



# Comparative Investigation of the Sensitivity of *Candida* Fungi Isolated From Vulvovaginal Candidiasis to Nystatin and *Teucrium polium* Smoke Product

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## Abstract

**Objectives:** The present study aimed to compare the antifungal activities of *Teucrium Polium* smoke product and nystatin in the treatment of *Candida* vaginitis in vitro.

**Materials and Methods:** In this quasi-experimental study, 105 subjects were diagnosed with *Candida* vaginitis. The data were collected through a collection form and the species were isolated by the germ tube, as well as CHROMagar chromogenic and chlamydo-spore formation tests.

**Results:** Based on the results of the germ tube, chlamydo-spore formation, and CHROMagar tests, 70.5%, 23.8%, and 66.6% of the species were *Candida albicans*, respectively. In addition, 99% of the samples were sensitive to nystatin. A significant relationship was also observed between the antifungal drug and the type of organism ( $P < 0.02$ ). Finally, all the 15 standard clinical samples were sensitive to *T. polium* smoke.

**Conclusions:** In general, the results confirmed the antifungal effects of *T. polium* and nystatin on the species isolated from 10 clinical samples obtained from *Candida* vaginitis as well as on five standard strains.

**Keywords:** Vulvovaginal candidiasis, Albicans, Nystatin, *Teucrium Polium*, Smoke, Persian medicine

## Introduction

Vaginal candidiasis infection (VVC) is a common problem among women and requires consultation and reference to primary healthcare centers (1). In addition, VVC infects the gastrointestinal tract and vagina by some *Candida* species. *Candida albicans* is reported as the cause of 85%-95% of the cases and *Candida glabrata* is mentioned as the most prevalent cause of non-*albicans* *Candida* vaginitis (2). Overall, in one study, the incidence of *Candida* infection was estimated as eight per 100 000 population (3), thus requiring adherence to epithelial cells. Based on some previous reports, *C. albicans* have the highest rate of adherence (4,5). A previous epidemiological study on the *Candida* species leading to VVC in Iran revealed that *C. albicans* (67%) was responsible for this infection, followed by *Teucrium polium* (18.3%), and *C. glabrata* (6. 8%). *Albicans* and *glabrata* species were also dominant among the infections involving several species simultaneously (6). Therefore, VVC mostly results from *C. albicans*. However, the attacks of non-*albicans* *Candida* species are increasing nowadays. Most non-*albicans* *Candida* species have greater resistance to the medicines which contain azoles and their resultant infection is difficult to treat as

well. Hence, physicians normally perform the treatment experimentally instead of routine laboratory techniques (7).

Although VVC is not life-threatening, it may cause complications for the patients and lead to their waste of time and money. Moreover, VVC is a psychosomatic disorder which results in stress in the patients, particularly its recurrent form (8). So far, the most common medicines for *Candida* include nystatin (polyene), clotrimazole, miconazole, ketoconazole (imidazole), and triazole which might have various side effects. In addition, considering their wide spectrum, they could have different sensibilities (9-12). Given the sensitivity of *Candida* isolates to antifungal drugs, particularly the azoles, as well as the side effects of nystatin, more attention is now paid to biologically active compounds which are obtained from the plants that were used in herbal medicine (13). Over the past decade, the use of complementary and alternative medicine has increased for treating female problems such as menopause, premenstrual syndrome, as well as sexual and menstrual problems and virginities (14-21).

*Teucrium polium* is regarded as one of the most important members of the *Teucrium* family which is

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abundantly found in South-Western Asia, Europe, the North of Africa, and the South and Northeast of Iran. Twelve species occur in Iran including three endemic species (22). The plant has 220 genera and almost 4000 species worldwide (23).

Further, *T. polium* in folk medicine and traditional medicinal plant is used for numerous ailments in Iran (24). For example, Khoshnood-Mansoorkhani et al reported that 28 compounds were identified in the essential oil of this plant with 99.75%, in addition to the combination of  $\alpha$ -pinene (12.52%), linalool (10.63%), caryophyllene oxide (6.69%),  $\beta$ -pinene (7.09%), and caryophyllene (6.98%) with 46.91% constitute the highest percentages of essential oil (25).

