The effects of *Ziziphus Spina* leaves' Hydro-Alcoholic Extract Vaginal Cream and Clotrimazole on *Candida albicans* in Wistar Rats

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ABSTRACT

BACKGROUND: *In vitro, Ziziphus Spina-Christi (ZSC) leaves have been shown to have antimicrobial and antifungal effects.* This study aimed to examine the effects of Ziziphus Spina leaves hydro-alcoholic extracts with Clotrimazole against Candida albicans in female rats.

METHODS: Four groups of rats were infected vaginally with *C.* Albicans, and 1 group not infected was considered negative control. The infected groups received the following treatments: 2 groups were treated with vaginal 5%, or 10%, of Ziziphus Spina extract creams. One group received 1% clotrimazole, and 1 group did not receive any treatment considered a positive control.

RESULTS: The mean number of colony-forming units (CFUs) before the intervention was 195.83 ± 395.126 in the 5% ZSC group, 346.33 ± 396.719 in the 10% ZSC group, 345.17 ± 507.431 in the clotrimazole group, 212.20 ± 148.304 in the positive control group ($P = .604$), and 0 in the negative control group ($P = .003$). After 1 week, the average number of CFUs considerably dropped to 65.14 ± 36.03 in the 5% ZSC group, 1.43 ± 3.60 in the 10% ZSC group, and 0.43 ± 1.13 in the clotrimazole group. The number in the positive control group remained unchanged (212.20 ± 148.304) ($P = .005$). After 2 weeks, the average number of CFUs was 0 in the 10% ZSC group, Clotrimazole and negative control groups and was 4.57 ± 23.99 in the 5% ZSC group ($P < .001$).

CONCLUSIONS: Our findings indicated that the effectiveness of Vaginal creams containing 10% Ziziphus Spina is similar to Clotrimazole in eliminating *C.* Albicans.

KEYWORDS: Clotrimazole, Candida albicans, vaginal cream, Ziziphus

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Introduction

Vulvovaginal candidiasis (VVC) is the second most common cause of infectious vaginitis. At least 75% of women have experienced VVC once in their life, and 5% more than 4 times a year.[1] Typically, 90% of Candida albicans-related infections are harmless and asymptomatic. However, VVC infections may be associated with severe itching, vaginal soreness, pruritis, pain, irritation, unpleasant odor, dyspareunia, dysuria, and burning during micturition.[2] Previous investigations revealed that in different provinces of Iran, the frequency of VVC varies from 4.8% to 73%.[3-6] Although it is not a life-threatening condition, it can cause physical and psychological problems and deteriorate marital relationships.[1] The limited number of effective antifungal drugs is an excellent issue in treating fungal infections. Many of these drugs have toxic effects, and since most of these drugs possess antifungal activities against fungi rather than killing fungal pathogens, they relapse in their treatment.[7] Echinocandins, caspofungin, micafungin, anidulafungin, and fluconazole are some of the drugs used to treat vaginal candidiasis.[8] Clotrimazole is a broad-spectrum drug mainly used to treat vaginal candidiasis and other fungal infections.[3] Abdominal pain, diarrhea, headache, rash, indigestion, allergic reaction, and severe skin reaction are all possible adverse effects of antifungal drugs.[4] On the other hand, long-time treatment with these drugs leads to acquired drug resistance.[7] Treatment of this disease is complex due to contributing factors and the risk of recurrence.[5] Some natural products, such as probiotic supplements,[6] organic apple cider vinegar,[9] and organic garlic, are commonly used to treat C. Albicans.[10]The Rhamnaceous family's Ziziphus Spina-Christi (L.) (ZSC) (Sider) is a tropical evergreen tree found mainly in the Sinai Peninsula. (Egypt). In other Middle Eastern countries, it is known as Nakba and

