ORIGINAL ARTICLE

Anti-*Candida* Activity of Pomegranate Peel Extract in Comparison with Curcumin Extract and their Synergism with Fluconazole and Nystatin

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ABSTRACT

Background: *Oropharyngeal candidiasis is an important sign that may reflect a serious systemic disease, especially in immunocompromised patients who face the intolerable side effects of the available antifungal drugs. This necessitates the development of safe and effective natural components.* ***Objectives:*** *to evaluate the in vitro activities of both pomegranate peel and curcumin extracts and to compare them with nystatin and fluconazole drugs against Candida species. As far as we know, this is the first study comparing between the antifungal potency of both extracts.* ***Methodology:*** *Different Candida species were isolated from patients with oropharyngeal candidiasis. The antifungal activities of methanolic extracts of pomegranate peel and curcumin were tested by disc diffusion method. Both extracts were added to each of nystatin and fluconazole discs to measure their synergistic effects.* ***Results:*** *Highly significant synergism was detected between both extracts and each of antifungal drugs. Curcumin extract was more potent than pomegranate extract.* ***Conclusion:*** *When used in combination with nystatin and fluconazole, curcumin and pomegranate peel extracts are promising and effective anti-Candida agents.*

INTRODUCTION

Oropharyngeal candidiasis is the commonest fungal infection in the human mouth[1]. It is defined as an opportunistic infection of the buccopharyngeal mucosa caused by *Candida* species, where *C. albicans* is the most important one, followed by other species such as *C. tropicalis*, *C. glabrata*, and *C. krusei*[2]. It is an important sign of systemic immunosuppression, such as diabetes mellitus, leukemia and acquired immunodeficiency syndrome (AIDS), however it may occur in healthy persons with poor oral hygiene or those using broad spectrum antibiotics for long durations[3].

Oropharyngeal candidiasis is associated with so annoying symptoms including soreness of buccal and esophageal mucosa, difficulty in swallowing, and difficulty in speech[4]. It can disseminate all over the body causing systemic candidiasis with a mortality rate of 71% to 79%[5]. Treatment of such infection, particularly in immunocompromised patients, is more difficult since reports of resistance to antifungal drugs in those patients are increasing[6]. Moreover, relapses after stopping treatment in those patients are common. Relapse rates has reached high levels (30%–50%) in severely immunocompromised patients[2].

Topical antifungal drugs are the recommended first line therapy for all cases of oropharyngeal candidiasis, while systemic drugs are added in severe cases only[3]. Nystatin is considered the most widely prescribed topical drug for the treatment of oropharyngeal candidiasis[7]. However, it has important side effects, including the prominent bad taste that causes nausea and vomiting[8]. Also, its oral rinse form has high level of sucrose content, that contraindicates it in cases of tooth decay and diabetes[9]. For severe infections or intolerance to local drugs, systemic triazoles such as fluconazole are effective[6]. Fluconazole is a fungistatic drug used to treat systemic mycoses due to its effectiveness with broad activity profile[10].

In general, local antifungal drugs often do not reach the recommended therapeutic concentrations in the mouth[11]. Hence, for systemic drugs like fluconazole, high doses are usually needed to successfully reach the oral tissues, this results in serious complications such as hepatotoxicity especially in old ages. Other limitations frequently encountered with fluconazole are drug interactions and the emergence of drug resistance[12]. These limitations related to conventional antifungal drugs have encouraged the use of herbal medicine that may introduce new therapeutic compounds with potent biological activity and no side effects, such as pomegranate and curcumin[13].

Pomegranate (Punica granatum L.) is a small tree originated from the Middle East and now cultivated in South Africa, India, China, and America[14]. It is one of the first cultivated plants by humanity. It is involved in

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old traditional medicine for the treatment of several diseases such as fever, diarrhea, skin ulcers and microbial infections[15]. It was used by ancient Egyptians for treating tapeworm and other parasitic infestations[16]. Pomegranate is a rich source of polyphenols, which have antimicrobial and antioxidant properties[17].

