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In vitro Antifungal Activity of *Cucumis melo* on *Candida albicans*

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Background: With respect to the emergence of susceptibility of some fungi to antifungal agents, making use of medicinal plants is progressing.
Objectives: The aim of this study was to verify the anti-fungal characteristics of mature and immature *Cucumis melo* fruit on *Candida albicans*.
Materials and Methods: In this descriptive study, antifungal activity of aqueous, ethnolic and methanolic extracts of *C. melo* fruits were tested on *C. albicans*; also results were obtained by disc and well diffusion methods.
Results: Minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) of mature *C. melo* fruit methanolic extract were 25×10^3 and 5×10^4 μg/mL, respectively. MIC and MFC of immature fruit methanolic extract was 25×10^3. For mature fruit ethanolic extract, MIC and MFC was 25×10^3 μg/mL and for immature *C. melo* fruit ethanolic extract MIC was 25×10^3 and MFC was 5×10^4 μg/mL. Gas chromatography/mass spectrometry (GC/MS) results showed that hexadecanoic acid was present in all of the extracts. The maximum size of inhibition zone for mature and immature methanolic extact in the disc method was 12 mm and in well method were 15 and 17 mm, respectively. In the disc method, for mature and immature ethanolic extracts maximum size of inhibition zones were 35 and 36 mm and in the well method, maximum size of inhibition zones for mature and immature ethanolic extracts were 28 and 15 mm, respectively.
Conclusions: These results indicated that *C. melo* extracts contain compounds with therapeutic potential and anti-fungal characteristics against *C. albicans*.

Keywords: Antifungal activity; *Cucumis melo*; *Candida albicans*; *In vitro*

1. Background

Opportunistic pathogens are accounted for a substantial morbidity rate and can result in hospitalization and expensive therapies, and they also reduce the survival rate of people with HIV infection. For immune-compromised patients, *Candida albicans* is an important opportunistic fungal pathogen and the major cause of oropharyngeal candidiasiss [1]. Nosocomial candidiasis is a worldwide problem. Candida is the fourth common nosocomial infections in USA [2, 3]. It causes urinary tract (UT) and blood infection in patients that admitted to the hospital [4, 5].

In (HIV)-infected patients, oropharyngeal colonization by Candida causes subsequent development of yeast infections; however, long-term therapy might lead to resistance development [6]. According to the literature, the investigation for natural products to be used against Candida species increased significantly in the last 10 years examining approximately 258 plant species from 94 families [2, 7]. Wild melon (Cucumis melo L.), belongs to the cucurbitaceae family, it is an annual plant, herbaceous, prostrates, creeping and its fruit is as large as 2 - 40 × 8 - 24 mm, it is ellipsoid and yellow and dark green veined with bitter pulps [8].

In various cucurbitaceous plants, a bitter material named cucurbitacine exists in different types (A, B, C, D, E, F, G, H, I, J, K, L). Cucurbitacine glycosides are tetra cycle three terpenic and have anti-tumor effects. This plant has medicinal use on common medicine. The plant's fruit is used as vomitive but in little amounts consumed with honey is tonic for stomach. Paste of its crushed grain along with *Cynodon dactylon* juice is used for curing and removing herpes grains and boils [9].

The anti-fungal characteristics of some medicinal plants on different species of fungi are already determined. Nadimi et al. reported that aqueous and ethanolic extracts of *Teucrium polium* had effect on *C. albicans* and Malassezia [9]. Marzouk et al. showed that *Citrullus colocynthis* had anti-bacterial and anti-candidal properties [10].

Kumar and Kamaraj reported that ethanolic fruit extract of *Cucumis anguria* have higher activity against both bacteria and fungi, also methanol chloroform and ethyl acetate extracts have moderate inhibitory effects on bacteria and fungi [11]. Because of the anti-fungus effects of some plants from Cucurbitaceae family, we set out to find any anti-fungal characteristics of both mature and imma-

ture wild melon on *C. albicans* by disc, well diffusion, MIC (minimum inhibitory concentration) and MFC (minimum fungicidal concentration) determination.

2. Objectives

The aim of this study was to verify the anti-fungal characteristics of mature and immature *C. melo* fruit on *C. albicans*.

