

Original Article

In Vitro Evaluation of Antifungal Activity of Three Traditionally Used Medicinal Plants; *Umbilicus Intermedius Boiss*, *Cuminum Cyminum* and *Zingiber Officinale* Extracts

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ABSTRACT

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Background and Aims: The study aimed to determine the effectiveness of three medicinal plant extracts on fungi with three methods and to compare methods.

Material and methods: This study examined the antifungal properties of cumin (*Cuminum cyminum L*), ginger (*Zingiber officinale Roscoe*) and Nafe Venus (*Umbilicus intermedius boiss*) extracts against fungi including, *Aspergillus* spp., *Penicillium* spp., *Mucor* spp., *Stemphylium* spp., *Drechslera* spp., *Alternaria* spp., *Cladosporium* spp., and *Aureobasidium pullulans*. Furthermore, 17 candida isolates including, *C. albicans*, *C. glabrata* and *C. dubliniensis* were tested. In the present study two methods of disc diffusion method, agar wells diffusion method were used for assay. Then, the mixing with culture medium method was used for assessment of the antifungal activity of extracts against *Alternaria* sp.(as black mold), *A. terreus* (as hyaline mold) and *C. albicans* (as yeast) to compare methods as well.

Results: No fungi were susceptible to extracts in disc diffusion method and agar wells diffusion method. But, this study showed that in mixing with culture medium method, cumin extract has valuable anti-fungal property and *Umbilicus intermedius boiss* has the inhibitory properties against the black fungi. Furthermore, it is found that mixing with culture medium method is more efficient than disc and agar well diffusion methods. *Alternaria* sp. and *C. albicans* were susceptible and resistant to all extracts.

Conclusions: it is found that mixing with culture medium method is more efficient than disc and agar well diffusion methods and inhibitory potency of the extracts varies according to the type of extraction and their concentration.

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Introduction

The use of medicinal plants for treating fungal and bacterial diseases has a long history. Today, the use of herbal medicines has increased due to microorganisms' resistance to chemical drugs and the side effects of these drugs. Most of the drugs have a chemical origin, but about one-third of all pharmaceutical preparations have plant origin or extractions which are modified. For example, approximately 25% of drugs in the United States are made of medicinal herbs [1, 2]. One of these herbs is Nafe Venus plant [3]. Nafe Venus (*Umbilicus intermedius boiss*, Genus: *Umbilicus*) is a hydrous perennial floral plant, which includes approximately 11 to 30 species. It belongs to the stonecrop family *Crassulaceae* (including approximately 1400 species and 34 or 35 genera). Nafe Venus is 20-35 cm high with simple green leaves including alternate, scutate, convex, lobate-crenate, stem leaves linear, wedge-shaped and serrate. It produces white, yellow flower clusters from March to June. The plant flowers are pendent in compact spike, urceolate-tubular, congregations [4]. Although *Umbilicus intermedius* has worldwide distribution, it is found more frequently in southern Africa, deserts of Israel, Lebanon and in mountains of Jordan and Saudi Arabia and in some regions of Iran such as Ilam [5]. The leaves of Nafe Venus is used freshly or dried mixed with yogurt for treating burned skin. Moreover, the decoction of the leaves was used to treat skin infections, carbuncles,

urinary tract infections and kidney stone disposal [5-7].

Cumin (*Cuminum cyminum L.*) is an aromatic flowering plant in the family *Apiaceae* or *Umbelliferae* [8, 9]. The important and effective components of *cumin* include: myrtenal, trans-carveol, O-cymene, cuminiquic alcohol and many other materials [9-11]. Several medicinal properties are attributed to *cumin*, including healing the common cold, jaundice, diarrhea, indigestion and bloating. Moreover, therapeutic properties against fungi, bacteria and viruses for *cumin* have been reported [8, 12].