Polyphenols are also present in another food additive which is curcumin. Curcumin is one of the chemical components of the common spice turmeric, that is derived from the rhizome of Curcuma longa plant[18], that is widely cultivated in many countries of Asia like China, India, and other tropical countries[19]. In ancient Asia, it was used for wound healing and relieving abdominal pain. Recently, curcumin has showed effective anti-inflammatory and anti-cancer activities[20].

The aim of this study was to assess the *in vitro* antifungal activities of pomegranate and curcumin extracts in comparison and in synergism with nystatin and fluconazole on different *Candida* species isolated from cases of oropharyngeal candidiasis. To the best of our knowledge, this is the first study that compares between the *in vitro* anti-*Candida* activity of pomegranate and curcumin extracts.

METHODOLOGY

Preparation of plant extracts:

Each pomegranate fruit (Punica granatum L. cv. Baladi) was rinsed with distilled water. The peels were collected, cut into small pieces, and then left to dry in air for 5 days[21]. The dried pieces were crushed into powder in a blender. Pomegranate methanol extract was prepared according to Mansourian et al.[22], where 10 grams of peel powder were soaked in 100 ml of absolute methanol in a flask to get a concentration of 10% (100 mg/ml). Regarding curcumin extract, one gram of purchased curcumin powder was dissolved in 100 ml of absolute methanol in another flask to get a concentration of 1% (10 mg/ml)[23]. Both flasks were put on a rotatory shaker for 48 hours at room temperature. The two solutions were then filtered using Whatman filter paper and stored at -20°C until use[22].

Fungal isolation:

This study included 60 patients clinically diagnosed as oropharyngeal candidiasis, who were admitted to Tropical Department, Tanta University hospitals, suffering from hepatic diseases complicated by oropharyngeal candidiasis. An informed consent was taken from each participant. This work was approved by the ethical committee of Faculty of medicine, Tanta University.

The oropharyngeal lesions were gently rubbed using sterile cotton swabs. The samples were transmitted immediately to Microbiology Department of Faculty of Medicine, Tanta University. The swabs were cultured on Sabouraud dextrose agar (Oxoid, UK) plates and incubated aerobically for 24-48 hours at 37°C. Isolates were identified to genus and species levels using colony morphology, Gram stain, germ tube test and confirmed by subculturing on chromogenic *Candida* agar (CHROMagar™Candida, Paris, France). According to manufacturer instructions, *C. albicans* showed green colonies, *C. tropicalis* showed blue colonies, while pink fuzzy colonies were identified as *C. krusei* (Fig. 1).

Antifungal susceptibility tests:

The anti-fungal susceptibility of *Candida* isolates was tested by disc diffusion method according to the Clinical and Laboratory Standards Institute (CLSI) guidelines[24]. The used antifungal discs were fluconazole 25µg (Oxoid, UK), nystatin 100 units (Oxoid, UK), and autoclaved sterile filter paper discs impregnated with 10µ of either pomegranate or curcumin extracts. For synergy testing, 10µ of each extract was added to both fluconazole and nystatin discs. One empty disc and one absolute methanol disc were used as negative controls. Zones of inhibition around discs were measured in millimeter using a ruler.

Statistical analysis:

Data were analyzed using SPSS 26. The diameter of inhibitory zones for all compared components were presented as mean ± standard deviation. Student's T-test was used to compare between the diameter of inhibitory zones of different components. *P*-value < 0.05 was considered significant and < 0.001 was considered highly significant.

RESULTS

Out of 60 samples tested, 61 *Candida* isolates were detected, since one sample yielded 2 *Candida* species (*C. albicans* and *C. tropicalis*). *C. albicans* (57/61, 93.44%) was the most frequent isolated species, followed by *C. tropicalis* (3/61, 4.92%), then *C. krusei* (1/61, 1.64%). In the present study, results of the antifungal activity of the tested components against *both C. albicans* and *C. tropicalis* are gathered in one table since both species have the same interpretative break points. According to CLSI[24], *C. albicans* and *C. tropicalis* breakpoints for fluconazole are (sensitive if ≥17 mm; resistant if ≤ 13 mm). *C. krusei* is intrinsically resistant to fluconazole[24], therefore testing of the synergistic activity against it was ruled out. There are no interpretative breakpoint criteria available from CLSI for nystatin.

