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Investigating the antifungal effect of alcoholic extract of Butternut squash on Candida albicans isolated from vaginal infection

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Original Article

Abstract

BACKGROUND: Vaginal candidiasis is one of the most important fungal diseases in humans. Butternut squash (B. squash) has antifungal effects. This study investigated the effect of alcoholic extracts of B. squash on Candida albicans (C. albicans).

METHODS: In this laboratory experiment, B. squash using convenient sampling was collected from Babol City, Iran, in 2019. Determining the sensitivity of C. albicans to alcoholic extracts was tested using the disk, well, and minimum fungicidal concentration (MFC) methods and through determining the minimum inhibitory concentrations (MICs). Gas chromatography (GC) was used to determine the effective substances of the chemical compounds of B. squash extract. SPSS software, two-way analysis of variance, multiple comparisons, and independent samples t-test were used for data analysis.

RESULTS: Around the disk with 70 g/ml of ethanol extract (10 mm) and methanol (11 mm), and the wells with 110 mg/ml of methanol extract (17 mm), the average growth halo was greater. The size of the non-growth halo and the increasing trend of halo diameter between different alcoholic groups increased with increasing extract concentrations, but this increase was not significant ($P < 0.05$). In all 3 extracts, MIC was observed in 5-9 tubes and MFC in 3-9. The highest chemical composition was related to 5-hydroxymethylfurfural with 83.62%.

CONCLUSION: Based on the results of this study, B. squash alcoholic extracts effectively inhibit C. albicans and can be used as a promising tool to control or treat fungal diseases.

KEYWORDS: Antifungal, Butternut squash; Candida albicans, Vaginal Infection

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Introduction

Candida albicans (C. albicans) are part of the natural flora of mucous skin areas, especially the genital tract of many people.[1] Types of C. albicans often have an internal origin (caused by invading the body's natural flora) and are related to factors such as suppression of the immune system, long-term use of antibiotics, malignancies, and malnutrition.[1,2] Candidiasis in humans is one of the most important fungal diseases that can be seen in cutaneous, mucocutaneous, systemic, and allergic forms.[3] Approximately 75% of all women experience a clinically significant episode of vaginal-vulva candidiasis at least once during their reproductive years.[1] The latter disease is a relatively benign condition that responds well to antifungal therapy. During pregnancy, the

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risk of vaginal thrush increases, probably due to changes in the production of hormones, which lead to an increase in the content of glycogen in the vagina.[4] Amphotericin B, nystatin, clotrimazole, itraconazole, fluconazole, and 5-flucytosine can be used to treat different forms of candidiasis in humans.[5] Medicinal plants have been considered an important source of natural antioxidants due to their effective compounds such as polyphenolic compounds, tannins, flavonoids, and phenolic acids.[6-8] The Cucurbitaceae family can be mentioned as the most effective of these plants.[9,10] Butternut squash (B. squash) seed oil is rich in antioxidants, phytosterols, polyunsaturated fatty acids, and trace elements such as zinc. In addition, B. squash seeds are a very good source of other minerals such as magnesium (effective in maintaining heart health), manganese, and phosphorus, and also a good source of iron and copper, which are among the most important vitamins in B. squash seed oil. Vitamin E, due to its antioxidant property, increases the shelf life of oil during storage.[11]

Various studies have also proven the antimicrobial effects of B. squash. Saddiq in 2012 showed that B. squash aqueous extract (0.5 to 2%) prevented the growth of Aspergillus flavus (A. flavus) after 6 days; 2% B. squash aqueous extract had the most effective inhibitory effect on the growth of A. flavus.[12] Park et al. in 2009 reported that the Pr-2 protein in B. squash has antifungal activity.[13] Moreover, the antimicrobial effects of B. squash flower extract on Curvularia lunata (C. lunata) and its antifungal activity, which was similar to fluconazole, were proven by Muruganantham et al. in 2016.[14]

The nutritional features of B. squash that increase its use as a traditional medicine include anti-diabetic, anti-blood pressure, anti-tumor, anti-microbial, soothing, anti-inflammatory, and anti-intestinal parasite properties. Thus, it has attracted the attention of many researchers. Today, in addition to the nutritional uses of B. squash, some of its medicinal and therapeutic properties such as antimicrobial, anticancer, antioxidant, and liver protection are also considered.[15] Considering the spread of antibiotic resistance in fungi and the importance of medicinal treatments with plants, as well as the importance of treating chronic fungal infection in women, this study aimed to investigate the antimicrobial effects of alcoholic B. squash extracts on C. albicans isolated from vaginal infections.

