Original Article

Use of checkerboard assay to determine the synergy between essential oils extracted from leaves of *Aegle marmelos* (L.) Correa and nystatin against *Candida albicans*

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Abstract

Background: *Candida albicans* is one of the most common pathogenic yeasts, responsible for causing candidiasis. The use of conventional antifungal agents for the treatment of *Candida* is reported to be less effective and hence alternative therapies for the treatment are needed. Essential oils of medicinal plants may serve as a strong candidate for natural products in modern therapies. **Aim:** The aim of this study was to determine the synergistic potential of essential oils extracted from leaves of *Aegle marmelos* (L.) Correa and a potent antifungal agent, nystatin, against three clinical isolates of *C. albicans* using checkerboard assay. **Materials and methods:** The antifungal activity of the essential oils of *A. marmelos* was screened against test cultures by disc diffusion technique. Antibiograms of the test organisms were developed. To determine the minimum fungicidal concentration of the essential oil and nystatin, the broth microdilution method was employed, and a checkerboard assay was used to investigate the synergistic potential of the essential oil and nystatin against the clinical isolates under study. The data were expressed as mean ± standard deviation. **Results:** The Σ fractional inhibitory concentration values were calculated as 0.12, 0.37, and 0.28 for three different strains of *C. albicans* used, respectively, which was <0.5, therefore, the synergy was demonstrated between essential oils and nystatin against the test cultures. **Conclusions:** Combinatorial therapy of the essential oils extracted from the leaves of *A. marmelos* and nystatin may be considered a line of treatment for candidal infections.

Keywords: *Aegle marmelos, Candida albicans*, checkerboard assay, essential oils, nystatin, synergy

Introduction

Among the various fungal infections seen in humans, candidiasis is most common, caused by *Candida* species of fungi. It can affect many body parts which include the oral cavity, skin, urogenital tract, gastrointestinal tract, and lungs. Immunocompromised patients are extremely prone to it.[1] There are numerous antifungal drugs such as fluconazole used to treat candidal infections.[2] Nevertheless, gaining resistance is a very common feature of the pathogen, which limits the use of certain therapeutics. Moreover, there are side effects associated with long-term intake of antifungal medicines.[3] Hence, it would be helpful to find an antifungal agent of natural origin, which can be advocated to use along with known potent antibiotics for better and quick relief with less or no side effects.

Aegle marmelos (L.) correa is one of the species of medicinal plant belonging to the family Rutaceae, commonly called *Bael*, cultivated across India, mainly near the temples because of its status as a sacred tree.[4] The tree of *A. marmelos* grows with a thick trunk, soft, and flaky bark and reaches a height of 7–8 m. The leaves of *A. marmelos* are pale green, trifoliate, and aromatic in nature.[5] It produces greenish-white flowers, which further develop into oval-shaped, sweet-tasting, soft fruit in a woody green shell that turns yellow on ripening.[6] *A. marmelos* is traditionally used to treat an array of ailments.[7] Along with antimicrobial, it shows anticancer, antidiabetic, and

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antioxidant activities.[8-11] Relevant literature has validated the antifungal properties of essential oils extracted from the leaf of *A. marmelos*.[12] Hence, the current study aims to confirm the antifungal nature of essential oils, extracted from leaves of *A. marmelos,* and to determine their synergistic potential with a well-known antibiotic, nystatin, against pathogenic strains of *Candida albicans*. This is mainly to limit the copious use of antibiotics with no or lesser side effects and to decrease the chances of gaining resistance from the pathogens against the antibiotics used.

