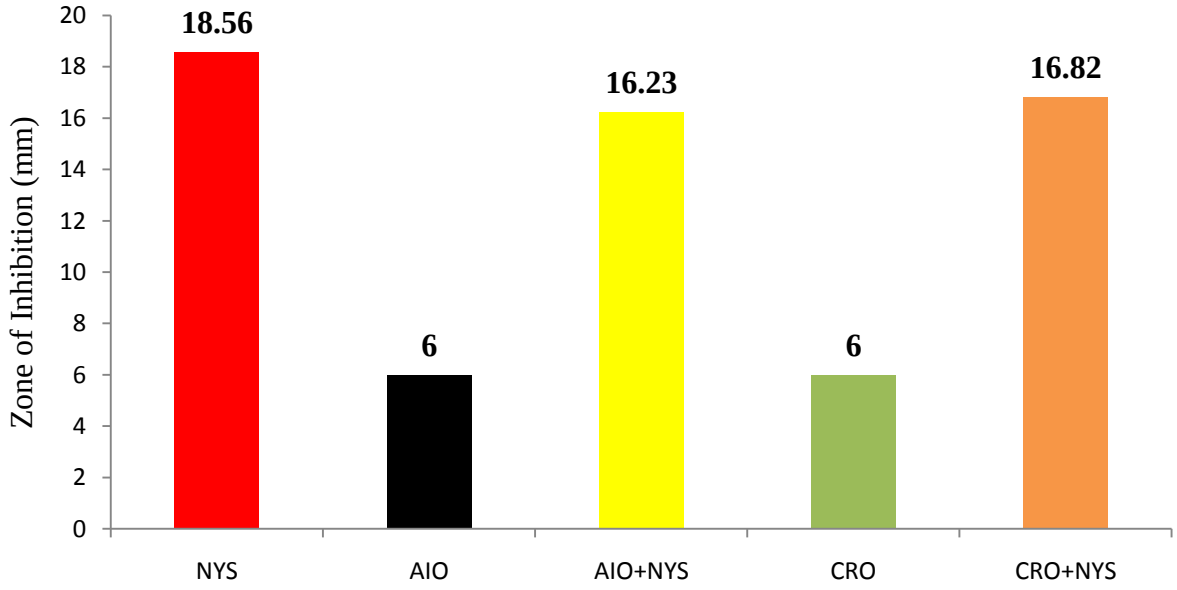

Fig 6B Mean values of zone of inhibition of Nystatin (NYS) alone and *Azardirachta indica* oil (AIO) and *Citrus reticulata* oil (CRO) and antibiotic in combination against 19 multidrug resistant *Candida* isolates

