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Minimum Inhibitory Concentration of *Peganum harmala* Extract Against Candida Species

Maryam Baharvand [1], Mojdeh Hakemi-Vala [2], Masoumeh Mehdipour [1], Leila Dehghani [3], Elnaz Jalali [4], Fahimeh Anbari [1]

[1] Department of Oral and Maxillofacial Medicine, School of Dentistry, Shahid Beheshti University of Medical Sciences, Tehran, Iran
[2] Department of Microbiology, Faculty of Medicine, Tehran University of Medical Sciences, Tehran, Iran
[3] School of Dentistry, Shahid Beheshti University of Medical Sciences, Tehran, Iran
4 Private Dental Practice, Oral & Maxillofacial Radiology Private Practice, Miami, Florida, USA

Corresponding author:
Fahimeh Anbari, Department of Oral and Maxillofacial Medicine, School of Dentistry, Shahid Beheshti University of Medical Sciences, Tehran, Iran

fahimeh.anbari@gmail.com

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Abstract

Background and Aim: There are some rich sources of antifungals in medicinal herbs. They have general acceptance due to their low cost and insignificant side effects. The efficacy of *Peganum harmala* (*P. harmala*) extract against Candida species was investigated in this study.

Materials and Methods: In this in vitro study, we used the maceration technique to obtain *P. harmala* aqueous and alcoholic extracts, and assess their antifungal activity against different species of *Candida albicans* (*C. albicans*), *Candida glabrata* (*C. glabrata*) and *Candida krusei* (*C. krusei*). We used the microdilution method to calculate the minimum inhibitory concentration (MIC). The tests were repeated in triplicate for higher accuracy.

Results: The aqueous and alcoholic *P. harmala* extracts showed antifungal activity against *C. albicans* in 100 mg/mL concentration. The antifungal efficacy of *P. harmala* alcoholic extract against *C. glabrata* (MIC: 100 mg/mL) was higher than the aqueous extract (MIC:200 mg/mL). The antifungal efficacy of *P. harmala* alcoholic extract against *C. krusei* (MIC: 1.56 mg/mL) was higher than the aqueous extract (MIC: 12.5 mg/mL).

Conclusion: The aqueous and alcoholic extracts of *P. harmala* were effective against all species of Candida particularly *Candida krusei*. The alcoholic extract of *P. harmala* was more effective than its aqueous extract against *C. glabrata* and *C. krusei*.

Key Words: *Candida albicans*; *Candida glabrata*; *Peganum harmala*

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Introduction

During the past decade, fungi surfaced as new life-threatening agents. The prevalence of these opportunistic infections in humans and animals has increased worldwide, challenging many medical centers in the world and becoming a significant cause of morbidity and mortality in immunocompromised patients residing in developing countries. Furthermore, the antifungal agents that are currently available have gradually lost their efficacy, highlighting the dilemma of acquired resistance by microorganisms. The resistant species of fungi continuously emerge, commanding the need for a comprehensive search and production of modified new drugs [1].

The Candida species particularly *Candidaalbicans* (*C. albicans*) as human

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commensals are among opportunistic microorganisms that can cause local and systemic infections. Under certain conditions, *C. albicans* and some other species can cause opportunistic oral, vaginal or systemic infections in immunocompromised hosts [2].

The non-albicans Candida species comprise approximately 50% of candidaemia cases [3]. They are also notorious for causing a variety of infections in immunocompromised patients, those with pacemakers or artificial joints, and bone marrow transplant recipients. Among the non-albicans Candida species, the highest mortality rate is caused by *Candida glabrata* (*C. glabrata*) (40-70%), which tends to infect immunocompromised and neutropenic patients. It also forms a biofilm in urinary catheters and on dental material surfaces [4]. Severe immunodeficiency is a major risk factor for development of *Candida krusei* (*C. krusei*) infection, particularly in patients with hematological malignancies, stem cell transplants, and interestingly, in patients receiving azole antifungals and corticosteroid therapy [5].