Materials and methods

Test microorganism

Three different clinical isolates of *C. albicans* were procured from the Microbiology Department of Brahma Kumari Hospital, Breach Candy Hospital, and KEM Hospital, respectively. These cultures were maintained on Sabouraud's dextrose agar media under refrigerator conditions. They were sub-cultured regularly, and a 25% glycerol stock of the test microorganism were stored at −20°C. Active cultures were obtained by inoculating a loopful of each strain on sterile Sabouraud's dextrose agar slants and incubated at 37°C for 48 h under aerobic conditions.[13]

Extraction of essential oils

The leaves of *A. marmelos* (*Bael*) were purchased from the local market of Mumbai and outsourced for authentication from Agharkar Research Institute, Pune. Obtained leaves were washed with tap water and air-dried for 3 days under sunlight. About 30 g of the air-dried leaves were powdered using a mixer grinder. The powder was subjected to hydrodistillation at 100°C using the Apparatus Clevenger for 1 h [Figure 1], and the procedure gave a yield of 0.3 mL of essential oil.[14] The extracted essential oil was stored at room temperature till further use.

Screening of antimicrobial susceptibility by paper disc diffusion method

To determine the antifungal activity of the essential oil extracted from the leaves of *A. marmelos*, a disc diffusion method was employed.[15] The initial optical density of each of the microbial cultures was maintained at 0.5 McFarland.[16] The media used for the antimicrobial study was Sabouraud's dextrose agar (HiMedia Pvt. Ltd., India). A total of 100 µL of the inoculum were swabbed evenly all over the surface of the media. Later, sterile Whatman filter paper number 1 discs were placed at the center of the agar and 20 µL of the essential oil was loaded onto the disc. Petri plates were subjected to incubation at 37°C for 48 h. The effect of essential oils was reflected by the appearance of a clear zone around the disc (6 mm in diameter). An antibiogram of the test microorganisms was developed using commercially available antibiotic discs (HiMedia Pvt. Ltd., India) by disc diffusion technique. The experiment was performed in triplicate.

Determination of minimum inhibitory concentration and minimum fungicidal concentration of the essential oils extracted from the leaves of *Aegle marmelos* against test microorganism

Overnight cultures of all three strains of *C. albicans* at 37°C were prepared. The culture density was adjusted to obtain turbidity comparable to the McFarland 0.5 standard. The initial undiluted form of the essential oil was considered 100 µL/mL. Later, an equal volume of essential oil and sterile double-strength RPMI 1640 containing 0.5% Tween 80 was mixed to obtain the final concentration of the plant extract at 50 µL/mL. This was further diluted through a two-fold dilution method using double-strength RPMI 1640 containing 0.5% Tween 80. The total system was 100 µL. Two-fold dilutions of the plant extract in sterile double-strength RPMI 1640 containing 0.5% Tween 80 were used for minimum inhibitory concentration (MIC).

Different concentrations of the essential oil were tested in the range of 50–0.39 µL/mL. Positive, negative, and media controls were also maintained. After incubation at 37°C for 48 h, 10 µL from each tube was plated onto sterile Sabouraud dextrose agar and incubated under standard conditions. The plates showing no growth with a minimum concentration of essential oils represented minimum fungicidal concentration.[17] The experiment was performed in duplicate.

Determination of minimum inhibitory concentration and minimum fungicidal concentration of nystatin against test microorganism

Overnight cultures of all three strains of *C. albicans* at 37°C were prepared. The culture density was adjusted to obtain turbidity comparable to the MacFarland 0.5 standard. The microdilution method was employed using a 96-well microtiter plate. The powder form of nystatin (HiMedia) was dissolved in 30% DMSO. The working stock of nystatin was made in double-strength RPMI 1640 at the concentration of 200 µg/mL. It was sterilized by membrane filtration. The media used was RPMI 1640 for MIC.

The antibiotic used for MIC ranged from 100 to 0.78 µg/mL. Positive, negative, and media controls were maintained. After incubation at 37°C for 48 h, 10 µL from each well was plated onto sterile Sabouraud dextrose agar plates and incubated for 48 h at 37°C. The plates showing no growth with a minimum concentration of nystatin were minimum fungicidal concentrations. The experiment was performed in duplicate.