Due to its antimicrobial and antioxidant properties, this herb is also traditionally utilized to treat fungal in Iran and some other countries (26). Other studies further indicated that *T. polium* has diuretic, antipyretic, diaphoretic, anti-spasm, tonic, anti-inflammatory, antihypertensive, appetizing, antibacterial, analgesic, and anti-glucose functions (27) and its extract demonstrates antihypertensive, anti-spasm, antibacterial, antipyretic, antimicrobial, and antifungal activities. Various studies have so far focused on the properties of *T. polium* and evaluated the plant extract which was used as an oil, essence, aqueous extract, volatile oil, decoction, and brewed (28). The chemical analysis of *T. polium* indicates that this plant contains various compounds such as iridoids, flavonoid, and cirziliol (29). Furthermore, *T. polium* is reported to include beta-sitosterol, campesterol, csterol, glucose, fructose, raffinose, rhamnose, limonene, linalool, cedrol, and alpha-phellandrene compounds (30). However, to the best of our knowledge, no study has investigated the effect of *T. polium* smoke on fungi in Iran. The present study sought to compare the sensitivity of *C. albicans* species to nystatin and *T. polium* smoke product 48 hours after culturing.

## Materials and Methods

The laboratory-experimental study was performed in the clinics affiliated to Shiraz University of Medical Sciences, Shiraz, Iran during 2007-2008. Using a randomized complete block design, the sample size was considered as 100 subjects. Thus, almost 1100 patients were evaluated for seven months, who referred to gynecology clinics of the selected hospitals in Shiraz. Among these patients, 450 ones had complaints related to genital infections out of whom, 280 patients were selected based on the history and characteristics of VVC. Finally, 105 subjects were diagnosed with VVC. Then, written informed consent was obtained for the isolation of *Candida* yeasts from all subjects.

In the first group, species were isolated from 15 clinical *Candida* samples in this study. Moreover, five *Candida* strains were examined, including *C. albicans*, *C. glabrata*, *C. tropicalis*, *C. krusei*, and *C. parapsilosis* that were

approved by the Mycology Group of Medical School for Training and Research, with *T. polium* smoke product in vitro.

In the second groups, species were isolated from 15 clinical *Candida* samples, as well as five *Candida* strains, confirmed by the Mycology Group with antifungal nystatin, were inoculated in the laboratory. The patients with *Candida* infection by three tests were identified by germ tube test, CHROMagar chromomeric medium, and chlamyospore formation test in gynecological clinics. At the end of incubation, the diameter of the inhibition zone around the disk drug nystatin and *T. polium* were compared as well.

## Germ Tube Test

The following steps were taken to perform the test of the germ tube, which is also referred to as the "Raynaud's phenomenon":

1. Organism passage for 24-48-hours;
2. Preparing human serum at 1 cc for each sample;
3. Removing a sterile ointment as much as a loop from the organism;
4. Introducing the organism into 1 cc of human serum;
5. Keeping the serum containing the organism at 37° incubator for 2-3 hours;
6. Taking one anus from a serum suspension of 30 lambdas, followed by placing a drop of each suspension on the labeled microscope slides to examine the germ tubes.

The gland tube vision was considered in favor of *C. albicans*. A germ tube test is a screening test which for t is an outgrowth which is produced by the spores of spore-releasing fungi during germination. Additionally, the germ tube test is regarded as the gold standard for the diagnosis of *C. albicans*. In this method, the intended yeast is inserted into human or rabbit serum diluted with normal saline (1/2) and is then kept in the incubator at 37°C for a few hours. Approximately most of the isolated *Candida albicans* develop the germ tubes when incubated in a proteinaceous medium. The observation of the germ tube is in favor of *C. albicans* (31,32)

## Chlamyospore Test

The purpose of this test is to differentiate *Candida* species from *albicans* and non-*albicans*, as well as to a large extent, to detect non-*albicans* such as *C. glabra*, *C. tropicalis*, and *C. krusei*. Using a chromogenic medium and the fungal samples after purification, it is cultured on this medium. They are incubated at 30°C for 48 hours and then the type of fungal is confirmed based on the colony color. Similar to the germ tube formation, this feature is also specific to *C. albicans*. In this study, the yeasts were cultured through a corn meal agar medium and then were kept at 25°C for a week. In addition to chlamyospore, *C. albicans* forms pseudohyphae in this medium (33).

## CHROMagar Test

Chromogenic *Candida* agar aims to differentiate *Candida* species based on the color which is produced on the media. In this study, fungal species were purified, cultured, and incubated at 30°C for 48 hours using a chromogenic medium. Then, eight colors were observed as green, light green, green with a blue border, bluish green, purple, blue, white, and white with blue spots. The first four colors were all categorized as green and were interpreted as *C. albicans* (33,34).