In recent years, resistance of different clinical isolates of Candida species to standard antifungal treatments has been frequently and extensively reported [6]. To overcome new and re-emerging infections, novel antimicrobial agents are required. Medicinal plants are inexpensive and harmless alternative sources of antimicrobial agents [1,2].

Peganum harmala (*P. harmala*), which is also known as the Syrian rue, is a flowering plant that belongs to the family of Zygophyllaceae. It can be found in different regions including the Mediterranean region, central Asia, and North Africa [7]. *P. harmala* is known as “Espand” in Iran.

The available pharmaceutical studies have revealed a variety of medicinal applications for *P. harmala* due to its antibacterial and antifungal properties and monoamine oxidase inhibition. Also, it has been reported that this plant has anti-tumor activity, vasorelaxant effects, and antipyretic, antinociceptive, antiprotozoal and anti-inflammatory properties. Traditionally, the seeds’ smoke is used as a disinfectant agent. The reported effects of *P. harmala*, at least partly, may be due to its beta-carboline alkaloids mainly found in the seeds, which include harmine, harmaline, harmalol and vasicine [8].

The antifungal activity of *P. harmala* and its inhibitory effects on Lactobacilli and *C. albicans* have also been reported [7,8] However, different effective concentrations have been reported, and only one type of extract (aqueous or alcoholic) has been evaluated in previous studies [8]. Furthermore, comparative effects of aqueous and alcoholic extracts of *P. harmala* on typical isolates of candida species have not been previously studied. Therefore, the aim of this study was to assess the antifungal effects of aqueous and alcoholic extracts of *P. harmala* on three typical yeast isolates as the causative agents of invasive candidiasis including *C. albicans*, *C. krusei*, and *C. glabrata*, to highlight the possible applications of *P. harmala* as a novel alternative therapeutic approach for fungal infections.

Materials and Methods

This study was approved by the ethics committee of Shahid Beheshti University of Medical Sciences (SBMU.161013/1394). In this in vitro study, *P. harmala* was collected in late spring from hills around Tehran, Iran and authenticated by the experts at the Medicinal Botanical Center, Faculty of Pharmacology. We used the maceration process to obtain aqueous and alcoholic extracts of *P. harmala*.

To prepare the aqueous extract, 100 g of air-dried *P. harmala* seeds was weighed with a digital scale and ground in an electrical grinder. This powder was then dissolved in 1000 mL of distilled water in an Erlenmeyer flask and heated for 10 minutes. The flask was covered with aluminum foil for 40 minutes. The

solution was then filtered by filter paper. By using water bath, the final extract with 10 gr/mL concentration was obtained [7].

To prepare the alcoholic extract, using the aforementioned method, 100 g of the ground *P. harmala* seeds was infused in 1000 mL of 96% ethanol in an Erlenmeyer flask. The flask was covered with aluminum foil and agitated on a shaker at 90 cycles per minute for 48 hours. The solution was filtered by filter paper, and the filtrate was condensed with a rotary evaporator to concentrate the extract. After thorough evaporation of the solvent, pure alcoholic extract with a concentration of 10 gr/mL was refrigerated at 4°C in an airtight container until use for antimicrobial assay [8].

The main fungal specimens included the standard Persian Type of Culture Collection (PTCC) strains of *C. glabrata* (PTCC 5295), *C. albicans* (PTCC 5027), and *C. krusei* (PTCC 5297) which were all obtained from the Iranian Research Organization for Science and Technology. To prepare fungal suspensions, 24-hour cultures of *C. albicans*, *C. glabrata*, and *C. krusei* were prepared on Mueller-Hinton agar medium at 37°C. Using the cultures on agar media, fungal suspensions were prepared containing approximately 1.5 x 10^8 colony-forming units per milliliter (CFUs/mL), by comparing them with the McFarland (turbidity) standard.