Elucidation of synergistic activity of essential oils of leaves of *Aegle marmelos* and nystatin

The stock solutions and serial two-fold dilutions of each drug to at least double the MIC were prepared as per the guidelines of the Clinical Laboratory Standards Institute (CLSI).[18] A total of 100 µL of RPMI 1640 media supplemented with 0.5% of Tween 80 was used in each of the wells of 96-well microtiter plates. According to the protocol of checkerboard assay, the stock solution of essential oils was diluted along the ordinate

and the stock solution of nystatin was diluted along the abscissa. Each of the wells was inoculated with 10 µL of the pure cell suspension of individual strains of *C. albicans* (optical density is adjusted to 0.5 McFarland unit). The plates were subjected to incubation at 37°C for 48 h under aerobic conditions. After the process of incubation, the growth was observed by removing 10 µL of the culture from each of the wells and spreading it on sterile Sabouraud's dextrose agar plates. The plates were incubated at constant temperature for a predetermined period and checked for the growth of *C. albicans*. The experiment was performed in duplicate.

According to the guidelines of CLSI for broth microdilution method, the MIC was known as the lowest concentration of the drug which inhibits the growth of the organism under study. As per the checkerboard assay,

Fractional inhibitory concentrations (ΣFICs) were calculated as follows: If A is essential oils and B is nystatin.

ΣFIC = FIC of drug A + FIC of drug B.

where, FIC of drug A = MIC of drug A in combination/MIC of drug A alone.

FIC of drug B = MIC of drug B in combination/MIC of drug B alone.

The combination is synergistic if ΣFIC is ≤0.5, indifferent if ΣFIC is >0.5 to <2, and antagonistic if ΣFIC is ≥2.[19]

Results

The overall yield of the essential oil extracted from the leaves of *A. marmelos* was found to be 0.01%. The sensitivity of the pathogenic strains of *C. albicans* procured from Brahma Kumari Hospital, Breach Candy Hospital, and KEM Hospital, respectively, were tested against essential oils extracted from leaves of *A. marmelos* and six different antifungal agents by paper disc diffusion method. All three strains of *C. albicans* appeared to be sensitive toward the essential oils and the zone of clearance shown was highest for nystatin among the six different antibiotics used. The zone of inhibition shown by essential oils against *C. albicans* obtained from Brahma Kumari Hospital, Breach Candy Hospital, and KEM Hospital was 15 mm, 17 mm, and 22 mm, respectively. [Figures 2-4] The antibiotic resistivity pattern was revealed [Figures 5-7] by determining the antibiogram of the test cultures as described in Table 1. To determine the minimum concentration of the essential oil to inhibit the growth of test cultures, the broth microdilution method was performed using 96-well microtiter plates. The results are shown in Table 2. Next, the results of the checkerboard assay to investigate the synergistic potential of the essential oils of the leaves of *A. marmelos* and nystatin against the test organism are depicted in Table 3.

Discussion

Antibacterial and antifungal activity of essential oils extracted from leaves of *A. marmelos* was previously reported.[12,15] Meena *et al*. found that methanolic, ethanolic, and aqueous leaf extract of *A. marmelos* showed minimal antifungal activity against the strain of *C. albicans* MTCC 183 as compared to that of fruit extract.[20] However, in the present study, essential oil obtained from leaves of *A. marmelos* showed satisfactory antifungal activity against all three clinical isolates of *C. albicans*; this may be due to the utilization of concentrated form of the essential oils and difference in the strain of the test microorganisms tested. Padalia *et al*. screened the synergistic potential of leaf, stem, and fruit extract of *A. marmelos* with two different antifungal agents amphotericin B and ketoconazole against *C. albicans* by paper disc method.[21] No synergy was reported in their work concerning the leaf extract of *A. marmelos* and that of amphotericin B, but synergy was observed with ketoconazole against *C. albicans*. However, in the current investigation, as test organisms were found sensitive to nystatin as well as essential oil, the checkerboard assay demonstrated synergy between the essential oil extract of *A. marmelos* and nystatin for all the clinical isolates screened. This could be due to the high affinity of nystatin toward ergosterol to inhibit the growth of fungi by making the cell membrane leaky and variation in the results reported by Padalia *et al*. may be attributed to the decoction method used for phytochemical extraction of the plant of interest as compared to the use of essential oil[21]. Nystatin is known to be one of the potent antifungal agents against *Candida* species. Taweechaisupapong *et al*. described the inhibitory effect of lemongrass oil and nystatin individually on a clinical isolate of *Candida dubliniensis*[17] In consensus with their study, the current work also depicted the antifungal activity of *A. marmelos* essential oils and nystatin against clinical isolates