3. Materials and Methods

In this descriptive study, mature and immature fresh fruits of *C. melo* were collected from different farms located in Mazandaran province, Babol city, North of Iran, Mid-September 2012. After collecting fruits, the skin of fruits was peeled; they were washed, then both mature and immature fruits were dried under shade at room temperature (27°C) for 1 month. After that dried, samples were grinded into fine powder [12, 13].

3.1. Fungal Cultures

C. albicans isolate (ATCC 10231) for this study was provided by Pasteur Institute of Iran. *C. albicans* isolate was located in lyophilized vials. It was cultured as linear culture on Sabouraud Dextrose Agar (SDA) medium (Merck, Germany) plates. Then they were incubated at 35°C for 2 days. Re-confirmation of *C. albicans* was performed at the laboratory of Mycology Department, Faculty of Veterinary Medicine, Islamic Azad University, Babol Branch, Babol, Iran. Laboratory diagnosis such as periodic acid-Schiff (PAS) staining, growth in SDA containing antibiotic (chloramphenicol and gentamicin to prevent the growth of bacteria), sugar fermentation and assimilation tests with Rap ID yeast kit (BioTeke, China), microscopic observation of long germ tubes, hyphae, blastoconidia and chlamydoconidia production was performed before the start of examinations [14-16].

3.2. Suspension Preparation

Fresh cultivated *C. albicans* colonies were suspended in 5 mL of 0.85% normal saline. Suspension was mixed for 15 seconds with a vortex. Then its concentration was adjusted to 1.5 × 108 CFU/mL based on a standard 0.5 McFarland [2, 17, 18].

Preparation of aqueous extract: Percolation method was performed to prepare the fruits' extracts. Twenty-five grams of dried samples powder was mixed with 250 mL of water, and it was shaken in Erlenmeyer flask continuously for 3 minutes, then it was stored at room temperature (22°C for 24 hours). In the next step the extract was filtered through Whatman No. 1 (9 mm diameter) filter paper (Behan, Iran); then the filtered extract was entered in Rotary for removing solvent. Then aqueous extract was dried in incubator at 40°C. In the end, 1 g of dried extract was mixed with 5 mL of 5% dimethyl sulphoxide (DMSO). Then it was filtered through a millipore syringe filters with 0.22 μ diameter [12, 17].

3.3. Preparation of Ethanolic and Methanolic Extract

For preparation of ethanolic and methanolic extracts, 20 g of mature and immature dried fruit of *C. melo* was mixed with 100 mL of 80% ethanol and 100 mL of 80% methanol, respectively. Then 100 mL of D-ethyl ether and N-hexane were added to them and then the mixture was wrapped in aluminum foil and stored at room temperature (22°C for 24 hours). Then extract was filtered through Whatman No. 1 (9 mm diameter) filter paper. Filtered extract was entered in Rotary for removing alcoholic solvent. Extract was dried in incubator at 40°C. Then 1 g of alcoholic dried extract was mixed with 5 mL of 5% DMSO and it was filtered through a millipore syringe filters with 0.22 μ diameter [2, 12, 17].

Determining antifungal activities using the disc diffusion method: The agar disc diffusion method was employed to determine antifungal activity in this study. Suspension of *C. albicans* (1.5 × 108 CFU/mL) through the lawn culture was planted on SDA plates by swap. Sterile blank discs with 6 mm diameter (Pattan Teb, Iran) were impregnated with 40 μL, 50 μL, 60 μL and 70 μL dilutions of plant extract, after drying, standard discs were put on agar media with sterile forceps that were inoculated by *C. albicans*. Positive controls were amphotericin B (10 μg), ketoconazole (15 μg), nystatin (50 μg) discs (Master Group Ltd, England) and negative control was a disc containing DMSO. Plates were incubated at 35°C for 48 hours. Principles that were used in this step were performed according to NCCLS standard (Table 1) [17, 19].