Sidr.[11,12] ZSC is utilized for its antimicrobial and antifungal properties in traditional medicine.[13-15] The leaves of ZSC are used to heal wounds, skin illnesses, inflammatory problems, sores, ringworm, fever, gonorrhea, sexually transmitted disease, and ulcers in Saudi Arabian traditional medicine.[16,17] In Iranian traditional medicine, the leaves of ZSC are used as an antibacterial, antifungal, and anti-inflammatory agent and for treating skin illnesses, including atopic dermatitis.[15,18,19] Metabolites such as flavonoids and alkaloids are the main antimicrobial agents which cause inhibition of bacteria growth. Detailed examination of ZSC showed that this plant contains flavonoids, alkaloids, saponins, lipids, proteins, and free sugar as the dominant chemical composition has antibacterial activity.[2,14] Analysis activities related to the ZSC, its methanolic and ethanolic extract indicated the preventing growth effect on different species of Gram-negative bacteria.[12,20]

Treatment of C. Albicans with Barhi date extract induced cell wall deformation, weakness, and partial collapse, according to Shraideh et al.[21] Cell lysis, cytoplasmic substance leakage, and cell death was detected at high doses. New antifungal tactics attempt to create drugs that combine lower manufacturing costs, high efficacy, local toxicity, and safety for humans, animals, host plants, and ecosystems. Furthermore, investigations have shown that ZSC leaves have antifungal properties.[13-15]

Objectives

There was insufficient data on the efficiency of ZSC leaf extract against Candida albicans. The goal of the present study was to compare the effects of ZSC leaves hydro-alcoholic extract and Clotrimazole on Candida albicans in Wistar rats.

Methods

Ethics statement

We conducted a double-blind, randomized, controlled study in female Wistar rats to examine ZSC leaves' effects on hydro-alcoholic extract compared to Clotrimazole on C. Albicans. The Medical Sciences ethics committee approved this study (Ref. No.: IR.SHOUSHTAR.REC.1398.009).

Preparation of C. albicans suspension

Candida albicans was isolated from a Candida vaginitis patient. (According to the DNA Data Bank of Japan, C. Albicans Code of Standard: Atcc10231) Moreover, it is used to infect rats. At 28°C-30°C, yeast extract peptone dextrose (YEPD) medium was used for cell culture. The cells were washed 3 times with sterile phosphate-buffered saline after (PBS) 48 hours. The OD was measured for the preparation suspension of 1×108 cells/mL (1×107 cells in 100 µL).

Induction vaginitis in Wistar rats

Wistar rats (n = 50) weighing 150 to 200 g were randomly put into 5 groups (10 per group). For 1 week, each group was placed in a separate cage with a sterile straw under standard temperature and light (12 hour light-dark cycle, temperature 20°F-25°F, and humidity 50%-60%).[22] To ensure the rats were not contaminated, the samples were cultured in 2 forms; with and without chloramphenicol. Candida albicans was cultured on a YEPD medium. After 18 hours, the cultured yeasts were washed 3 times with *PBS*. This mixture was centrifuged, and 5×10^7 to 1×10^8 yeast cells were suspended in PBS. Two estradiol valerate doses were injected subcutaneously in the lower abdomen during the first and third days to suppress the rats' hormonal systems.[4] After the second estradiol injection, 0.1 mL yeast suspension was injected into the vagina, and the rats were kept upside down for about 1 minute to ensure the inoculum.

Preparation of ZSC *leaf extract*

Fresh Iranian Z. Spina-Christi (sidr) with green leaves were collected. Leaves were washed and then air-dried in the shade at 30°C ± 2°C for 4 days and then transferred to the pharmacognosy lab. The dried green sidr leaves were pulverized, and a maceration procedure was used to make an extract. The powdered green sidr was mixed with 80% ethanol and kept at room temperature (27°C-32°C) for 3 days, stirring occasionally. Using a vacuum flask, the mixture was filtered using Whatman paper. The extract was freeze-dried to a fine bulk powder after being dried in a vacuum rotary evaporator at 45°C. The powder was stored at a temperature of less than 4°C.

The extract was added to a suitable cream basis (an emulsion of water in oil) at concentrations of 5% and 10% (w/w) to make ZSC green leaves cream.[23] The same base was utilized at the pharmacy to manufacture 1% clotrimazole cream. All finished solutions had the same appearance, and an authorized color was utilized to assure consistency. The produced formulations were subjected to microbiological and physicochemical testing (uniformity, physical stability, distribution, and acidity). 5% and 10% ZSC leaves and clotrimazole creams were put in identical canisters and coded A, B, and C by a blinded third party. The texture and appearance of all the creams were the same. Creams were administered, and a blinded researcher prepared samples.