Table 1: Antibiogram of different clinical isolates of *Candida albicans*

Antibiotics	Zone of inhibition (mm) ± SD		
	C. albicans (Brahma Kumari Hospital)	*C. albicans* (Breach Candy Hospital)	*C. albicans* (KEM Hospital)
Amphotericin-B (AP - 100U)	15	14	14
Clotrimazole (CC - 10 µg)	10	12.5	9
Fluconazole (FLC - 25 µg)	-	19	-
Itraconazole (IT- 10 µg)	-	9	-
Ketoconazole (KT - 10 µg)	11	21	14
Nystatin (NS - 100U)	14	23	14

SD: Standard deviation, *C. albicans: Candida albicans*

Figure 1: Collection of essential oils by Clevenger Apparatus

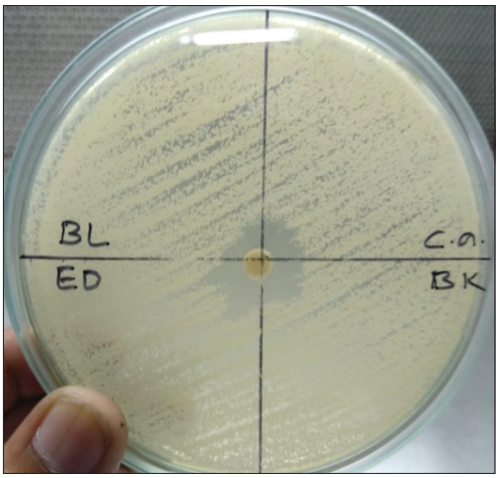

Figure 2: Screening of the antimicrobial activity of essential oils of *Aegle marmelos* against *Candida albicans* (Brahma Kumari Hospital)

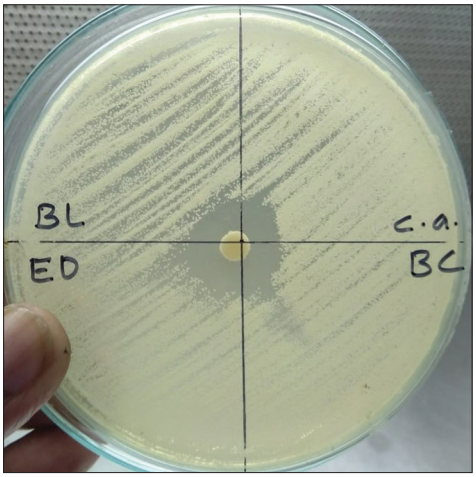

Figure 3: Screening of the antimicrobial activity of essential oils of *Aegle marmelos* against *Candida albicans* (Breach Candy Hospital)

Figure 4: Screening of the antimicrobial activity of essential oils of *Aegle marmelos* against *Candida albicans* (KEM Hospital)

Table 2: Determination of minimum inhibitory concentration/minimum fungicidal concentration of essential oils and nystatin against test organisms

Test microorganism	MIC/MFC of Eos (μL/mL)	MIC/MFC of nystatin (U/mL)
C. albicans (Brahma Kumari Hospital)	12.5	100
C. albicans (Breach Candy Hospital)	12.5	25
C. albicans (KEM Hospital)	25	50

Eos: Essential oils, MIC/MFC: Minimum inhibitory concentration/minimum fungicidal concentration, *C. albicans: Candida albicans*

of *C. albicans*. Therefore, nystatin can be considered the most possible antifungal drug to treat candidial infections along with essential oils of plant origin. Yoosuf *et al*., in their research, investigated synergistic interaction between fluconazole and essential oils of *Ajwain* (*Trachyspermum ammi* (L.) Sprague ex Turril) and coriander against clinical strains of *Candida* species by checkerboard assay[22] and they reported synergy.