3.4. Determining Antifungal Activities Using the Well Diffusion Method

SDA medium was inoculated with *C. albicans* suspension by swap. Then 5 mm wells were caved in it by Pasteur

Table 1. Susceptibility of *C. Albicans* to Antifungal Drugs According to NCCLS Disk Diffusion Method (mm)

Drug μg/mL	Sensitive	Dose-Dependent	Resistance
Amphotericin-B	≥ 15 mm	10 - 14 mm	≤ 10 mm
Ketoconazole	≥ 28 mm	21 - 27 mm	≤ 20 mm
Nystatin	≥ 15 mm	10 - 14 mm	Without inhibition zone

pipette. Number of wells was 5 on plates, being 25 mm apart and having a 15 mm space from the plate wall. Bottom of wells was closed with medium, aseptically. Then 30 μL of 80 μL, 90 μL, 100 μL and 110 μL of each extract were added to wells. A cavity containing 100 U/Disc amphotericin B (Sigma, USA) that was dissolved in 5% DMSO was considered as positive control and a cavity containing DMSO was considered as negative control. Positive and negative controls indicating the validity of the test and eliminating positives and negatives false. These plates were incubated at 35°C for 48 hours. After incubation, inhibition zone was observed and it was determined in millimeters. Each experiment for disc and well method was repeated 3 times and the mean of results was recorded [2, 20].

3.5. MIC Determination

MIC determination was performed using the serial dilutions method. At first, suspension of *C. albicans* according to standard 0.5 McFarland was provided in sabouraud dextrose broth (SDB) (Merck, Germany). Eleven tubes were considered. One millimeter of SDB was added to them. In the next step 1 mL of extract was added to tubes according to serial dilutions procedure. Also 20 μL of *C. albicans* suspension was added to tubes. Then they were incubated in 35°C for 24 hours. Tube number 11 was considered as the control tube (1 mL SDB + 20 μL *C. albicans* suspension). After incubation, with observation of turbidity or lack of turbidity in tubes, level of MIC was determined.

Determination of the MFC in prepared extracts: To determine the MFC, tubes without darkness were taken and then were cultured in SDA by sowap. Plates were incubated at 35°C for 48 hours. MFC was considered as colony-forming unit (CFU) that is an estimate of viable or fungal numbers. Minimum concentration that inhibited the growth of *C. albicans* was considered as MFC [13, 21].

3.6. Gas Chromatography-Mass Spectrometry (GC-MS) Procedure

In this research, for determination of constituent compounds of extracts, at first aqueous and alcoholic extracts were prepared using mature and immature wild melon. Components of different extracts of *C. melo* were detected and separated using the GC/MS chromatograph system (HP 6890, model) with HP-5MS column (240 m × 0.25 mm). One microliter of extracts was injected into the GC/MS system. Injector was programmed as follows: 6°C at 1 minute, then gradually increased to 240°C. Helium was at a flow rate of 1 mL/mn. Injection temperature was 250°C. Compounds of each extract were identified after analyzing the chromatogram [22].

3.7. Statistical Analysis

In order to analyze the information, descriptive and inferential methods were utilized. Student's *t*-test was used in our study for analysis of information. All of our analysis was done by SPSS-16. P-Values < 0.05 were considered statistically significant.

4. Results

C. albicans showed no sensitivity to the aqueous extract of mature and immature wild melon in disc and well diffusion methods and *C. albicans* colonies were observed in all parts of plate.

Alcoholic extracts of mature and immature fruit of *C. melo* in the disc diffusion method showed less effect comparing to well diffusion method. Also ethanolic extract of immature wild melon in disc method showed a better effect on *C. albicans* than well diffusion method.

In this study, by increasing the amount of alcoholic extract on disc and well diffusion methods, inhibition zone around the disc and wells was increased. Various sizes of inhibition zones were measured by a ruler in millimeters. The maximum size of inhibition zone for mature and immature methanolic extact in the disc method was 12 mm (70 μL, resistant) and in well method was 15 and 17 mm (110 μL, sensitive), respectively.

For mature and immature ethanolic extracts in the disc method, maximum size of inhibition zones was 35 and 36 mm (70 μL, sensitive) respectively. In the well method, maximum size of inhibition zones for mature and immature ethanolic extracts were 28 and 15 mm (110 μL, sensitive), respectively (according to Table 1 inhibition zones that are ≥ 15 and ≥ 28 mm show sensitivity and inhibition zones that are ≤ 10 and ≤ 20 show resistant) (Table 2). The MIC and MFC of methanolic extract of mature *C. melo* fruit were 25×10^3 and 5×10^4 μL/mL, respectively. Also MIC and MFC of methanolic extract of immature *C. melo* fruit was 25×10^3 μL/mL. Results of MIC and MFC determination of ethanolic extracts of mature *C. melo* fruit was 25×10^3 μL/mL. MIC and MFC of ethanolic extract of immature *C. melo* fruit were 25×10^3 and 5×10^4 μL/mL (Table 3).