The agar dilution method was carried out according to Diba et al. [8]. The primary extract concentration of 100 mg/mL was chosen. The following eight decreasing titers of *P. harmala* were then prepared: 50.0, 25.0, 12.5, 6.25, 3.125, 1.56, 0.78, 0.39 mg/mL. To prepare these dilutions, 8 sterile capped micro-plates containing 100 µL of Mueller-Hinton broth were used. The aqueous and alcoholic extracts of *P. harmala* (100 mg/mL) were diluted with 2% dimethyl sulfoxide. Then, 100 µL of each solution was transferred into the first micro-plate containing 100 µL of substrate, mixed and transferred into the next micro-plate in the same manner up to the 8th well. Accordingly, the aforementioned eight titers of *P. harmala* were prepared.

Using distilled water, the prepared Candida suspensions were diluted 1:20. Then, 10 µL of each Candida suspension was inoculated into the serially diluted micro-plates and incubated at 37°C for 24 hours. Consequently, each micro-plate contained a concentration of 5 x 10^8 CFUs/mL of the fungal suspension.

Considering the Clinical and Laboratory Standard Institute guidelines, the microdilution method was adopted to calculate the minimum inhibitory concentration (MIC) [9]. Due to colored nature of the extract, MIC determination by visual assessment of Candida growth and turbidity in the micro-plates was not possible. To overcome this problem, the content of each micro-plate was cultured on Mueller-Hinton agar medium. The prepared media were re-incubated at 37°C, and were investigated for fungal growth after 24 hours. The Mueller-Hinton medium with Candida inoculum was used as positive control, and the culture medium with or without the extract was used as negative control for each micro-plate. The lowest concentration of extract that inhibited the growth of Candida was recorded as the MIC [9]. Since a minimum of 24-hour time interval was required for the MIC of the plant extract against fungal growth, the effective time interval of 24 hours was chosen in this study [8].

Since the fungicidal effect of the extracts was not observed clearly on *C. albicans* and *C. glabrata*, the plant extracts were prepared with a higher concentration in another antimicrobial assay from which serial dilutions were made. Therefore, the primary concentration was considered to be 800 mg/mL to include a broad range of various concentrations of *P. harmala* extract with the following titers: 400.0, 200.0, 100.0, 50.0, 25.0, 12.5, 6.25, and 3.125 mg/mL. The serial dilution process was performed according to the aforementioned protocol. During our experimental process, MIC

was evaluated for three times to reduce systematic errors.

Results

The results of micro-dilution assay (Fig. 1) showed that 24-hour treatment with both aqueous and alcoholic extracts of *P. harmala* were effective against all tested Candida isolates in the following concentrations: MIC of 100 mg/mL of both types of extracts for *C. albicans* (Fig. 2a and 2b), MIC of 12.5 mg/mL and 1.56 mg/mL of aqueous and alcoholic extracts of *P. harmala* for *C. krusei*, respectively (Table 1), and MIC of 200 mg/mL and 100 *harmala* for *C. glabrata*, respectively (Fig. 3a and 3b).

As shown in Table 1, for each Candida isolate, the MIC of aqueous and alcoholic extracts of *P. harmala* was totally different. In the present study, we obtained the same MIC value in the three replica trials; thus, there was no need to perform statistical analysis among the experimental groups.