In the present study, encouraging results were obtained concerning the synergistic ability of essential oils of leaves of *A. marmelos* and nystatin against clinical isolates of *C. albicans*. This suggests that essential oil is one of the major constituents of traditional medicine and can be effectively employed for the betterment of human health to treat certain diseases along with known drugs since finding a medicinal component from natural sources is always preferable due to its lesser side effects.[23]

Conclusions

Acquiring resistance by pathogenic microorganisms against antibiotics is of major concern in global health care. However, by combining green medicine and allopathy, we could be able to treat various infections caused due to various pathogens including *Candida* species. *A. marmelos* is considered a medicinally important plant according to the Natural Medicinal Plants Board of the Government of India,[24] and therefore, the present line of research strongly inspires the use of *A. marmelos*

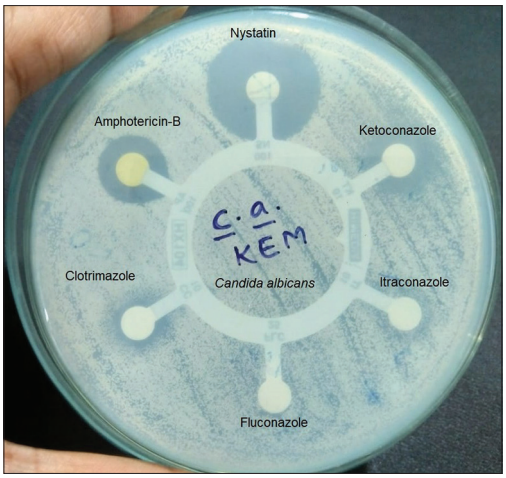

Figure 5: Antibiogram of *Candida albicans* (KEM Hospital)

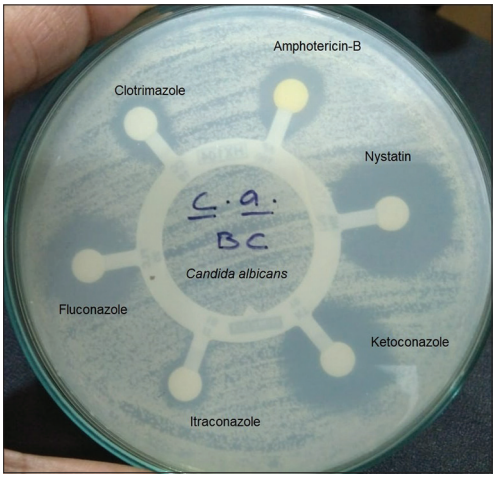

Figure 6: Antibiogram of *Candida albicans* (Breach Candy Hospital)

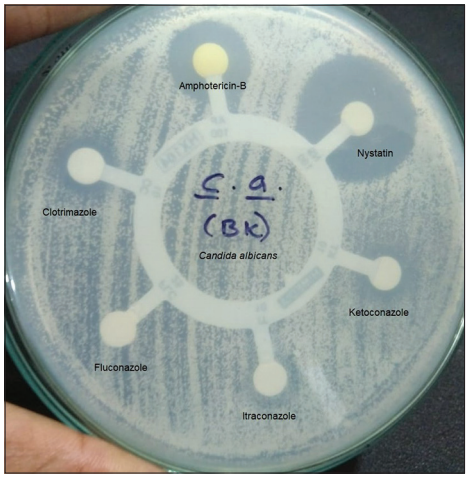

Figure 7: Antibiogram of *Candida albicans* (Brahma Kumari Hospital)

as a potent candidate for combinatorial therapy of modern medicine. Thus, it can be concluded that essential oils extracted from the leaves of *A. marmelos* and a potent antifungal agent nystatin against pathogenic strains of *C. albicans* were found to be synergistic with each other as per the checkerboard assay. The present study indicates that the combined effect of medicinal plant extracts and known antifungal agents offers significant potential for the development of modern era of clinical therapies for the treatment of candidal infections.