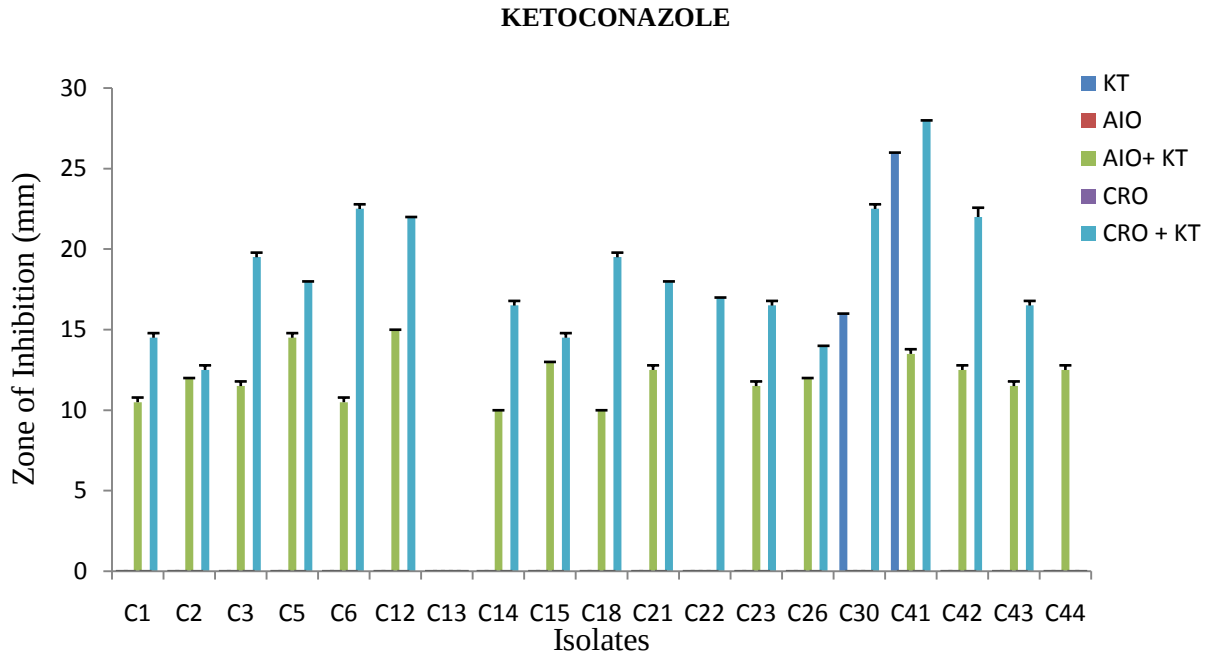

Fig 7A Synergistic antifungal activity of *Azardirachta indica* oil (AIO) and *Citrus reticulata* oil (CRO) with Ketoconazole(KT) antibiotic against 19 multidrug resistant *Candida* isolates

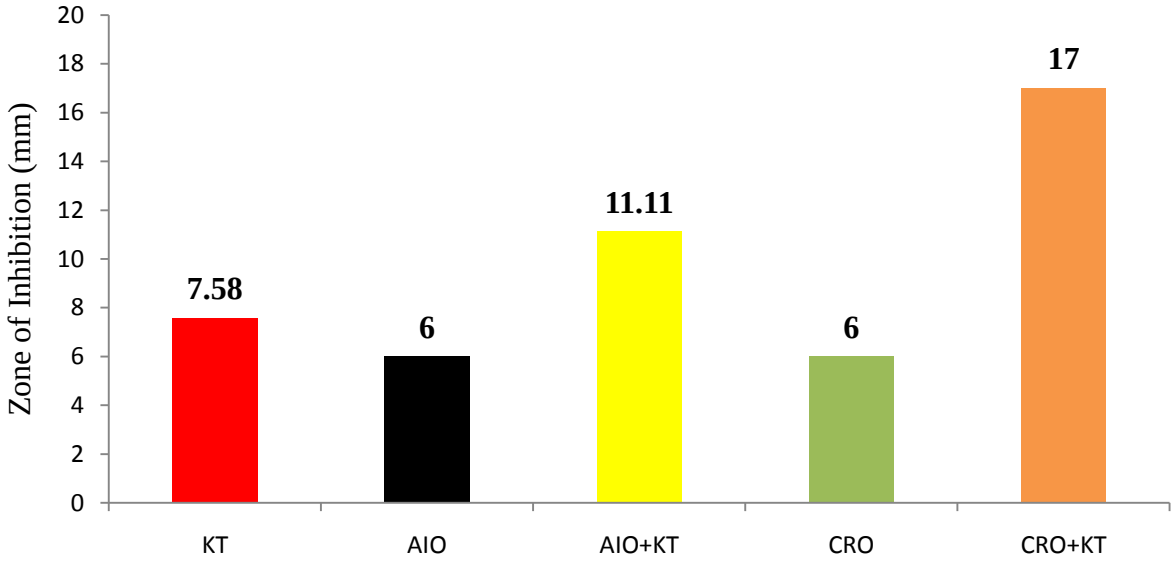

Fig 7B Mean values of zone of inhibition of Ketoconazole (KT) alone and *Azardirachta indica* oil (AIO) and *Citrus reticulata* oil (CRO) and antibiotic in combination against 19 multidrug resistant *Candida* isolates