Ginger consists of thick squamate root of the monocotyledonous plant *Zingiber officinale*, belonging to the family *Zingiberaceae* [13]. Various studies have shown that *ginger* has many therapeutic properties including antidiabetic, anti-arthritic, anti-microbial activities against various bacteria, fungi, and nematodes, anti-inflammatory and anti-thrombotic. *Ginger* (*Zingiber officinale*) is known throughout history as a medicinal plants [14-18].

Saprophytic fungi include some of the most common indoor and outdoor molds which can cause different infectious diseases in immunocompromised patients [19].

Furthermore, some fungi are resistant to routinely used antifungals and some patients do not tolerate chemical antifungals. For this reason, the present study aimed to investigate the antifungal activity of methanol-chloroform, alcoholic and aqueous extract of

Umbilicus intermedius boiss and alcoholic extract of *ginger* and *cumin* against fungi such as *Aspergillus*, *Penicillium* and *Mucor* species. In addition, some dematiaceous fungi (*Stemphylium* sp., *drechslera* sp., *Alternaria* sp., *Cladosporium* sp., *Aureobasidium pullulans* sp.) and *Candida* species (*C. albicans*, *C. glabrata* and *C. dubliniensis*) were used to compare methods.

Materials and Methods

Plant Preparation

The *Umbilicus* plant (herbarium code: 296) was collected from the mountains of Ilam and *Cumin* (herbarium code: A170106OFP) and *Ginger* (herbarium code: A173211ORP) plants were purchased from groceries in Ahvaz and Yazd, respectively. Plants were completely washed with cold water and then dried in a dark place. Then, they were completely powdered by a household mill and stored at a dark and dried place at the room temperature until use.

Preparation of alcoholic, aqueous and metanol chloroform extracts

Ten grams of plant powder were added to 250 ml of 80% ethanol in a dark vessel and completely mixed on a shaker at 25-30°C for 72 hours at 150 rpm. The mixture was filtered twice using a filter paper (Whatman No-1) and filtrate was placed in an oven at 40°C until the elimination of the alcohol. Then it was stored at 4°C until use [6, 20].

Ten grams of plant powder were added in 50 ml of boiling distilled water, while shaking once every few minutes for 72 hours.

Finally, the extract was prepared as explained above [21].

In a dark bottle, 25 ml of 80% methanol was mixed with 25 ml of chloroform; it was then added to 7.87 grams of powdered plant. Later, it was shaken in a refrigerator incubator in 10°C at 100 rpm for 72 hours. Finally, supernatant was filtrated twice by using a filter paper and extract was prepared as mentioned above [22].

Preliminary preparation of fungal samples

Twenty nine fungal strains were collected in the laboratories of mycology department of Ahvaz Jundishapur University of Medical Sciences. The strains were confirmed by using standard methods [23-25]. All strains were cultured in Sabouraud dextrose agar (SDA) (Bio life, Italian) and incubated at 35°C for 24 hours for yeasts and filamentous fungi at 25°C for 3-7 days.

Preparation of standard fungal suspension

The fungi, mostly collected in autumn and spring, were re-cultured on SDA. A suspension of the yeast strains were adjusted according to CLSI M44-A using a spectrophotometer in a concentration of $1-5 \times 10^6$ (a 0.5 McFarland standard). In addition, the suspensions of filamentous fungi were prepared spectrophotometrically to optical densities ranging from 0.09 to 0.11 in accordance with CLSI M51-A [26].

Disc diffusion method: One hundred μ L of fungal suspensions were spread on SDA medium and kept at room temperature for 10-15 minutes. After the microbial suspensions were completely absorbed, blank discs (diameter of 6.4 mm, PADTAN TEB Co.,

Iran) impregnated with 25 µL different concentrations (200, 100 and 50 mg/ml) of the extracts, were placed on the plate's surface. Yeasts were then incubated at 35°C for 24 hours and saprophytic fungi at room temperature for 3-7 days [6]. Finally, diameter inhibition of zone by extracts around each disc was measured.