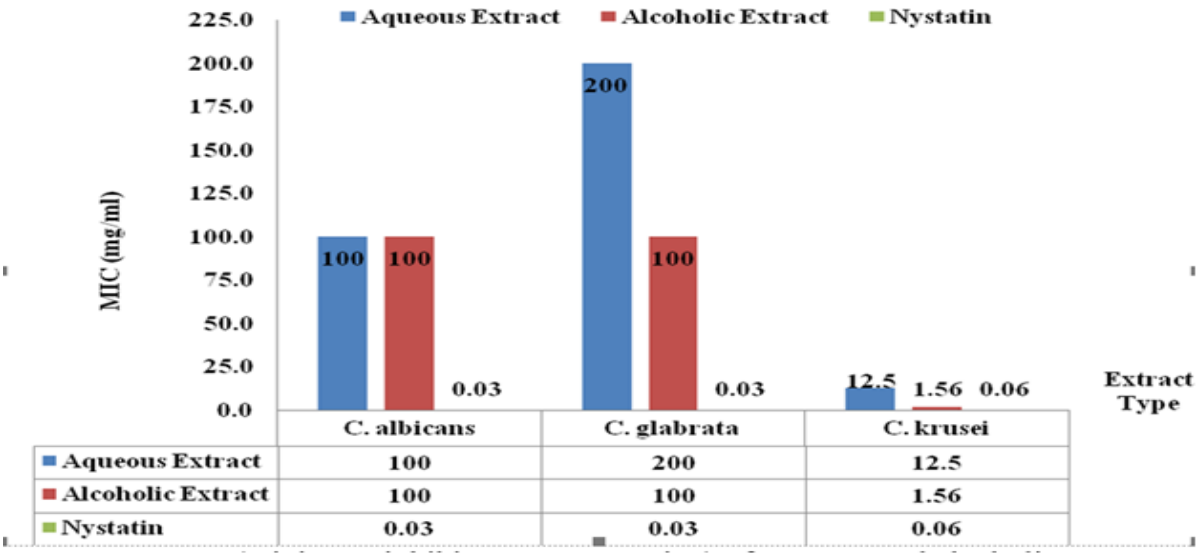

Figure 1. MIC of aqueous and alcoholic extracts of *P. harmala* compared with that of nystatin against *C. albicans*, *C. glabrata*, and *C. Krusei*

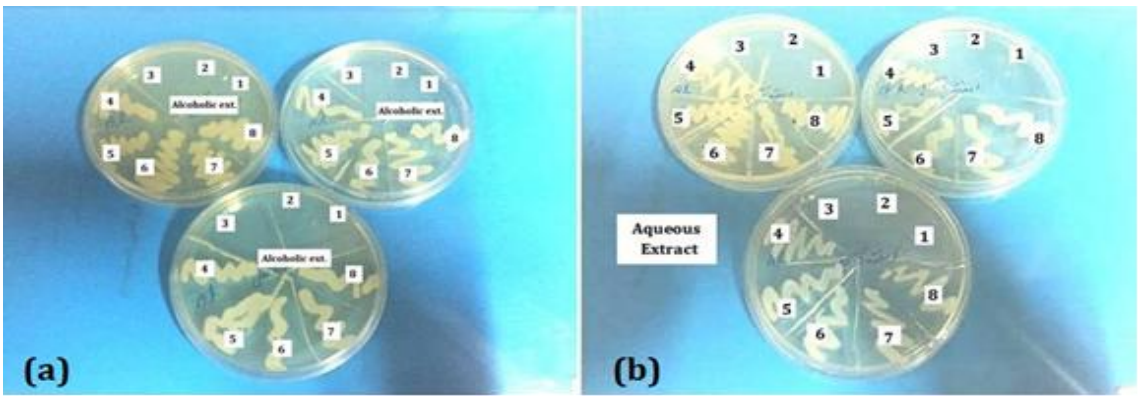

Figure2. (a). MIC of alcoholic extract of *P. harmala* against *C. albicans* in titers of 400.00 to 3.125 mg/mL
(b). MIC of aqueous extract of *P. harmala* against *C. albicans* in titers of 400.00 to 3.125 mg/mL

Table 1. MIC of aqueous and alcoholic extracts of *P. harmala* against *C. albicans*, *C. glabrata*, and *C. Krusei*