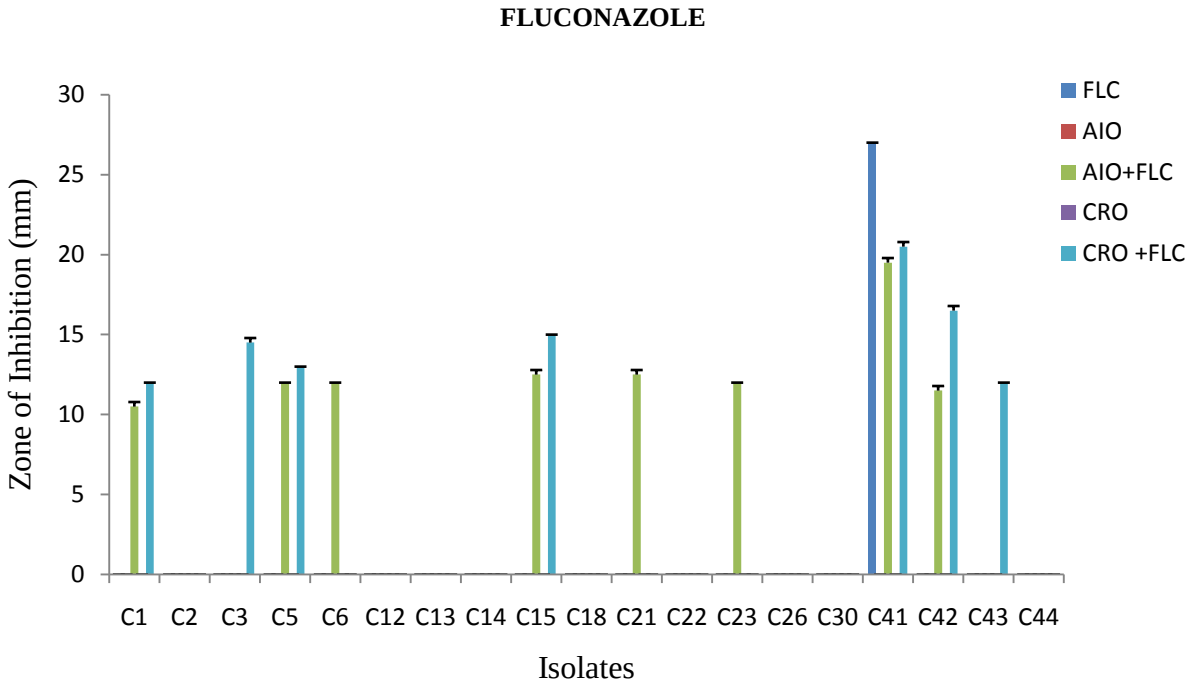

Fig 8A Synergistic antifungal activity of *Azardirachta indica* oil (AIO) and *Citrus reticulata* oil (CRO) with Fluconazole (FLC) antibiotic against 19 multidrug resistant *Candida* isolates