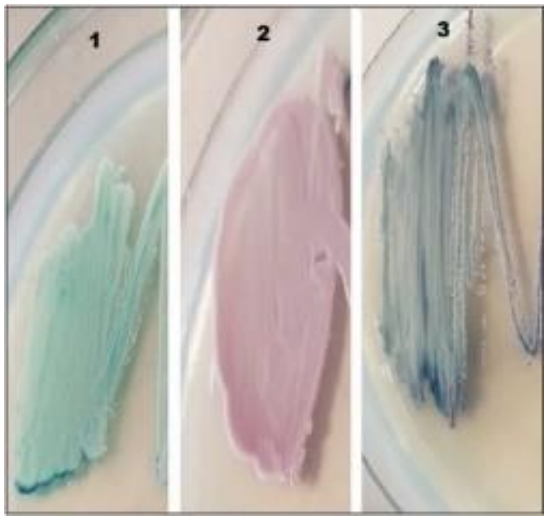

Fig. 1: Identification of isolated *Candida* species on CHROM agar.
1: Green colonies of *C. albicans*; **2:** Pink colonies of *C. krusei*; **3:** Blue colonies of *C. tropicalis.*

As shown in table 1, the mean of inhibitory zones of pomegranate and curcumin extracts against *C. albicans* and *C. tropicalis* isolates were 11.5 ± 1.05 mm and 13.5 ±1.39 mm, respectively. We noticed smaller diameters of the inhibitory zones of pomegranate and curcumin extracts in comparison with nystatin and fluconazole zones (20.17 ± 0.41 and 33 ± 0.63, respectively). However, these diameters are significantly increased by the synergism effects of each of pomegranate and curcumin when added to each of nystatin and fluconazole with highly significant differences between all couples (Fig. 2, Fig. 3).

Table 1: Comparison between the inhibition zone diameters exhibited by different tested components against both *C. albicans* and *C. tropicalis* (60 isolates):

Mean ± SD of the inhibitory zones in millimeter		***P*-value**
Nystatin alone	**Pomegranate + Nystatin**	0.000**
20.17 ± 0.41	21.67 ± 0.52	
Nystatin alone	**Curcumin + Nystatin**	0.000**
20.17 ± 0.41	22.83 ± 0.75	
Fluconazole alone	**Pomegranate + Fluconazole**	0.001**
33 ± 0.63	34.67 ± 0.52	
Fluconazole alone	**Curcumin + Fluconazole**	0.000**
33 ± 0.63	37.5 ± 0.55	

* Statistically significant at $P < 0.05$.
** Statistically highly significant at $P < 0.001$.

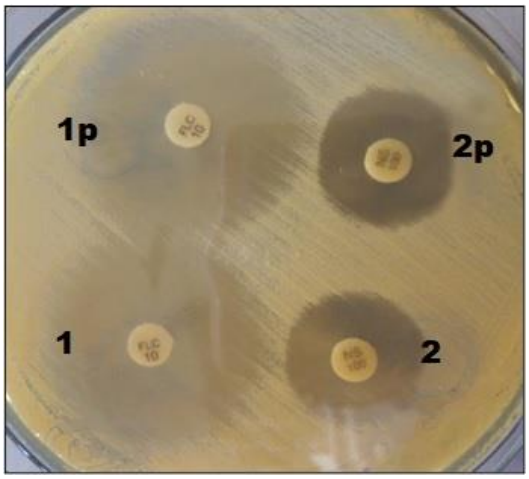

Fig. 2: Synergism between pomegranate extract and each of fluconazole and nystatin against *Candida albicans*
1: Zone inhibition of fluconazole; **1p:** Zone inhibition of fluconazole combined to pomegranate;
2: Zone inhibition of nystatin; **2p:** Zone inhibition of nystatin combined to pomegranate.