Grouping

The rats were divided into 5 groups of 10 rats each.:

1- C. Albicans-infected control group that was not treated (positive control)
2- An uninfected control group that was not treated (negative control)
3- An infected group underwent therapy with a hydro-alcoholic extract of 5% ZSC leaves.
4- An infected group underwent therapy with a 10% hydro-alcoholic extract of ZSC leaves
5- An infected group underwent therapy with a 1% clotrimazole.

Table 1. Results before and after treatment in groups.

GROUPS	BEFORE INTERVENTION	1 WEEK AFTER THE INTERVENTION	2 WEEK AFTER THE INTERVENTION	*P*-VALUE*
A	195.83 ± 395.126	65.14 ± 36.03	4.57 ± 23.99	.001
B	345.17 ± 507.431	0.43 ± 1.13	0.00 ± 0.000	
C	346.33 ± 396.719	1.43 ± 3.60	0.00 ± 0.000	
D	212.20 ± 148.304	178.00 ± 173.551	174.80 ± 164.970	
*P***	.604	.005	>.001	

A, 5% of Zizyphus Spina leaves extracts group; B, 1% clotrimazole group; C, 10% of Zizyphus Spina leaves extracts group; D, positive control group; E, negative control group.
*Group and time interaction in the GEE model.
***P*-value between groups.

Assessment of infection

A vaginal sample was taken from each rat before commencing therapy (6 days after the initial injection of yeast suspension into the vagina of rats). To screen for bacterial infection, the samples were cultured on Sabouraud dextrose agar with chloramphenicol (SC). An additional yeast dosage was administered to the vagina if the infection was not discovered. According to references, the number of yeast colonies in a sample is usually assessed by criteria of 7 to 8 colonies per swab.[24] A colony counter machine counted the number of colonies growing on the plate surface, and the direct sample revealed more yeast colonies.

Intervention

After the rats' infections, the case groups received 5% or 10% hydro-alcoholic ZSC extraction. The concentrations of ZSC extraction were chosen based on the previous study to find whether the increasing drug resistance of Candida Albicans over time is efficient.[4]

Statistical analysis

Descriptive statistics, including mean and standard deviation (SD), were utilized to describe the fracture resistance values. The normality of data distribution was assessed using Shapiro ilk and Kolmogorov Smirnov tests. Data analysis was performed using Kruskal-Wallis and post hoc tests. The significant level was considered as $P < .05$. The collected data were analyzed with IBM.SPSS statistics software 24.0 Version.

Results

Before the intervention, all samples were evaluated for confirmation of CFUs accumulation (Figure1A0, B0, C0, D0). 1 week later, the mean number of CFUs significantly reduced (P-Value = .005) in 3 groups, including 5% ZSC group (65.14 ± 36.03), 10% ZSC group (1.43 ± 3.60), and clotrimazole group (0.43 ± 1.13) (Figure1A1, B1, C1, D1) (Table 1). After 2 weeks, the mean number of CFUs was 0 in both the 10% ZSC leaves group and Clotrimazole and negative control groups and 4.57 ± 23.99 in the 5% ZSC leaves group ($P < .001$) (Figure1A2, B2, C2, D2) (Table 1).

Discussion

Despite the existence of various treatments for opportunistic infections, nevertheless, the refractory to treatments for these pathogens are increasing, such as Candida albicans. The drug resistance against Candida albicans in vulnerable patients such as diabetic and cancerous patients is a critical concern.[25,26] This issue causes a proliferation of studies intending to recognize the new therapeutic agents with the lowest side effects.[27] Despite considerable evidence in this field, still, there are inconsistent results due to influencing factors such as type of herbal, concentration, and used methods.[22] Additionally, following the recognized mechanism of drug-refractory of candida Albicans, it was understood that the traditional treatments are becoming outdated.[28] In this regard, the present survey aims to investigate the efficacy of ZSC hydro-alcoholic extraction in growth inhibition of Candida albicans compared with standard treatment by Clotrimazole. Our data revealed that the 10% concertation of ZSC hydro-alcoholic extraction had the highest therapeutic efficacy, the same with Clotrimazole.