Methods

Preparation of B. Squash samples: In this experimental laboratory study, B. Squash fruit was prepared (Babol City, Mazandaran province, Iran, 2019) and transported to Islamic Azad University, Babol Branch. One B. squash was selected for testing; the B. squash was healthy and the effects of decay were not observed in it. All tests were performed by researchers and laboratory experts. B. squash samples were collected using convenient sampling, and then, they were completely washed several times with clean water. After the complete removal of water, they were placed layer by layer in a warm and shaded space. After 1 month, it was completely dried with hot air flow, and then, the samples were powdered separately using an electric mill so that the extraction process could be done better and more easily. These powder samples were taken to the laboratory for extraction.

C. albicans sampling and preparation: A C. albicans sample was obtained from 1 of the medical diagnosis laboratories in Babol city, and after re-cultivation and final confirmation in the mycology laboratory of Islamic Azad University, Babol Branch, a study was conducted on it. For identification, a direct test was performed with 10% KOH (Padtan Teb, Iran). The sample was incubated on Sabouraud dextrose agar (SDA) (QUELAB, Canada) containing 50 µg/mg chloramphenicol (Padtan

Teb, Iran) for 48 hours at 20-30°C. Moreover, with the use of culture on Candida chrome agar, the colony grown in 48 hours at 37 °C was identified based on color and morphology. Candida isolates were identified by the test of tube mass formation in horse serum and chlamydospore formation test on cornmeal agar medium (QUELAB, Canada) containing 1% Tween 80. After the cultivation and identification of C. albicans, the 0.5 McFarland turbidity of 1.5×10^8 was prepared from the bacteria and adjusted using a spectrophotometer (Cintra-GBC, Australia). Then, dilution was done and the amount of McFarland was reduced to 1.5×10^6.

Extraction steps: To obtain the alcoholic extract, the first two series of 300 grams (g) of dry plant powder were mixed separately in 1200 ml of 96% methanol to prepare methanolic extract and 1200 ml of 96% ethanol to prepare ethanolic extract for 2 hours. The mixtures were placed at laboratory temperature (25 °C) for 24 hours. The next day, the solvent containing the extract was separated from the mixture using filter paper No.1, and added to the solvent again. This operation was carried out for 5 days and all the solvents were mixed after filtering, they were transferred to the vacuum distillation machine to be distilled. After the extract was concentrated, it was placed under a hood to dry completely. The dry extract was kept in the refrigerator until the experiment.[16]

Test to determine the sensitivity of C. albicans to extracts through disk method: To determine the antifungal effect of the alcoholic extract using disk diffusion method, concentrations of 40, 50, 60, and 70 g/ml were prepared from each extract separately, and 15 microliters of each were placed on empty standard sterile disks (Padtan Teb, Iran) imported by the sampler. Then, the disks were placed in a 45 °C oven to dry. In the next step, a thin layer of olive oil was added to the SDA with a sterile swab and the prepared fungal suspension was cultured on it. Then, the disks were placed with sterile forceps at a distance of 1.5 cm from the edge of the plates, with a 2 cm distance between each disk. The plates were incubated for 48 hours at 32 °C. The positive control was a 122-unit nystatin disk (Padtan Teb, Iran), and the negative control was a disk containing 15 microliters of dimethyl sulfoxide (DMSO), which was poured onto the disk by a sampler.[17]