Some materials that were included in the Sabouraud dextrose agar, Corn Meal Agar, disc diffusion clotrimazol, fluconazole, and nystatin were purchased from Himedia in India.

The inclusion criteria included being married, not having vaginal bleeding and chronic diseases, not using herbal and chemical drugs for the treatment of genital infection in the recent 2 weeks, as well as not performing vaginal douching, creams, or suppositories within the past 48 hours. The exclusion criteria were the negative fungal culture in Sabouraud dextrose agar medium and negative direct test in the lam staining.

In order to collect the samples, the patients were required to lie in lithotomic position and the samples were gathered from vaginal discharges using a speculum and sterile swab. For each patient, 2 swabs were used for preparing direct smear and culturing the sample in Sabouraud dextrose agar medium under a sterile condition. It should be mentioned that the number, date, and other characteristics of the samples were recorded on the plates containing the culture media. The plates were daily transferred to the mycology laboratory of the school of medicine and kept in the incubator at 30°C for 24-48 hours. Next, the colonies were passaged for purification and were then kept in sterile distilled water at -20°C for further experiments.

In order to prepare *T. polium* smoke, the plant from the Asteraceae family was identified and approved by a specialist in medicinal plants. Then, it was transferred to the pharmacognosy laboratory of the school of pharmacy. In the laboratory, first, 1000 g of the plant was powdered using a grinder. Afterward, it was heated in a device with a 1000 cc balloon at the bottom and a converter on the top for one hour. In this way, the plant was turned into smoke, moved upwards, collected in the converter, and changed into a liquid. In addition, the solvent of the liquid was extracted using nitrogen and the pure smoke was prepared to perform drug sensitivity tests. At this stage, *T. polium* smoke product was prepared in pure concentration and a soluble form was created in the polar solutes. Further, disks were provided containing different concentrations of *T. polium* smoke product (i.e., 10, 20, 30, 60, 90, 120, 180, 210, and 240 lambdas). The researchers also applied nystatin disks containing 100 unit/disk medicine made by Himedia Company, India. The concentration of the fungal suspension was determined based on McFarland 0.5 turbidity standard

and was cultured on Sabouraud dextrose agar medium by a sterile swab. The aforementioned disks were located on the medium for 48 hours and the inhibition zones around the disks (representing the antifungal effects of the drugs) were evaluated accordingly. In addition, the effect of *T. polium* smoke product was assessed on five standard *Candida* strains including *C. albicans*, *C. glabrata*, *C. tropicalis*, *C. krusei*, and *C. parapsilosis*, as well as 10 clinical *Candida* species which were isolated from VVC in the present study. Then, the antifungal effect of *T. polium* smoke product was determined according to the presence or absence of the inhibition zone around the disks.

At the end of the incubation stage, the diameters of the inhibition zones around the nystatin and *T. polium* disks were measured and compared as well. It should be noted that the sensitivity or resistance to the antifungal drugs was determined based on the measures mentioned in the brochure provided by the company manufacturing the disks (Himedia, India). Accordingly, the inhibition zones with <15 mm diameters were considered resistant while those with ≥15 mm diameters were found to be sensitive to nystatin.

Based on the investigation of the antifungal effects of *T. polium* smoke product, no inhibition zones were formed in the disks containing 10, 20, 30, 60, and 90 λ medicine. However, the inhibition zone started to form around the disk containing 120 λ medicine, which indicates that the antifungal activities of *T. polium* smoke product began at this concentration. Although the inhibition zone was yet larger around the disks containing 180 and 210 λ medicine, they were not different from those which were created around 240 λ disk. Therefore, the amount of medicine increased no more, and 180 λ disks were considered as the minimum concentration preventing the fungal growth. Finally, the collected data were entered the SPSS statistical software (version 16) and analyzed using the chi-square test and descriptive statistics.

## Results

According to the results of the present study, the mean age of the patients was 32 ± 9.49 years, and the highest proportion of infection was related to 26-35 years age-group. Among the 450 patients with the clinical symptoms of VVC, 102 cases showed positive fungal colony cultures, and 2 fungal species grew in three cases. Thus, a total of 105 cases (23.3%) were culture positive. Based on the germ tube test and chlamyospore test, 70.5% and 23.8% of the cases were *C. albicans* (Table 1). In CHROMagar test, 66.6%, 21.9%, 8.6%, and 2.9% of the samples were also *C. albicans*, *C. glabrata*, and the other non-*albicans*, *C. tropicalis*, and *C. krusei*, respectively. Based on the diagnostic test for chlamyospore test, 54.3%, 21.9%, and 23.8% of cases were reported as *Candida albicans*, yeast, and of *Candida non-albicans* as well.