Table 3: Determination of fractional inhibitory concentration index

Test organisms	FIC of Eos	FIC of nystatin	ΣFIC
C. albicans (Brahma Kumari Hospital)	0.06	0.06	0.12
C. albicans (Breach Candy Hospital)	0.12	0.25	0.37
C. albicans (KEM Hospital)	0.03	0.25	0.28

Eos: Essential oils, FIC: Fractional inhibitory concentration, *C. albicans: Candida albicans*

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Conflicts of interest

There are no conflicts of interest.

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हिन्दी सारांश

कैंडिडा अल्बिकन्स के प्रतिकार में *एगले मार्मेलोस* (एल.) कोरिया की पत्तियों से निकाले गए वाष्पशील तेलों और निस्टैटिन के बीच सहक्रिया निर्धारित करने के लिए चेकरबोर्ड परीक्षा का उपयोग

प्रमोद आनंद कांबले, मंजू फडके

पृष्ठभूमि: *कैंडिडा एल्बिकैंस* (सी-पी. रॉबिन) बर्खौट (1923) प्रमुख रोगजनक यीस्ट में से एक है, जो कैंडिडिआसिस पैदा करने के लिए जिम्मेदार है। कैंडिडा के उपचार के लिए पारंपरिक एंटिफंगल एजेंटों का उपयोग कम प्रभावी बताया गया है और इसलिए उपचार के लिए वैकल्पिक उपचारों की आवश्यकता है। औषधीय पौधों के वाष्पशील तेल, आधुनिक उपचारों में प्राकृतिक उत्पादों के लिए एक महत्वपूर्ण विकल्प के रूप में काम कर सकते हैं। **उद्देश्य:** चेकरबोर्ड परीक्षा का उपयोग करके कैंडिडा अल्बिकन्स के तीन नैदानिक आइसोलेट्स के खिलाफ *एगले मार्मेलोस* (एल.) कोरिया, की पत्तियों से निकाले गए वाष्पशील तेलों और एक शक्तिशाली एंटीफंगल एजेंट, निस्टैटिन की सहक्रियात्मक क्षमता का निर्धारण करना। **सामाग्री एवं विधि:** *एगले मार्मेलोस* के वाष्पशील तेलों की एंटीफंगल गतिविधि को डिस्क प्रसार तकनीक द्वारा संवर्धन परीक्षण के विरुद्ध जांचा गया था। परीक्षण किए गए जीवों के प्रतिजैविक विकसित किए गए। वाष्पशील तेल और निस्टैटिन की न्यूनतम कवकनाशी सांद्रता निर्धारित करने के लिए, मांसरस सूक्ष्म-तनुकरण विधि को नियोजित किया गया था, और अध्ययन के तहत नैदानिक पृथकीकरण के प्रति वाष्पशील तेल और निस्टैटिन की सहक्रियात्मक क्षमता की जांच करने के लिए एक चेकरबोर्ड परीक्षा का उपयोग किया गया। सांख्यिकीय विश्लेषण के लिए डेटा को मध्य ± एस.डी. (मानक विचलन)के रूप में व्यक्त किया गया। **परिणाम:** उपयोग किए गए *कैंडिडा अल्बिकन्स* के तीन अलग-अलग उपभेदों के लिए ΣFIC (फ्रैक्शनल इनहिबिटरी कन्सन्ट्रेशन) मानों की गणना क्रमशः 0.12,0.37 और 0.28 के रूप में की गई थी। जो कि 0.5 से कम है, इसलिए परीक्षण संवर्धन के विरुद्ध वाष्पशील तेलों और निस्टैटिन के बीच सहक्रिया स्थापन किया गया। **निष्कर्ष:** *एगले मार्मेलोस* की पत्तियों से निकाले गए वाष्पशील तेल एवं निस्टैटिन की संयुक्त चिकित्सा को कैंडिडल संक्रमण के उपचार के लिए एक विकल्प के रूप में माना जा सकता है।

मुख्य शब्द: *एगले मार्मेलोस, कैंडिडा अल्बिकन्स,* चेकरबोर्ड परीक्षा, वाष्पशील तेल, सहक्रिया निस्टैटिन।