Numerous studies have attempted to improve therapeutic strategies against prevalent infections, such as Candida albicans, to reduce treatments' side effects. The common inhibitory mechanisms of herbal agents with synthetic drugs in infection restriction cause the proliferation of studies in this field. Previous investigations report the most antifungal of Lawsonia inermis, Pelargonium graveolens, Camellia sinensis, Mentha piperita, and Citrus latifolia against Candida albicans[29]; whereas our findings indicated the 10% of Zizyphus Spina leaves' hydro-alcoholic extract has favorable outcome as the same with Clotrimazole. Following our results, Lashin et al[30] have shown that Zinc and selenium oxide nanoparticles biosynthesized using callus extract of ZSC have potential antimicrobial activity and antioxidant activity.

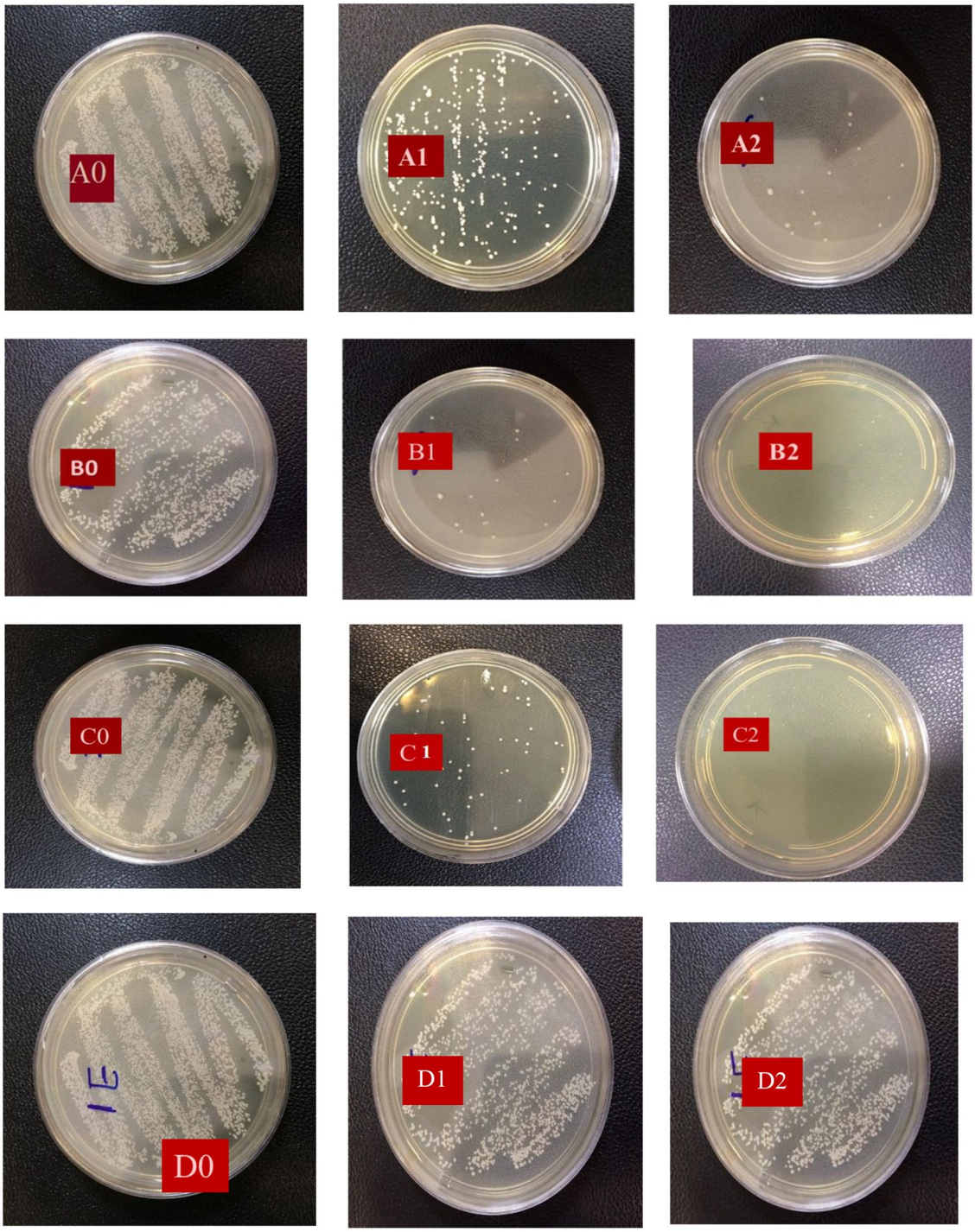

Figure 1. (A): 5% of Zizyphus Spina leaves extracts group, (B): 1% clotrimazole group, (C): 10% of Zizyphus Spina leaves extracts group, (D): positive control group. 0: before the intervention, 1: 1 week after intervention, 2: 2 weeks after intervention.

Similar to our aim, the results of Soliman et al[31] demonstrated that Saussurea costus Root extracts have a therapeutic effect in Candida albicans treatment. Additionally, it was shown that herbal products are more efficient in treating drug-resistant candida sp.[32]

For Candida albicans growth inhibition, critical mechanisms for surviving Candida albicans must be targeted; including inhibition of hyphae transformation, biofilm formation, cell wall or cytoplasmic membrane biosynthesis, ROS production, and over-expression of membrane transporters.[32] Zizyphus Spina containing saponin disrupts the cell wall of Candida albicans.[33] Additionally, Zizyphus Spina has endophytic A. Alternaria, which has potent antioxidant activity that can cause cell membrane disruption.[34] Finally, the tannins in Zizyphus Spina, by eliminating Iron, reduce the pathogen growth and proliferation.[35]

In a study by Butassi et al[36] it was reported that Phytolacca tetramera from Argentina has antifungal activity against Candida albicans and Candida glabrata. After examination of 12 Phytolacca tetramera extracts, they found that dichloromethane extract from berries, by altering the plasma membrane, has the most effective therapeutic activity.[36]