Agar well diffusion method

According to disc diffusion method, medium was prepared and inoculated with fungi. The wells were made using a 6.0 mm diameter glass tube. Then, wells were poured with 50 µL of the extracts with mentioned above concentrations. Phosphate buffered saline (PBS) solution was used for negative control and fluconazole (Bioanalyse co., Turkey), and amphotericin B (Bioanalyse co., Turkey) for positive control. Finally, diameter inhibition of zone around each well was measured [3].

Mixing with culture medium method (MCM)

Firstly, several dilutions (2000, 1600, 800, 400, 200 and 100 mg/ml) of tested extracts were prepared using PBS, and 100 µL of each concentration was mixed with 10 ml of Potato Dextrose Agar (PDA) (45-50°C) and poured into an 8 cm plate. A PDA without any extract was prepared as control. A piece of fresh fungus colonies (1 x 1 cm) was then placed in the center of the plate and incubated at a temperature of 25-27°C for 3-7 days (filamentous fungi) and 35°C for 24 hours (Yeast). Growth diameter of the samples and controls were measured and percent growth inhibition were calculated with the following equation [27-29].

$$IP = \frac{C - T}{C} \times 100$$

IP = Percentage of growth inhibition

C = Mean diameter of fungus colony in control

T = Mean diameter of fungus colony in the tested concentration of extracts

Results

The inhibitory efficacy of extracts was evaluated by disc diffusion, agar wells and mixing the extracts with culture medium methods against several molds and yeasts pathogens. In this study, it was found that all three extracts have no inhibitory effect on tested fungi using both methods, disc and agar wells diffusion methods (Fig. 1). In disc diffusion method, fluconazole 10, 50 and amphotericin B 100 Mcg were used as controls; *C. albicans* were sensitive to fluconazole 50 and amphotericin B 100, *A. terreus* sensitive only to fluconazole 50 (Fig. 2). The extracts inhibited the black and hyaline fungi growth when used MCM method. Specially, *Umbilicus intermedius boiss* showed a better inhibitory effect than the other two extracts (Fig. 3). The inhibitory values of *ginger*, *cumin* and average different extracts of *Umbilicus intermedius boiss*, against *Alternaria* sp., in concentration of 2000 mg/ml per plate were 24.56%, 38.46%, 54.33% respectively, whereas, the inhibitory values against *A. terreus* were 23.4%, 12% and 21.7% for *ginger*, *cumin* and average different extracts of *Umbilicus intermedius boiss*, respectively. It is indicated that all extracts are more effective on black fungi than others. However, *C. albicans* was resistant to this method as well (Table 1). In this study,

Umbilicus extract decreased the pigment produce in the *A. niger* and the *A. flavus* in

agar-diffusion disc method (Fig. 4).

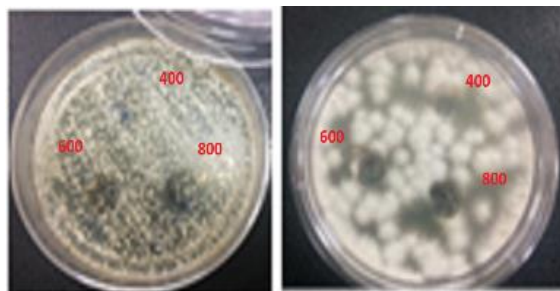


Fig. 1. Evaluation of *C. albicans* and *A. terreus* sensitivity to plant extracts with agar wells diffusion method

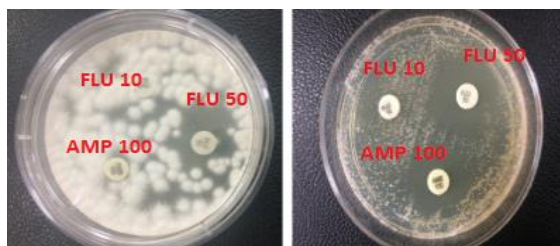


Fig. 2. Evaluation of *C. albicans* and *A. terreus* sensitivity to fluconazole and amphotericin B discs (as Control)