Test to determine the sensitivity of C. albicans to extracts using the well method: For this purpose, first, wells with a length of approximately 1.5 to 1.5 cm were created in the SDA, with a distance of 1.5 cm from the edge of the plates, and 2 cm distance between each sample. The wells were filled with 15 microliters of each alcoholic extract at the concentrations of 80, 90, 100, and 110 microliters. After adding the extract to the wells, the plates were placed at room temperature for several hours so that the extract penetrated completely into the culture medium. The plates were incubated for 48 hours at 32 °C. After the incubation, the development or lack of development of the non-growth halo diameter around the well was checked. The positive control was 15 microliters of nystatin drops of 100000 international units/ml I.U/ml (EIPICO, Egypt), and the negative control was the disk containing 15 microliters of DMSO, which was poured into the respective wells by the sampler.[16,17]

Determining the minimum inhibitory concentration (MIC) of the extract on C. albicans: In 11 test tubes, 1 ml of liquid SD broth medium was poured. Into the first tube, ml of the extract solution was introduced. Therefore, 1.2-fold dilutions were made of the desired extract, in such a way that only the last tube (tube no. 11) had no extract and was considered as a control for the growth of the C. albicans (1 milliliter (ml) of the contents of the ninth tube was added to the tenth tube. But, 1 ml of the contents of the tenth tube was not added to the eleventh tube and discarded. The eleventh tube had no extract and was used as control).

Table 1. The amount of extract in each tube at the minimum inhibitory concentration (mg/ml)

Tube number	1	2	3	4	5	6	7	8	9	10	11
Amount of extract (mg/ml)	$10^4 \times 5$	$10^3 \times 25$	$^{102} \times 125$	6250	3125	1562.5	781.25	390.62	195.31	97.65	0

In the next step, 10 microliters of fungal suspension containing 1 × 10^4 fungi per ml were added to all the tubes. After this period of incubation, the turbidity in the tubes was compared with the control sample, the eleventh tube, and the results were checked. According to the dilution method in the liquid medium, the amount of active substance in the first tube was 104 × 5 g/ml, but the amount of active substance in the second tube was 25 × 103 g/ml. In the same way, a certain amount of active ingredients was made in different tubes (Table 1).[16]

Determining the minimum fungicidal concentration (MFC): After incubation, to determine MFC, 10 microliters were taken from the MIC tube and other tubes were cultured in SDA without turbidity, and incubated at 25-30 °C. After the required time in the incubator, the colony-forming unit (CFU) was determined. In this way, the colonies were counted and the dilution factor (1/10) was multiplied in the photo, and the number of fungi in the plates was obtained. The lowest concentration in which no growth was observed was considered as the MFC.[16,17]

Determining the chemical composition of B. squash extract: Gas chromatography (GC) was used to determine the effective substances of the chemical compounds of B. squash extract and their percentage.

Statistical analysis: Statistical Package for the Social Sciences (SPSS) software (version 21; IBM Corp., Armonk, NY, USA), two-way analysis of variance (ANOVA), multiple comparisons, and independent samples t-test were used to investigate the effect of the concentration and types of extracts on the non-growth halo diameter around the C. albicans ($P < 0.05$). Quantitative values were reported as mean ± standard deviation (mean ± SD). (Ethics code: IR.IAU.BABOL.REC.1399.002)

Results

The results of determining the sensitivity of C. albicans to extracts using the disk method: Around the disks containing 40, 50, 60, and 70 g/ml of ethanol extract, the average growth halo was equal to 7.33, 8.67, 9.33, and 10 mm, respectively. Moreover, around the disks with 40, 50, 60, and 70 g/ml of methanolic extract, the average non-growth halo diameter was equal to 6.67, 7.33, 9, and 11 mm, respectively. The non-growth halo diameter was higher in the ethanolic extract (40, 50, and 60 g/ml) compared to the methanolic extract (40, 50, and 60 g/ml), but this difference was not significant ($P < 0.05$). In the case of methanolic and ethanolic extracts from 40 to 70 g/ml concentration, an increasing trend was observed in the lack of growth, but this increase was not significant ($P < 0.05$). The volume of 70 g/ml showed a larger non-growth halo diameter compared to the other concentrations, but no significant difference was observed ($P < 0.05$) (Table 2).