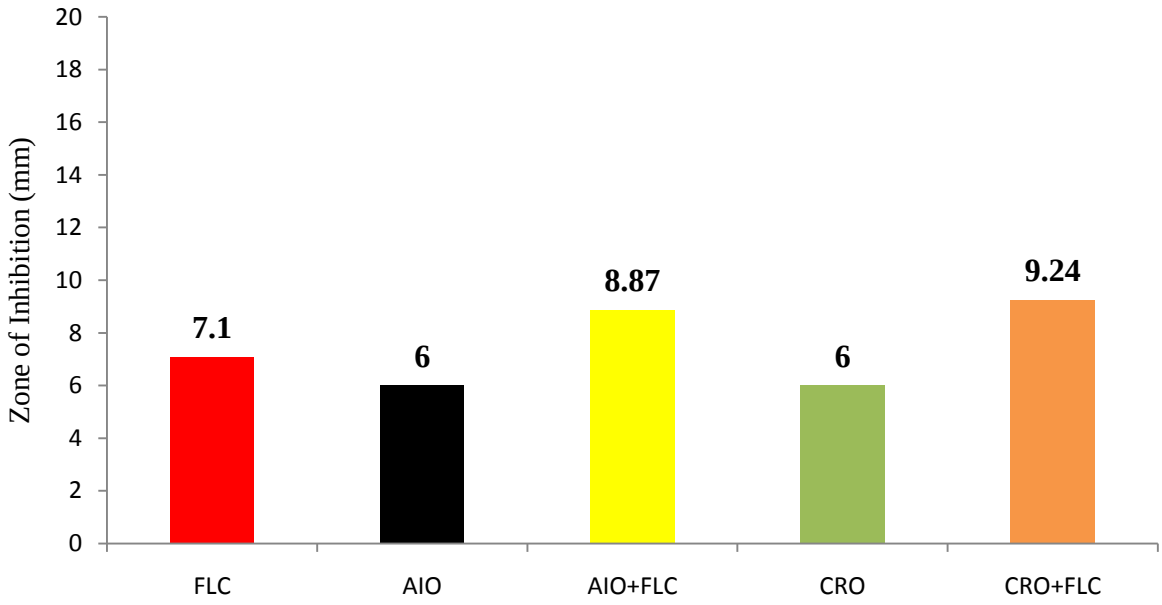

Fig 8B Mean values of zone of inhibition of Fluconazole (FLC) alone and *Azardirachta indica* oil (AIO) and *Citrus reticulata* oil (CRO) and antibiotic in combination against 19 multidrug resistant *Candida* isolates

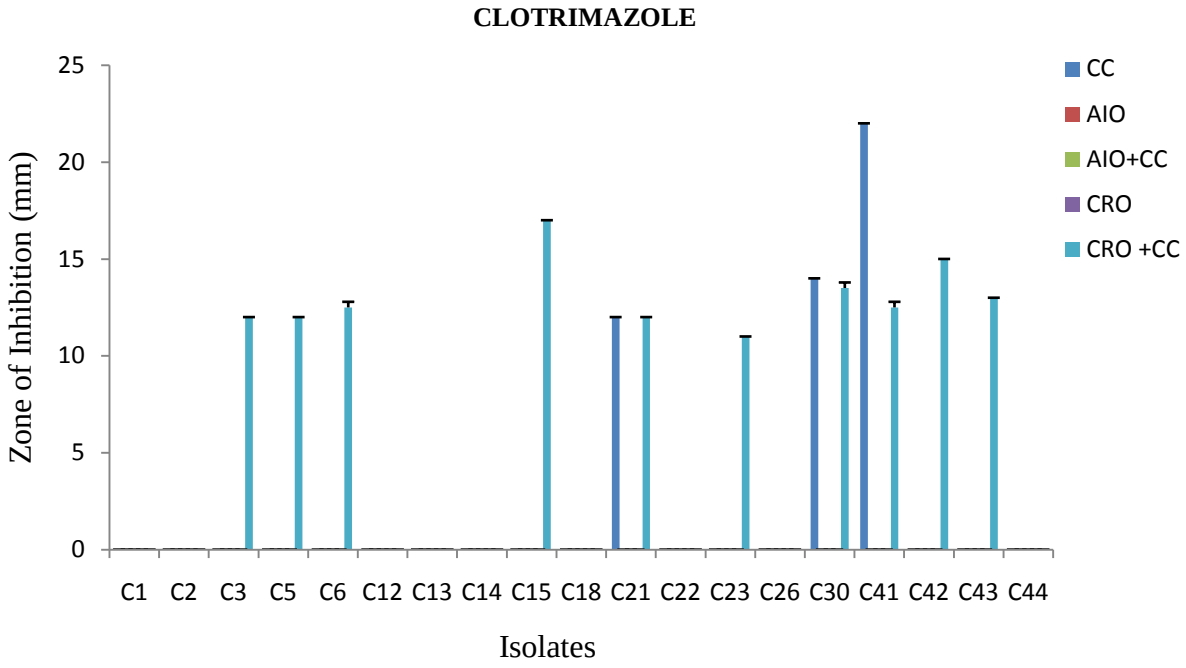

Fig 9A Synergistic antifungal activity of *Azardirachta indica* oil (AIO) and *Citrus reticulata* oil (CRO) with Clotrimazole (CC) antibiotic against 19 multidrug resistant *Candida* isolates