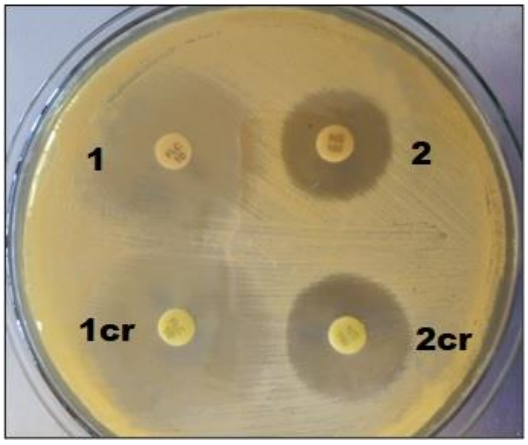

Fig. 3: Synergism between curcumin extract and each of fluconazole and nystatin against *Candida albicans*
1: Zone inhibition of fluconazole; **1cr:** Zone inhibition of fluconazole combined to curcumin; **2:** Zone inhibition of nystatin; **2cr:** Zone inhibition of nystatin combined to curcumin.

Table 2 shows that curcumin extract was more potent than pomegranate peel extract against *Candida* isolates ($P = 0.017$), and the synergism between both extracts was highly significant compared with the activity of each one tested alone ($P = 0.000$).

Table 2: Comparison between the potency of pomegranate and curcumin extracts against both *C. albicans* and *C. tropicalis* (60 isolates):

Mean ± SD of the inhibitory zones in millimeter		*P*-value
Pomegranate alone	**Curcumin alone**	0.017*
11.5 ± 1.05	13.5 ±1.39	
Pomegranate alone	**Pomegranate + Curcumin**	0.000**
11.5 ± 1.05	17.33 ± 0.82	
Curcumin alone	**Pomegranate + Curcumin**	0.000**
13.5 ±1.39	17.33 ± 0.82	

* Statistically significant at $P < 0.05$.
** Statistically highly significant at $P < 0.001$.

Regarding the single *C. krusei* isolate, the diameters of inhibitory zones of nystatin, pomegranate and curcumin extracts were 15 mm, 8 mm, and 10 mm, respectively. Higher diameters were noticed when nystatin combined with pomegranate and curcumin extracts (17 mm and 19 mm, respectively). No inhibitory zones around the methanol disc and the blank filter paper disc (negative controls) were seen.

DISCUSSION

In the present study, there was a highly significant synergistic antifungal activity when methanolic pomegranate extract was added to nystatin against *C. albicans* and *C. tropicalis* ($P = 0.000$). This potent activity of pomegranate was supported by other studies, Da Silva et al.[25] observed a synergistic activity by the combination of nystatin and punicalagin (an ellagitannin isolated from pomegranate) compared with the activity of each compound tested alone, using broth microdilution checkboard method. In addition, Bassiri-Jahromi et al.[14] compared between the *in vitro* activity of different parts of the fruit against five species of *Candida*, including *C. albicans*, *C. parapsilosis*, *C. tropicalis*, *C. glabrata*, and *C. krusei*. They demonstrated that all parts had high antifungal activity, but peel extract was the most potent part.

Moreover, Wang et al.[26] reported that the methanolic extract of pomegranate peels exhibited the maximum antifungal inhibition in comparison with other extracts; ethyl acetate, ethanol, acetone, and water. Furthermore, an *in vivo* study was conducted by Bassiri-Jahromi et al.[27] in rats, they observed that pomegranate peel extract had a strong activity comparable to nystatin, against *C. albicans*, and no side effects were noticed in the examined rats. On the contrary, Bonjar et al.[28] showed that pomegranate had no activity against *C. albicans*. This may be related to the low concentration they used (20 mg/ml), but in our study, the used concentration was 100 mg/ml of pomegranate extract.

On the other hand, the combination of pomegranate and fluconazole yielded a highly significant synergistic activity against *C. albicans* and *C. tropicalis* isolates in the present study. This was in agreement with Endo et al.[15], they proved this synergism by detecting the antifungal inhibition effect of this combination, in addition to its effect on the morphology and ultrastructure of *C. albicans* using electron microscope. An irregular budding and pseudohyphae were found in treated isolates.