The study results showed that 99% of the samples were sensitive to nystatin and a significant relationship was

**Table 1.** Candidates Based on a Variety of Diagnostic Tests in Women With Vaginal Candiditis

Tests	Germ Tube Test No. (%)	Chlamyospore Formation No. (%)	CHROMagar Candida Test No. (%)
Albicans	74 (70.5)	57 (45.3)	40 (66.6)
Non-albicans	31 (29.5)	48 (45.7)	35 (33.4)

observed between the antifungal drugs and the types of organisms ( $P \leq 0.02$ ). Moreover, all the 15 standard clinical samples were sensitive to *T. polium* smoke. (Table 2). The inhibition zone around 105 nystatin disks ranged from 13.5 to 37 mm with an average diameter of  $21 \pm 2.98$  mm. Additionally, the inhibition zones were formed around all the nystatin disks and none of the samples showed 100% resistance to this medicine (Table 3).

On the other hand, *T. polium* smoke product saturated with 180  $\lambda$  medicine was used for five standard *Candida* strains including *glabrata*, *tropicalis*, *krusei*, *parapsilosis*, and *albicans*, as well as for 10 samples which were obtained from the study patients. According to the results, the mean diameter of the inhibition zone was 13 mm.

## Discussion

The results revealed that 66.6% of the species were *C. albicans* while 33.4% of them were non-*albicans*. In the study by Moreira et al, 63% of the patients with the clinical diagnosis of *Candida* showed positive cultures, 95% of which were related to *C. albicans* (35). In another study in India, 65.73% of the *Candida* cultures included non-*albicans* species as well (36). In addition, Panahi et al found that 50.8% of the cases were positive among which, 68.8% were related to *C. albicans* (37). Based on the reports of Mirhendi et al, 66.5% of the cases were also *C. albicans* whereas the rest of them were *Candida* non-*albicans* (38).

Similarly, the results of the study by Mucci et al showed that the presence of *C. albicans* and *C. dubliniensis* and the absence of *C. africana* in pregnancy were significant so

**Table 2.** Distribution of the Susceptibility of Candida Species Isolated From the Vagina to the Drugs

Drugs	Nystatine No. (%)	<i>Teucrium polium</i> Smoke Product No. (%)
Sensitive	104 (99)	15 (100)
Resistance	1 (1)	0 (0)
P value <sup>a</sup>	0.02	0.00

<sup>a</sup> Chi-square test.

**Table 3.** Relationship Between VVC Species, the Germ Tube Test and Chlamyospore Test

Chlamyospore	Positive	Yeast	Pseudohyphae	Negative	P Value*
Germ tube	No. (%)	No. (%)	No. (%)	No. (%)	
Albicans	58 (55.2)	5 (4.8)	11 (10.4)	0 (0)	0.001
Non-albicans	0 (0)	18 (17.1)	9 (8.6)	4 (3.8)	
Total	58	23	20	4	

VVV: Vaginal candidiasis infection; \*Chi-square test.

that about 80.7% (42 cases) of the samples had *C. albicans*, which was observed in 7.5% (3 cases) with other species of the genus. This is in line with the results of the present study regarding the prevalence of a candidate (39).

Richter et al concluded that azoles were less effective in non-*albicans Candida* species while nystatin was effective in 90% of the patients (7). Despite the increase in the incidence of non-*albicans* species, they demonstrate more resistance to azoles and their resultant infections are difficult to treat accordingly (7, 40). The higher prevalence of non-*albicans* vaginitis might be justified by an excessive increase in the utilization of tropical azoles in some countries since 1992 (7). Moreover, the high prevalence rate of *C. albicans* might be attributed to its higher capability in adherence to the vaginal epithelium (5).

In the present study, 2 fungal species were isolated from 3 cases. In the study by Richter et al (7), compound infections (mostly *C. albicans* and *C. glabrata*) were isolated from 27 out of 429 cases with VVC as well (5%). However, Fan reported that more than one *Candida* species was responsible for VVC in only 0.02% of a total of 1070 patients (41).