Hexadecanoic acid compounds were found in all of the extracts of both mature and immature fruits. But in methanolic extract of the mature fruit, 9, 12-octadecadienoic acid with 36.38% and hexadeconoic acid with 26.46%, in ethanolic extract of the mature fruit, hexa octadecadienoic with 25.62% and in methanolic extract of immature fruit different compounds of benzene with 64.26% were found. Hexadeconoic acid with 12.71% and 9, 12-octadecadienoic acid with 27%, 09% and n-hexadecanoic acid with 19.27% were most of the compounds in the ethanolic extract of the immature fruit.

Mean results showed that effect of ethanolic extract was more than methanolic extract. Since amount and distribution of extracts were different, disc and well methods results do not support each other but in total there was a significant relation between inhibition zone diameters with increasing extracts in disc and well methods P=0.02 (Table 4).

Table 2. Inhibition Zones (mm) Against Different Amounts of *C. melo* Alcoholic and Aqueous Extracts (μL) by Disc and Well Diffusion Methods

Fruit Type	Methods	Concentration, μL	Inhibition Zone of Methanolic Extract [a]	Inhibition Zone of Ethanolic Extract[a]	Inhibition Zone of Aqueous Extract[a]
Mature	Disc	40	8	26	0
		50	9	28	0
		60	11	33	0
	Well	70	12	35	0
		80	12	24	0
		90	13	25	0
		100	14	26	0
		110	15	28	0
Immature	Disc	40	9	30	0
		50	10	31	0
		60	11	34	0
	Well	70	12	36	0
		80	11	12	0
		90	13	13	0
		100	16	14	0
		110	17	15	0

[a] Values are expressed as mm.

Table 3. MIC and MFC Determination of the *C. melo* Extracts on *C. albicans* [a]

Fruit Type	Methanolic Extract		Ethanolic Extract	
	MIC	MFC	MIC	MFC
Mature	25×10^3	5×10^4	25×10^3	25×10^3
Immature	25×10^3	25×10^3	25×10^3	5×10^4

[a] Values are expressed as μg/mL.

Table 4. The Mean and Standard Deviation of Inhibition Zones (mm) Against Different Amounts of *C. melo* Extracts (μL) by Disc and Well Diffusion Methods [a]

Methods	Concentration, μL	Extract	Mature	Immature
Disc	40	Methanolic	8 ± 1	9 ± 3
		Ethanolic	26 ± 3	30 ± 3
	50	Methanolic	9 ± 2	10 ± 2
		Ethanolic	28 ± 2	31 ± 2
	60	Methanolic	11 ± 1	11 ± 2
		Ethanolic	33 ± 2	34 ± 2
Well	70	Methanolic	12 ± 2	12 ± 2
		Ethanolic	35 ± 3	36 ± 2
	80	Methanolic	12 ± 1	11 ± 3
		Ethanolic	24 ± 3.6	12 ± 2.66
	90	Methanolic	13 ± 1	13 ± 2
		Ethanolic	25±4.36	13 ± 4.36
	100	Methanolic	14±2.62	16 ± 1.72
		Ethanolic	26 ± 4	14 ± 1
	110	Methanolic	15 ± 3	17 ± 1
		Ethanolic	28 ± 2.62	15 ± 3.61

[a] Data are presented by mean ± SD.

5. Discussion

In present study, both mature and immature ethanolic and methanolic extracts of *C. melo* fruit showed anti-Candida effects. So these extracts had compounds that were effective against *C. albicans*. However, some extracts had better antifungal effects compared to others.