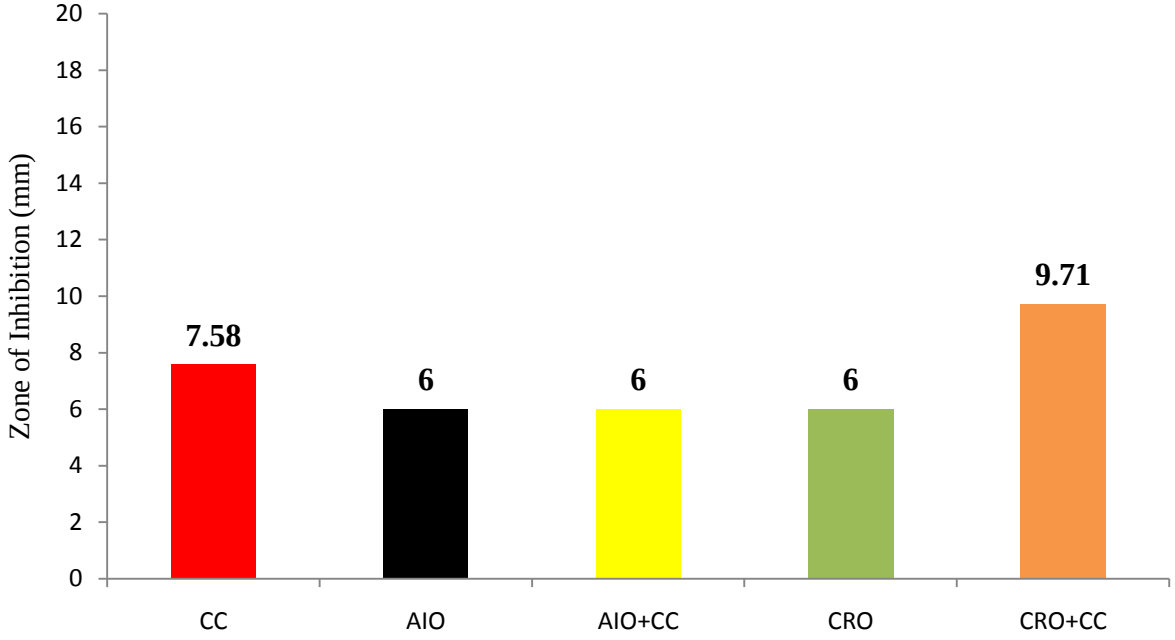

Fig 9B Mean values of zone of inhibition of Clotrimazole (CC) alone and *Azardirachta indica* oil (AIO) and *Citrus reticulata* oil (CRO) and antibiotic in combination against 19 multidrug resistant *Candida* isolates