The main components in the pomegranate extract are polyphenols and ellagitannin such as punicalagin. Punicalagin was originally isolated from the peel of pomegranate fruit and is reported to have different antimicrobial activities[29]. Also, polyphenols form soluble complexes with high molecular weight proteins. These polyphenols inhibit the fungal enzymes of protein nature (oxidoreductases) in *Candida* cell wall and cytoplasm. They may also interact with the adhesins, so they can interfere with the function of fungal surface receptors[16]. One of the important parameters determining the concentration of the polyphenols in pomegranate fruit is its peel color, where the darker red color showed higher concentrations than the light-colored fruits[30].

As regards curcumin in the current study, we observed a potent antifungal activity of curcumin. Similarly, Martins et al.[31] reported a dramatic antifungal effect of curcumin against different types of fungi. They found that *C. albicans* was highly susceptible to curcumin. In addition, they explored the ability of curcumin to prevent the adhesion of *Candida* to buccal epithelial cells, and they found that curcumin was more potent than fluconazole in inhibiting the adhesion of different *Candida* species to the epithelium of buccal mucosa. In addition, Sharma et al.[32] reported a 16- and 32-fold drops in the MIC_{80} values of the nystatin and fluconazole, respectively when curcumin was used in combination with both drugs, supporting the synergistic interaction between curcumin and each of nystatin and fluconazole. Moreover, Garcia-Gomes et al.[33] demonstrated the synergistic effect between curcumin and fluconazole and showed that curcumin was able to restore the sensitivity to fluconazole when curcumin was applied to fluconazole-resistant isolates of *C. albicans*.

Curcumin or diferuloylmethane is a polyphenolic compound that has wide range of biological activities against *Candida*. It can raise the level of oxygen radicals in *Candida* cells stimulating the fungal apoptosis[34]. Also, by using scanning laser microscopy, Raja et al.[35] demonstrated that methanolic extract of Curcuma Longa can disrupt the cell membrane and the cell wall of *Candida*. Moreover, curcumin can reduce the expression of genes responsible for formation of fluconazole efflux pumps on *Candida* plasma

membranes, this explains the ability of curcumin to decrease the drug resistance against fluconazole[36].

The current study showed that curcumin extract was significantly more potent than pomegranate peel extract against *Candida* isolates. Unfortunately, there are no similar studies comparing between the antifungal activities of both extracts. This made it difficult to compare our results with other ones and to conclude that there are no different results.

Finally, it is important to shed the light on the safety and adverse effects of both extracts. Several studies have been performed on different parts of pomegranate, but no side effects have been reported for the examined dosages[37]. As regards curcumin, it is considered generally safe as approved by Food and Drug Administration (FDA)[38]. However, some dose-related adverse effects were reported. Therefore, the European Food Safety Authority (EFSA) and The Joint United Nations and World Health Organization Expert Committee on Food Additives (JECFA) have recommended daily dose of curcumin to be 0–3 mg/kg body weight[39]. Higher daily doses for one to four months may cause mild symptoms such as diarrhea, yellow stool, rash, and headache[40].

CONCLUSION

In conclusion, our *in vitro* results highlight the effect of pomegranate peel and curcumin extracts as effective antifungal agents in treating oropharyngeal candidiasis, giving much more potent synergistic inhibitory effects than the sole use of the commercial antifungal drugs, either nystatin or fluconazole. Also, curcumin extract showed a greater activity against *Candida* than pomegranate peel extract.

- The authors declare that they have no financial or non financial conflicts of interest related to the work done in the manuscript.
- Each author listed in the manuscript had seen and approved the submission of this version of the manuscript and takes full responsibility for it.
- This article had not been published anywhere and is not currently under consideration by another journal or a publisher.

Authors contribution:

Sara Y. Maxwell and Azza M. Hassan contributed to the study design. Sally Elnawasany collected the samples. Sara Y. Maxwell and Marwa M.E. Abd-Elmonsef contributed to laboratory experiments. All authors contributed to the data interpretation. Marwa M.E. Abd-Elmonsef and Sara Y. Maxwell drafted and revised the manuscript. All the authors read and approved the final manuscript.

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