MIC (mg/mL)	*C. albicans*		*C. glabrata*		*C. krusei*	
	Aqueous Extract (+/-)	Alcoholic Extract (+/-)	Aqueous Extract (+/-)	Alcoholic Extract (+/-)	Aqueous Extract (+/-)	Alcoholic Extract (+/-)
400.00	-	-	-	-	-	-
200.00	-	-	-	-	-	-
100.00	-	-	+	-	-	-
50.00	+	+	+	+	-	-
25.00	+	+	+	+	-	-
12.50	+	+	+	+	-	-
6.25	+	+	+	+	+	-
3.12	+	+	+	+	+	-
1.56	+	+	+	+	+	-
0.78	+	+	+	+	+	+
0.39	+	+	+	+	+	+

(-= No Growth, + = Growth)

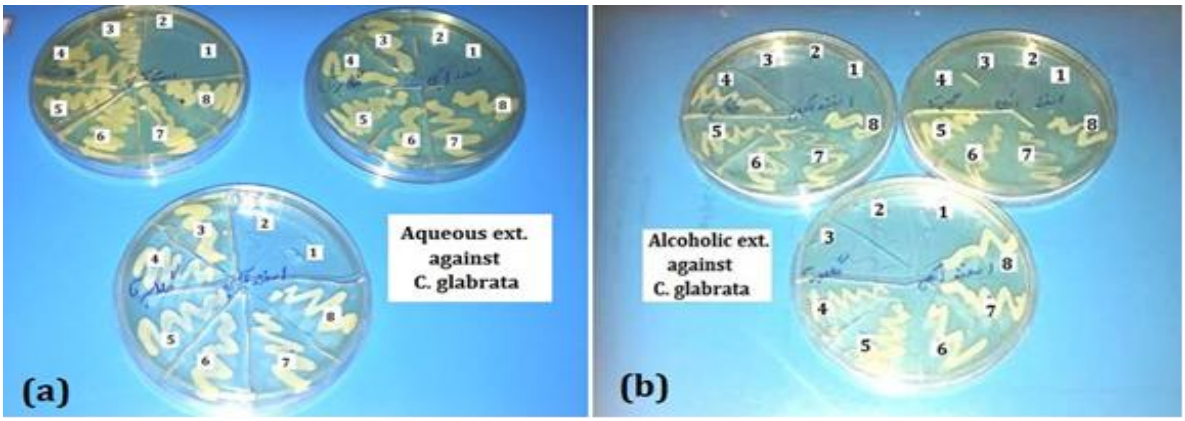

Figure 3 (a). MIC of aqueous extract of *P. harmala* against *C. glabrata* in titers of 400.00 to 3.125 mg/mL
(b). MIC of alcoholic extract of *P. harmala* against *C. glabrata* in titers of 400.00 to 3.125 mg/mL

Discussion

In this study, using the micro-dilution method, antifungal effects of different concentrations of aqueous and alcoholic extracts of *P. harmala* in 2% dimethyl sulfoxide solvent on growth of three Candida sub-species of *C. albicans*, *C. krusei*, and *C. glabrata* were investigated to evaluate the MIC values.

Despite the development of antibiotics, presence of multidrug resistant strains poses a great challenge in medicine against fungal and bacterial infections. Due to their availability, better biodegradability, low cost and harmless nature, ethno medicinal plants may offer a great potential as alternative anti-fungal agents. From the ancient times, *P. harmala* seeds have been considered as a plant with medicinal properties. Previous studies confirmed the antifungal, antibacterial, antipyretic and anti-inflammatory effects of *P. harmala* [7,8]. Although scientific evidence indicates that the alkaloid extract of *P. harmala* has in vitro antifungal activity against Candida species, to use this medicinal property in the clinical setting, primarily the proper concentration of this extract against Candida needs to be ascertained. The results of this investigation confirmed the antifungal activity of both aqueous and alcoholic extracts of *P. harmala* in MIC of 100 mg/mL against *C. albicans*. However, the alcoholic extract of *P. harmala* in MIC of

100 mg/mL caused more growth inhibition against *C. glabrata* species than the aqueous extract in MIC of 200 mg/mL. Also, the inhibitory effect of the alcoholic extract in MIC of 1.56 mg/mL against *C. krusei* was higher than the aqueous extract (MIC of 12.5 mg/mL).