In recent years, interest in the application of essential oils in the treatment of infectious diseases has notably increased. In this study, we evaluated the anti *C. albicans* activity of essential oils from 16 medicinal plants in Iran. *C. albicans* is responsible for the majority of infections in humans. Also medicinal plants are used in different disease treatments and they are popular all over the world [7]. Different studies showed anti-fungal activity of medicinal plants against *C. albicans* [1]. Nchu et al. in South Africa showed that the leaf extracts of *Markhamia obtusifolia* had effects against *C. albicans in vitro* [23]. In Iran, Amini et al. reported that three medicinal plant essential oils of *Zataria multiflora*, *Thymus vulgaris* and *Thymus kotschyanus* are effective against mycelial growth of *P. aphanidermatum*, *R. solani*, *F. graminearum* [12]. In our study methanolic extract of mature and immature, also ethanolic extract of mature and immature *C. melo* fruit showed anti-fungal effects against *C. albicans*. Bioactivity of phytochemical constituents of different parts of plants that are used in different studies may be similar, so extracts of different plant parts can have anti-fungal affects against fungal strains [18]. Varalakshmi et al. in India reported that *Garcinia indica* fruit extract had both anti-fungal (*C. albicans, A. niger*, Fusarium spp. and Penicillium spp.) and antibacterial properties [20]. Also in our study MIC values confirmed inhibitory effects of *C. melo* fruits on *C. albicans*. It was verified that both of mature and immature wild melon fruits had anti-fungal effects on *C. albicans*. In the other hand, based on the ability of different plant compounds to inhibit fungi, results of studies may be different [20, 23, 24].

In other study Sharma et al. in India showed that ethanolic extracts of *Rumex nepalensis* and *Jacquinia ruscifolia* had a broad spectrum of activity against fungal pathogens [25]. Mature and immature ethanolic extracts in the disc method in our research showed that maximum size of inhibition zone was 35 and 36 mm, respectively. In the well method, maximum size of inhibition zone for mature and immature ethanolic extracts was 28 and 15 mm, respectively. But maximum size of inhibition zone for mature and immature methanolic extracts in the disc method was 12 mm and in well method were 15 and 17 mm, respectively. So ethanolic extract was more effective in compared to mature and immature methanolic extracts. Also by increasing the amount of ethanolic extract of mature and immature wild melon fruit using the disc and well methods, the inhibition zone was increased. So it might be with increasing the amount of extract, diffusion of extract in medium rises and it shows greater antifungal effect [2]. Steenkamp et al. in South Africa showed that crude methanol and water extracts of 32 plant species inhibited *C. albicans* growth [1]. In Uruguay, Diaz-Dellavalle et al. determined that aqueous extract of 10 plant species exhibit anti-fungal activity against Alternaria spp. [26]. Aqueous extract of both mature and immature fruits of our study had no effect on *C. albicans* compared to alcoholic extracts, so it may be some plant compounds such as phenols and alcohols have more anti-fungal potency than other types of extract [2].

Kumar and Kamaraj in India using GC/MS demonstrated the chemical composition of *Cucumis anguria* (such as 9-hexadeconoic acid, n-hexadeconoic acid, hexadeconoic acid, ethyl ester and phytol) and they also proved that ethanolic extract of *Cucumis anguria* had anti-bacterial and anti-fungal activities [22]. In our study GC/MS results showed that hexadecanoic acid compound, 9, 12-octadecadienoic acid, different compounds of benzene and n-hexadecanoic acid were most of the compounds. Indeed, the plant contains many types of polyphenols, ranging from monomers to oligomers which may induce various anti-microbial effects [7]. Different components in plants act as anti-oxidants and by activating H_2O_2 they cause oxidative-stress-related responses in mold cells [27].

Pozzatti et al. in Brazil showed the effects of essential oils of rosemary in inhibition of *C. albicans* and *C. dubliniensis*. The primary mechanism of antimicrobial activity of essential oils is associated with their lipophilicity and consequent interactions with the microbial cell membrane. These interactions may result in changes and losses in the enzymatic and structural components of fungal cells [24]. For example studies showed that anti-microbial effect is due to chemical reaction of plant compounds with thiol groups of various enzymes [28].

These studies demonstrated that different parts of plants have anti-fungal activity and are worthful for further investigations in order to identify their active compounds and their clinical applications for treatment of mycosis [2, 18]. According to the widespread activity of natural anti-fungus substances in plants, we hope that results of this study would help to increase the identification and use of medicinal plants.

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Authors' Contributions

Issa Gholampour-Azizi carried out the study, collected data, performed the statistical analysis and prepared the manuscript. Samaneh Rouhi helped in writing the manuscript. Fahimeh Yahyayi supervised the study and participated in designing and conducting the study and also manuscript preparation. All authors have studied and approved the content of the present manuscript.

Conflict of Interest

The authors declare no conflict of interest.

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