The findings of the current study indicated that *T. polium* smoke had desirable effects on preventing the growth of *C. albicans* and non-*albicans*. Similarly, Qabaha demonstrated that the extracts of 5 medicinal plants (i.e., grape seed, *R. officinalis*, *P. guajava*, *P. granatum* peel, and *T. polium*) had antimicrobial effects on three Gram-positive microorganisms (i.e., *S. aureus*, *B. subtilis*, and *M. luteus*), 3 gram-negative cases (i.e., *E. coli*, *P. aureuginosa*, and *K. pneumonia*), and finally, 2 fungal species (*C. albicans* and *A. niger*) (26). In addition, the other researcher suggested that these medicines could be used as natural fungistatic, fungicidal, bactericidal, bacteriostatic, and antioxidant components in natural preservatives, as well as pharmaceutical products (30). Furthermore, the findings of another study performed in Iran demonstrated that *T. polium* could be an appropriate choice for the treatment of ulcerative colitis and Crohn's disease (42). Tepe et al also examined the effect of *T. polium* and *T. chamaedrys* on trophozoites and cysts in vitro and revealed no viable trophozoites at 32 mg/mL concentration within 48 hours (43).

In the study by Kremer et al. on chemical and other compounds, especially *Teucrium arduini* L. (Lamiaceae), the results represented that the plant had the properties of antimicrobial activity on *Staphylococcus aureus*, *C.*

*albicans*, and several other microorganisms (44).

Likewise, Taheri et al. reported that different extracts of *A. sieberi* affected *C. albicans* and prevented its growth (21). Thus, they suggested more examinations in order to survey the composition of this plant and more extensive use in human domains. The results corroborate with the findings of the current study. In both studies, the herbal drug stopped the growth of *Candida albicans*. Further, Gholampour-Azizi I et al investigated the in vitro antifungal activity of *Cucumis melo* in *Candida albicans* by utilizing the disc and well diffusion and found that *C. melo* extrusion has a therapeutic and specific anti-fungal potential against *C. albicans* (20). In another study, Nadimi et al indicated that *Teucrium polium* extract has a significant effect on *C. albicans* and highlighted the need for further research in order to identify the effects of this plant in the treatment of *Candida* infections (45).

Therefore, Bahramikia and Yazdanparast reviewed over 100 articles evaluating the effects of pharmacology and toxicology extracts and the compounds isolated from *T. polium*, which were published during 1970-2011. They reported that these compounds have a wide range of pharmacological effects including antioxidant, anti-cancer, anti-inflammatory, blood sugar, protect the liver, lipid-lowering, anti-bacterial and antifungal activities (22).

Considering the comparison of the effects of *T. polium* and nystatin, the utilized disks were not of a similar type. Nystatin was prepared based on the unit while *T. polium* was provided based on  $\lambda$ . Thus, a quantitative comparison was impossible. Qualitatively, however, *T. polium* showed relative antifungal sensitivity as compared to nystatin. The relative effect of *T. polium* smoke might be because the components and derivatives of this plant should be separated in order to determine its active ingredient. Furthermore, due to the lack of studies on *T. polium* smoke in Iran, the results could not be compared to those of the other studies. On the other hand, such studies investigated other forms of *T. polium* such as essence, oil, and decoction, which also restricts the comparison.

Financial restrictions were considered as one of the limitations of this study. In addition, the researchers faced with the lack of laboratory experts when providing *T. polium* smoke, and therefore, extended the duration of the projects.

#### Suggestions for Further Research

1. Conducting research on human subjects rather than the medium;
2. Using a liquid medium instead of the disk drug for determining the sensitivity tests, particularly in the case of fluconazole;
3. Preparing *T. polium* powder instead of a liquid for assessing the effect of the antifungal plant;
4. Preparing aqueous and alcoholic extracts instead of *T. polium* smoke for antifungal effect;
5. Analyzing *T. polium* smoke and more precise

identification of the antifungal compounds of the plant.

#### Conclusions

Overall, the findings of this study showed the antifungal effects of *T. polium* on the species isolated from 10 clinical specimens of *Candida* vaginitis obtained in the present study, as well as on five standard strains of *Candida* including *C. albicans*, *C. glabrata*, *C. tropicalis*, *C. krusei*, and *C. parapsilosis* which had a relatively good sensitivity as compared to the chemical drug nystatin. To the best of our knowledge, this is the first research that has been conducted in this area in Iran and after extraction of the compounds of this plant, stronger antifungal effects might yet be achieved in future studies.

#### Conflict of Interests

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

#### Ethical Issues

This research project was approved by the Ethics Committee of Shiraz University of Medical Sciences and written informed consent was obtained from all the participants.

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