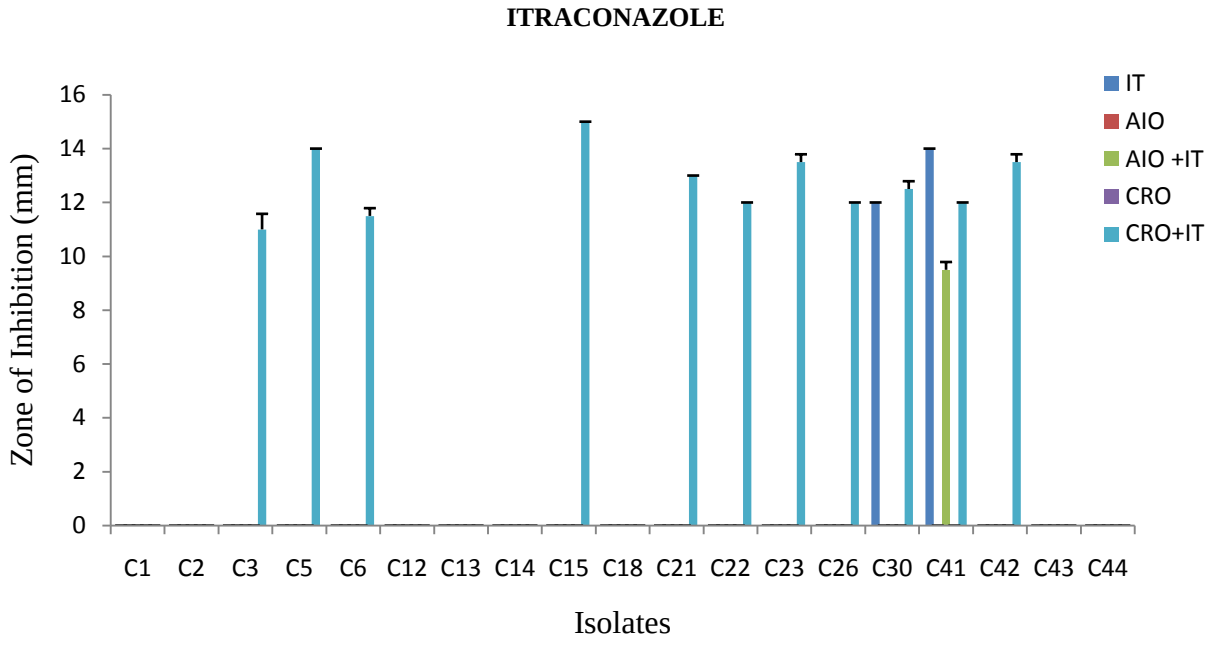

Fig 10A Synergistic antifungal activity of *Azardirachta indica* oil (AIO) and *Citrus reticulata* oil (CRO) with Itraconazole (IT) antibiotic against 19 multidrug resistant *Candida* isolates

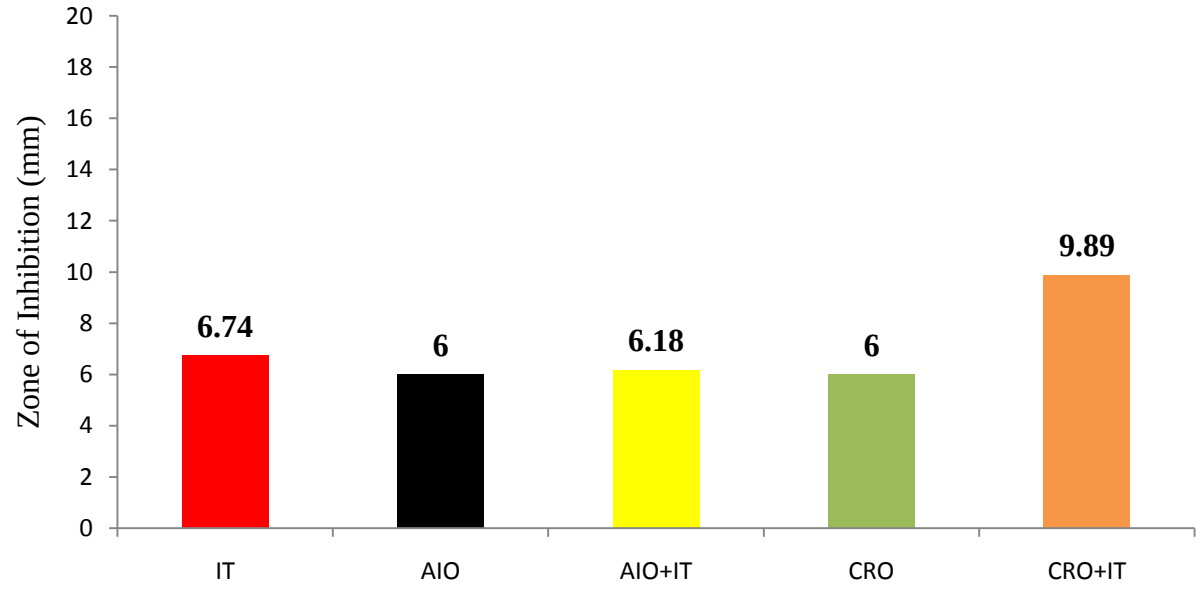

Fig 10B Mean values of zone of inhibition of Itraconazole (IT) alone and *Azardirachta indica* oil (AIO) and *Citrus reticulata* oil (CRO) and antibiotic in combination against 19 multidrug resistant *Candida* isolates