Fig. 3. Effect of alcoholic extract of *Umbilicus intermedius boiss* on *Alternaria* fungi in the extract MCM method [From above, dilutions of 100, 200, 400 and 800mg/plate extracts are mixed with the medium, and the last plate is control (no extracts)]

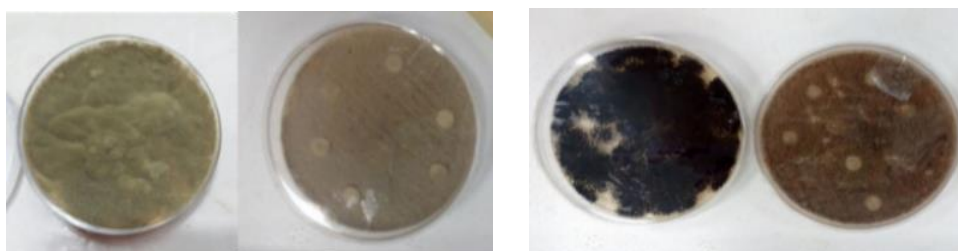


Fig. 4. Effect of extract of *Umbilicus intermedius boiss* on *A. flavus* (left) and *A. niger* (right) inhibiting of pigment production (no sensitive to discs containing extracts)

Table 1. Percent growth inhibition of *cumin*, *ginger* and *Umbilicus intermedius boiss* extract using the MCM method against fungi_strains

Fungi	Herbal extract	Concentrations (mg/mL)						Growth time (day)
		100	200	400	800	1600	2000	
<i>Alternata sp.</i>	IP:Gi	20.51	25.64	30.76	27.54	28.8	28.56	3
		12.2	14.03	15.78	17.5	22.8	24.56	7
	IP:Cu	28.2	30.77	33.33	35.89	38.46	38.46	3
		14.03	17.54	21.05	28.07	31.57	33.33	7
	IP.U.C-M	5	56.4	56.4	53.8	56.5	56.7	3
		1.7	42.1	44	42.1	43.2	44.1	7
	IP.U.AQ	10.2	48.7	53.8	51.3	51.45	52.2	3
		3.5	42.1	45.6	47.3	47.3	47.5	7
	IP.U.AL	7.6	53.8	56.4	53.8	54	54.1	3
		0	36.8	38.6	40.4	40.4	40.5	7
<i>A. terreus</i>	IP:Gi	0	10.7	21.4	21.5	23.1	23.4	3
	IP:Cu	7.1	10.7	7.1	10.2	10.6	12	3
	IP.U.C- M	0	3.5	7.1	14	26.7	30.1	3
	IP.U. AQ	10.7	17	7.1	14	17.2	17.8	3
	IP.U.AL	3.5	14	17	17	17.1	17.3	3
<i>C. albicans</i>	IP:Gi	-	-	-	-	-	-	Up to 3
	IP:Cu	-	-	-	-	-	-	Up to 3
	IP.U.C-M	-	-	-	-	-	-	Up to 3
	IP.U. AQ	-	-	-	-	-	-	Up to 3
	IP.U.AL	-	-	-	-	-	-	Up to 3

IP:Gi=Inhibitory percentage of *Ginger officinal* extract; IP:Cu=Inhibitory percentage of *cumin cyminum L* extract; IP.U.C-M=Inhibitory percentage Methanol- Chloroform extract of *Umbilicus intermedius boiss*; IP.U.AQ=Inhibitory percentage aqueous extract(watery) of *Umbilicus intermedius boiss*; IP.U.AL=Inhibitory percentage of alcoholic extract of *Umbilicus intermedius boiss*

Although the growth inhibition of extracts against *Alternaria sp.* fluctuated in various concentration, the extracts were more effective on the third day than the seventh day of growth (Table 1).