C. albicans is the major species of Candida in the oral cavity. Other Candida species can be found in other parts of the oral cavity. Presence of Candida species in the oral cavity is less frequent (40-60%) in healthy subjects compared with HIV/AIDS patients (62-93%) [10] and hence, they have higher sensitivity towards antifungal drugs.

Compared with others, higher fungicidal effect of ethanolic extract is mostly related to the higher extraction efficiency of alcohol, especially for non-polar compounds [11]. The potential antimicrobial activity of a medicinal plant may not only be related to one single active ingredient but also to synergistic interactions when applied as binary mixtures of different alkaloids [12]. The main active ingredients of *P. harmala* seeds include harmaline and harmine, which are responsible for different pharmacological and therapeutic effects of this traditional plant [8]. Although in this study, isolation of active alkaloids of *P. harmala* was not conducted, the antifungal activity of this plant is attributed to its main active alkaloid constituents including beta-carbolines, harmine, harmaline and harmalol [12]. The analogous β-carboline alkaloid harmine of *P. harmala* is capable of denaturing bacterial DNA [13]. Further antibacterial and antifungal effects of *P. harmala* have been reported by Hajji et al [14]. Hashem [11] used the crude extracts of five Egyptian medicinal plants to investigate their antifungal properties against dermatophytes and emerging fungi. The results showed a MIC of more than 100 mg/mL for ethanolic and chloroform extracts of *P. harmala* while the MIC of ethyl acetate extract of *P. harmala* was reported to be 125 mg/mL [11]. The MIC value of the alcoholic extract of *P. harmala* against *C. albicans* in our study was also in accordance with the study by Hashem (100 mg/mL) [11]. Although the methodology and collection site of *P. harmala* differed in these studies, the ethanolic extract of this plant was used against *C. albicans* in both studies. Furthermore, since in both studies the season of collection was late spring and similar parts of the plant were utilized, the minimal detectable difference in MIC seems to be related to the elimination of agar in the present study and applying the micro-dilution method while in Hashem's study the broth micro-dilution method was performed.

Diba et al. [8] evaluated the fungicidal effect of *P. harmala* in the form of alcoholic extract on some hospital isolates of Aspergillus and Candida in vitro. The results of the broth micro-dilution assay in this study showed that the minimum fungicidal concentration of the alcoholic extract for Candida isolates ranged from 1.40 to 1.160 mg/mL. They found the highest fungicidal activity against *C. glabrata* at a MIC of 0.0625 g/dL, and the lowest MIC (0. 25 g/dL) against *C. albicans*. In general, the alcoholic extract was active against all the yeasts tested in this study [8]. The alcoholic extract of *P. harmala* in this study similarly exhibited antifungal properties against *C. albicans* and *C. glabrata*.

In a study carried out by Falahati et al., [15] the antifungal effects of *P. harmala* alkaloid extract against Aspergillus niger, Microsporum gypseum, and *Candida albicans* was evaluated in vitro. The MIC range for the alkaloid extract of *P. harmala* was not broad and reported to be in the range of 1.00 to 3.20 mg/mL. Furthermore,

comparison between the effects of alkaloid extract and miconazole showed a great discrepancy and although the alkaloid extract initially seemed to be a weak candidate to exhibit antifungal properties, the final results proved otherwise [15]. Similarly, in the present study, both aqueous and alcoholic extracts of *P. harmala* showed antifungal activities. Additionally, in both studies the antifungal effect of *P. harmala* extract was evaluated by determining the MIC value for *C. albicans*. Yet, in contrast to the study by Falahati et al. [15] that the alkaloid extract of *P. harmala* was used, the aqueous and alcoholic extracts of the plant were prepared in the present study.