Table 1 Chemical composition, retention time and % area, compound name and structure of *Azardirachta indica* seed essential oil

No.	Retension Time	% Area	Compound Name	Structure
1	7.309	0.36	D-Limonene	
2	11.032	1.07	Monomethyl phthalate	
3	14.198	1.78	Phthalic acid	
4	18.661	2.12	Methyl hexadecanoate	
5	18.962	1.33	Tetradecanoic acid	
6	19.639	8.56	10-Octadecenoic acid	

7	20.825	3.62	Heptadecanoic acid	
8	20.889	2.26	13-Docosenoic acid	
9	21.273	2.05	Nonadecane	
10	21.506	7.58	Palmitin	
11	22.549	0.60	n-Triacontane	
12	22.959	2.30	Triacontane	
13	23.370	1.04	Oleic acid	
14	24.162	2.20	Linoleoyl chloride	
15	24.585	12.24	n-Pentatriacontane	
16	24.679	2.92	Hexatriacontane	

17	24.924	2.85	Methanamine	
18	25.657	3.40	Heptacosane	
19	26.368	4.78	1,3-Naphthalenediol	
20	27.052	3.67	Tetracosane	
21	29.166	14.07	2-Methylcoumaran-3-one	

Table 2 Chemical composition, retention time, % area, compound name and structure of *Citrus reticulata* fruit peel essential oil

No	Retention Time	% Area	Compound Name	Structure
1	7.338	37.44	Limonene	
2	7.403	3.07	Octanal	
3	8.716	7.66	Linalool L	
4	9.442	2.06	cis-p-Mentha-2,8-dien-1-ol	

5	9.756	2.47	Cyclohexanol	
6	10.464	6.42	Decanal	
7	10.669	2.90	trans-Carveol	
8	11.193	2.36	Dodecanal	
9	11.920	3.36	2,6 Di-Tert-Butyl Phenol	
10	13.243	2.32	1,2 Benzenedi-carboxylic acid,	

11	14.846	1.84	Octacosane	
12	20.634	4.40	Pentatriacontane	
13	26.361	4.34	Heptacosane	

Table 3 Increase in fold area (IFA) of six different antibiotics with *Azardirachta indica* seed and *Citrus reticulata* fruit peel essential oils

Isolates No.	IFA values of *Azardirachta indica* seed oil plus antibiotics						IFA values of *Citrus reticulata* fruit peel oil plus antibiotics					
	AMP	NYS	FLC	KT	CC	IT	AMP	NYS	FLC	KT	CC	IT
C1	-	-	2.06	2.06	-	-	-	-	3.34	3	-	-
C2	-	-	3	-	-	-	-	-	3.34	-	-	-
C3	-	-	2.67	-	-	-	-	-	9.56	4.84	3	2.36
C5	-	-	4.84	3	-	-	-	-	8	3.69	3	4.44
C6	-	-	2.06	3	-	-	-	-	**13.06**	-	3.34	2.67
C12	1.78	**9.03**	**5.25**	-	-	-	**2.06**	**6.56**	12.44	-	-	-
C13	-	-	-	-	-	-	-	-	-	-	-	-
C14	-	-	1.78	-	-	-	-	-	6.56	-	-	-
C15	-	7.51	3.69	**3.34**	-	-	-	0.34	4.84	5.25	**7.03**	**5.25**
C18	-	-	1.78	-	-	-	-	-	10.56	-	-	-
C21	-	-	3.34		-	-	-	-	8	-	3	3.69
C22	-	-	-	-	-	-	-	-	7.03	-	-	3
C23	-	-	2.67	3	-	-	-	-	6.56	-	2.36	4.06
C26	-	-	3	-	-	-	-	0.27	4.44	-	-	3
C30	-	-	-	-	-	-	-	-	0.94	-	-	3.34
C41	-	-	-	-	-	-	-	-	0.16	-	-	3
C42	-	-	3.34	2.67	-	-	-	-	12.44	**6.56**	5.25	4.06
C43	-	-	2.67	-	-	-	-	-	6.56	3	3.69	-
C44	-	-	3.34	-	-	-	-	-	-	-	-	-

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