Conversely, Mardani et al compared Lawsonia inermis and ZSC Christi by nystatin and fluconazole, inhibiting Candida activity from liver transplant patients.[37] Their findings indicated that ZSC Christi unripe fruit and L. inermis leaf have potential anti-Candida activity.[37]

Understanding the mechanism of ZSC in drug-resistance candida Albicans inhibition can improve our knowledge in promoting the efficacy of the therapeutic effect of ZSC. The main limitation of the present survey is no measurement of various concentrations of ZSC against both sensitive and resistant candida Albicans. Since vulnerable patients are the main concern for fungal infection, drug interaction should be considered in these patients. In this regard, further clinical trials to evaluate the efficacy of ZSC in candida Albicans treatment are highly recommended in vulnerable patients.

Conclusion

The results of our study indicated that with increasing drug-refractory of candida Albicans, extraction of ZSC can be helpful in candida Albicans restriction. Recently, it was demonstrated that nanobiotechnology had shown the beneficial effect of improving herbal products' efficacy in infection treatment. Hence, it is highly recommended that more investigations consider this issue.

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Authors' Contributions

Azam Honarmandpour and Amir Masoud Keshavarzzade were involved in the study design. Azam Honarmandpour, Hossein Kamali, Mahnaz Fatahinia and Amir Masoud Keshavarzzade were involved in data collection. Azam Honarmandpour and Foroogh Namjoyan were responsible for drug development. Elham Maraghi and Azam Honarmandpour were responsible for data interpretation. Azam Honarmandpour was responsible for writing and finalizing manuscript in English.

Availability of Data and Materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics Approval and Consent to Participate

The Medical Sciences ethics committee approved this study (Ref. No.: IR.SHOUSHTAR.REC.1398.009). All methods were performed in accordance with the relevant guidelines and regulations the study is reported in accordance with ARRIVE guidelines.

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