Saadabi [16] investigated the chloroform, methanolic, and aqueous extracts of 11 medicinal plants in traditional medicine in Saudi Arabia, including *P. harmala*, for in vitro activity against four pathogenic fungi: *Aspergillus flavus, Aspergillus fumigatus, Aspergillus niger*, and *C.albicans*. He showed that the methanolic extract had the highest antifungal activity, followed by aqueous and chloroform extracts. In his study, phytochemical analysis of the most active antifungal plant samples was carried out and the antifungal activity of *P. harmala* was shown to be mostly related to its rich alkaloid content [16]. The present study similarly indicated that the alcoholic extract was more effective than the aqueous extract against fungal growth. In both studies, the antifungal activity of *P.harmala* extracts against *C. albicans* was evaluated. However, the studies differed in timing and site of collection of the plant, the protocol for plant extract preparation, and the methodology. While Saadabi [16] used methanol to prepare the alcoholic extract of *P. harmala*, ethanol was utilized in the present study.

Ahmed et al. [17] evaluated the effect of the mixture of natural honey and *P. harmala* seeds against *C. albicans* to compare the antifungal activity of aqueous extract of *P. harmala* alone and in combination with six honey types from different regions of Algeria against *C. albicans*. The activity of extract of the plant was calculated by measuring the clear zone around each well in millimeters. Various concentrations from 3.10 to 100% of *P. harmala* extract were tested in order to determine the MIC. The extracts were shown to have antifungal activity with zones of inhibition varying from 1 to 6.5 mm with the highest activity for 12.5% concentration with the inhibition zone diameter of 6.5 mm. Furthermore, the range of inhibition zone diameter of 6 different types of honey was reported to be between 1.5 and 9 mm, and between 1.7 and 13 mm for the mixture of honey and *P. harmala*. Therefore, the results showed that adding honey to *P. harmala* increased the antifungal activity [17]. Although the methodology of their study differed from the present study, the antifungal activity of the aqueous extract of *P. harmala* alone against *C. albicans* was well proven.

Al-Izzy [7] evaluated the antimicrobial activity of aqueous and alcoholic extracts of *P. harmala* against Lactobacilli spp. and Candida spp. isolated from saliva. Al-Izzy applied the agar diffusion technique to investigate the antimicrobial effect of the fungi on Mueller–Hinton agar. Twenty isolates of Lactobacilli and the same for Candida were used and the well-plate method was employed by making holes that were 5 mm in diameter and 4 mm in depth. The study concluded that the aqueous and alcoholic extracts of *P. harmala* were effective for inhibition of growth of these microorganisms. Also, it was shown that the alcoholic extract of *P. harmala* was more effective against both fungal isolates than the aqueous type [7]. Although the methodology of Al-Izzy's study and the

current one differed in some aspects, the results of both studies confirmed the antifungal properties of aqueous and alcoholic extracts of *P. harmala* and identified the alcoholic extract to be more potent. Hence, the present study highlighted the potential of *P. harmala* as a novel antifungal agent and provided a basis for future ethno pharmacological use of the alcoholic and aqueous extracts of this plant against mycotic infections. Further in-vivo clinical trials and human metabolism experiments should be carried out to ensure lack of cytotoxicity and minimal side effects of *P. harmala* to justify the safe use of this herbal drug.

Conclusion

This in vitro study showed that both aqueous and alcoholic extracts of *P. harmala* seeds possessed antifungal properties against *C. albicans*, *C. glabrata*, and *C. krusei* and exhibited a stronger fungicidal activity against *C. krusei*. The alcoholic extract of *P. harmala* caused greater growth inhibition of *C.* glabrata and *C. krusei* species than the aqueous extract.

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