Discussion

In the present study, antifungal susceptibility of three plant extracts were surveyed against various fungi, e.g. *Alternaria sp.* (cause allergic respiratory diseases) [30], *A. terreus* (resistance to amphotericin B) [31] and *Candida sp.* (cause opportunistic yeast infections) [32]. *Cumin* and *ginger* are widely

used as a spice in the Indian subcontinent and some other Asian, African and Latin American countries. *Umbilicus intermedius boiss* (Nafe venus), distributed in the West of Iran such as Ilam province, grows in arid areas and is traditionally used to treat various infectious diseases [5-7]. In recent years, due to drug resistance, using medicinal plants has been widely considered. *Umbilicus intermedius boiss* extract has not yet been tested against various mold fungi.

Mahmoudi et al.[33] demonstrated that the essential oil of *cumin* has a good inhibitory

effect on *Pseudomonas syringae*. Moreover, Rozegar et al. [6] surveyed *in vitro* inhibitory effect of alcoholic and aqueous extracts of *Umbilicus intermedius boiss* on *Staphylococcus aureus* and *P. aeruginosa* and showed aqueous extract having a higher antibacterial effect compared with the alcoholic extract.

Baljeet et al. [34] showed that fungi are sensitive to *cumin* and *ginger* extracts which is consistent with our study. They reported that the diameter of inhibition zones of extracts, of *cumin* and *ginger* range from 16.3 to 18.3 mm against *C. albicans*, but *Rhizopus azygosporus* is resistant to these. However, in our study, *C. albicans* isolate was resistant to all three herbs by all methods, probably because the tested *C. albicans* isolates were isolated from women with *Candida vaginitis* and indeed these isolates are resistant to these compounds but the extracts mentioned can be effective on non-clinical strains. On the basis of the study data, alcoholic extract of *cumin* inhibited only 12% growth of *A. terreus* but Mohammadpour and et al. reported the *C. cyminum* L. oil kill more than 60% of the spores of the four *Aspergillus* species tested within 12 hours [11]. In a study performed by Kedia et al. (2014), *C. cyminum* showed a fungicidal effect against *Alternaria* sp. that amounted to 100% mycelial inhibition at the dose of 0.6 µl/ml concentration [21]. Skrinjar and Nemet in a study indicated that *ginger* and *cumin* bear weak and medium inhibitory effects against *Aspergillus* spp., *Cladosporium* spp. and many other fungi [35]; our study shows that *ginger* has different effects and almost similar inhibitory effect on tested fungi.

Interestingly, in the present study, *Zingiber officinale* was effective on all of the tested fungi except *Candida* species. Srinivasan et al. [36] in study on antimicrobial activity employed fifty medicinal plants used in folkloric medicine and concluded that *Zingiber officinale* shows reaction against all the tested pathogenic bacteria and *candida* sp, having, however, no effect on *Aspergillus* species. Whereas in our study, *Zingiber officinale* was effective on *Aspergillus* spp, it had no effect on *Candida* species. Romagnoli et al. showed that *cumin* is active against all tested fungi, in particular on the dermatophytes. They also reported that inhibitory effect of *cumin* extract on *Alternaria* sp. is 19.6 % and 81.4% for 5 and 20 µ per disc respectively [37]. But in our study, disc diffusion method showed no inhibitory effect, while the inhibitory percentages of 100 and 2000 mg/ml per 10 ml of culture medium (plate) were 14.03% and 33.3% respectively. In this study, as a secondary outcome, it was observed that *Umbilicus* extract decreased the pigment produce in the *A. niger* and the *A. flavus* in agar-diffusion disc method that proved to be interesting (Fig. 4). These natural compounds (pigment) can bear pathogenicity as well as mutagenic and carcinogenic potential [38].

Conclusion

The study suggests that *cumin* and *ginger* plant extracts can partially inhibit the growth of fungi but *umbilicus intermedius boiss* can only prevent the growth of some fungi. This amount of inhibitory strength of extracts fails to be dependent on the type of extraction,

whereas it is dependent on concentration of the extract and the test method. These findings may depend on the nature and type of chemical compounds of the plant extract and on how it is distributed in the culture medium; this should be considered in determining the inhibitory effect of plant extracts.

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Conflict of Interest

No potential conflict of interest was reported by the authors.

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