Table 2. Average non-growth halo diameter (mm) in ethanolic and methanolic extracts at different concentrations (g/ml) in the disk method

Concentrations (g/ml)	Extracts		P-value
	Growth halo (mm) of methanolic extract (mean ± SD)	Growth halo (mm) of ethanolic extract (mean ± SD)	
40	6.67 ± 0.58	7.33 ± 0.58	0.23
50	7.33 ± 1.15	8.67 ± 1.15	0.23
60	9.00 ± 1.73	9.33 ± 2.31	0.85
70	11.00 ± 1.73	10.00 ± 3.46	0.67

The mean ± SD of the non-growth halo for nystatin was 21.6 ± 2.40 mm, which was significantly higher than methanolic and ethanolic extracts (P > 0.0001).

The results of determining the sensitivity of C. albicans to the extracts in the well method: Around the wells with concentrations of 80, 90, 100, and 110 g/ml of ethanol extract, no non-growth halo was observed. Around the wells with 80, 90, 100, and 110 mg/ml of methanolic extract, the average non-growth halo was equal to 12.00, 13.00, 14.00, and 17.00 mm, respectively. Non-growth halo diameter around the well containing nystatin was 30 mm. The non-growth halo diameter of the methanolic extract showed an increasing trend with increasing volumes used, but this increase was not significant (P < 0.05). The mean ± SD of the halo of non-growth halo diameter for nystatin was 20.5 ± 2.1 mm, which was significantly higher than the aqueous and alkaline extracts (P < 0.0001) (Table 3).

Table 3. The average halo of non-growth halo diameter (mm) in different concentrations (g/ml) of the methanolic extract in the well method

Concentrations (g/ml)	Methanolic extract (mean ± SD)	P-value
80	12.00 ± 0	0.30
90	13.00 ± 0	
100	14.00 ± 0	
110	17.00 ± 0	

The results of MIC and MFC: In the case of the ethanolic extract, turbidity was seen in all three repetitions in tube number 3 (125 × 10^2 g/ml). Turbidity was observed in the methanolic extract in the first, second, and third iterations, respectively, in tubes number 3 (125 x 10^2 g/ml), 3 (125 x 10^2 g/ml), and 4 (6250 g/ml). The amount of MFC of the ethanolic extract in the first, second, and third repetition was equal to tube number 2 (25 × 10^3 g/ml). Furthermore, the amount of MFC of the methanolic extract in the first, second, and third repetitions is equal to tube number 2 (25 × 10^3 g/ml), 2 (dilution 25 × 10^3 g/ml), and 3 (dilution 125 × 10^2 g/ml), respectively. In the MIC (P = 0.317) and MFC (P = 0.317), there was no significant difference between the alcoholic groups (Table 4).

The results of the chemical composition of B. squash extract: The highest percentage of compound was related to 5-hydroxymethylfurfural with 83.62% (Table 5).

Discussion

In this research, the antifungal effect of B. squash was evaluated on C. albicans isolated from vaginal infections. Antioxidant activities along with bioactive compounds that have antimicrobial activity have been reported in different parts of B. squash.[18,19] B. squash ethanolic and methanolic extracts at different concentrations showed antimicrobial activity on the C. albicans. Park et al. reported that based on their biological nature and activity, the known antifungal proteins from the gourd family are ribosomal inactivating proteins (RIPs) and vanillin-like proteins.[13] These proteins significantly inhibited the growth of Botrytis cinerea (B. cinerea), Fusarium solani (F. solani), Fusarium oxysporum (F. oxysporum), and Trichoderma harzianum (T. harzianum) at a concentration of 10-20 µmol/l.[13] Park et al. also reported that a novel antifungal protein of 40 kDa was isolated from B. squash skin, and then, purified by anion exchange chromatography and high-performance liquid chromatography (HPLC). This protein inhibited the growth of several fungi including B. cinerea, F. oxysporum, F. solani, Rhizoctonia solani (R. solani) as well as C. albicans at a concentration of 10-20 µl.[20]

Table 4. Average MIC and MFC for alcoholic extracts (g/ml)

Test	Ethanolic extract (g/ml)	Methanolic extract (g/ml)	P-value
MIC	1041.67 ± 3608.44	16666.76 ± 7216.88	0.317
MFC	20833.33 ± 7216.88	33333.33 ± 14433.76	0.317

Table 5. Chemical compounds of B. squash extract

Components	The percentage of constituents	Inhibition index
2, 4-Dihydroxy-2,5-dimethyl-3(2H)-furan-3-one	2.78	5.814
4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl	9.88	8.650
5-Hydroxymethylfurfural	83.62	10.013
Benzene, 1-chloro-2-methoxy-Hydrazine	1.89	12.127
1-Deoxy-d-altritol.beta.-D-Glucopyranose	0.77	15.579
4-Quinolinamine, 7-chloro-Lidocaine	2.95	17.798

The results of these studies are in line with the results of our study.[13,20] In another review, Vassiliou et al. reported that several antimicrobial proteins were isolated from B. squash; the 3 essential proteins microtubule-associated protein 2 (MAP2), MAP4, and MAP11 have been isolated from B. squash seeds that inhibit the growth of yeasts.[21] According to the study by Wang and Ng, Cucurmoschin is an antifungal peptide isolated from B. squash seeds, which is rich in arginine, glutamate, and glycine.[22] This peptide inhibits the growth of B. cinerea, F. oxysporum, and Mycosphaerella.[22] Moreover, Barbieri et al. showed that ribosome-inactivating protein derived from B. squash has antibacterial activity against phytopathogenic bacteria.[23] In our study, some different compounds were identified by GC that can have antimicrobial effects.[21-23] In a study conducted by Jarungjitaree and Naradisorn, the antioxidant and antifungal activities of B. squash were investigated. B. squash skin was extracted with 3 different solvents: methanol, ethanol, and acetone. 50% crude methanol extract of B. squash skin was diluted to 95%, 70%, and 50% for antifungal assays, in vitro and in vivo. Methanolic extract of B. squash skin at all concentrations significantly delayed the growth of Colletotrichum musae (C. musae) during the incubation period for 6 days compared to the control group. Based on the results of our study, the methanol extract of B. squash in the well method prevented the growth of C. albicans to some extent, so it showed an increasing trend with the increase in the volumes used.[2] Muruganantham et al. showed that there was no non-growth halo around C. lunata and C. albicans in the disk method.[14] Ethanolic extract of B. squash with a concentration of 10 mg/ml was used in the study by Muruganantham et al.[14] The results of this study were not in agreement with the results of our study. In our study, the non-growth halo around the disks containing volumes of 40, 50, 60, and 70 g/ml of ethanol extract was equal to 7.33, 8.67, 9.33, and 10 mm, respectively.[14] The study of Mohammed and Suhail Najm, which was designed to describe and evaluate different organic solvents (distilled water, ethanol, hexane, and petroleum ether) of B. squash leaf extract against some pathogenic fungi, showed that B. squash leaf extract can inhibit the growth of Aspergillus fumigatus (A. fumigatus), Aspergillus niger (A. niger) and C. albicans, and was comparable to ketoconazole.[24] In our study, B. squash was also comparable to nystatin.[24] In the present study, the growth of C. albicans was prevented by the ethanolic extract of B. squash. These results are in line with the results of previous studies. According to Jadhav et al., ethanolic extracts of B. squash leaves, which contain saponin, alkaloid, tannin, and phenol, synergistically lead to antimicrobial effects.[25] Based on the results of this study, B. squash alcoholic extracts effectively inhibit C. albicans and can be used as a promising tool to control or treat fungal diseases. In addition, more research is needed in this field to obtain an alternative method for the practical use of this extract in medicinal compounds. This study had no specific limitations, but it is suggested that different parts of this plant should be

investigated separately and its antimicrobial effect on more microbial species should be tested and compared.

Conclusion

Around the disks containing ethanolic and methanolic extracts, the average non-growth halo was seen. Around the wells with methanolic extract, a non-growth halo was observed. In the case of MIC and MFC tests, no significant difference was observed between different alcoholic groups. Comparison of non-growth halo in the case of ethanolic extract compared to methanolic extract was not significant in any of the case volumes.

Conflict of Interests

Authors have no conflict of interests.

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