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In vitro antifungal effect of herbal mixture (*Nigella sativa, Foeniculum vulgare* and *Camellia sinensis*) against *Candida* species isolated from denture wearers

Alireza Naeini[1], Seyed-Shojaddin Shayegh[2], Hojjatollah Shokri[3*], Ali Davati[4] , Ali Khazaei[5], Abdollah Akbari[6]

[1]Department of Parasitology and Mycology, Faculty of Medicine and Traditional Medicine Clinical Trail Research Center, Shahed University, Tehran, Iran
[2]Department of Prostodontics, Dental School, Shahed University, Tehran, Iran
[3]Department of Pathobiology, Faculty of Veterinary Medicine, Amol University of Special Modern Technologies, Amol, Iran
[4]Department of Social Medicine and Health, Faculty of Medicine, Shahed University, Tehran, Iran
[5]Dentist, Health Network, Noorabad, Lorestan, Iran
[6]Dentist, Qom University of Medical Sciences, Qom, Iran

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ABSTRACT

Introduction: Due to antimicrobial and dental plaque control activities, herbal mouthwashes lead to an improvement in oral health. Although chemical mouthwashes have demonstrated the greatest antimicrobial and anti-inflammatory effects, their usage has been limited because of their numerous side effects. The aim of this study was to assess the antifungal activity of herbal mixtures containing *Nigella sativa*, *Foeniculum vulgare* and *Camellia sinensis* against oral isolates of *Candida* from denture wearers.

Methods: We selected 93 individuals wearing complete denture prosthesis. Samples were collected from oral mucosa and dentures and cultured onto Sabouraud dextrose agar (SDA). The antifungal activities of *N. sativa*, *F. vulgare* and *C. sinensis* and their mixtures (no. 1, 2 and 3) against oral isolates of *Candida* were determined using punch-hole test.

Results: The oral cavities of all denture wearers were colonized with yeasts. Among the *Candida* species, *Candida albicans* was the most frequently recovered species (45; 48.4%), followed by *C. tropicalis* (14; 15%), *C. krusei* (9; 9.7%), *C. glabrata* (6; 6.5%), *C. dubliniensis* (4; 4.3%) and *Candida* spp. (15; 16.1%). Among the tested plants, *N. sativa* (mean value: 12.3 mm) and *F. vulgare* (mean value: 7.9 mm) showed positive results against all *Candida* isolates. The results exhibited that all herbal mixtures were active against various tested *Candida* isolates, ranging from 7.8 to 15 mm, 7.6 to 15.5 mm and 7 to 15 mm inhibition zones for herbal mixtures no. 1, 2 and 3, respectively.

Conclusion: The results indicated that *C. albicans* was the most prevalent *Candida* species. *N. sativa* and *F. vulgare* were good antifungal agents against oral species of *Candida* isolated from individuals wearing complete dentures, hence, there is a possible usefulness as therapeutic agents.

Implication for health policy/practice/research/medical education:
The herbal mixtures of *N. sativa*, *F. vulgare* and *C. sinensis* are able to act as antifungal agents against oral species of *Candida* isolated from individuals wearing complete dentures. Hence it might be a candidate for preparation of drug.

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Introduction

Among the several hundred species of microorganisms in the oral cavity, yeasts, especially members of the genus *Candida*, are representative of the few fungi considered to be commensal oral flora. *Candida albicans* is the most common species isolated from the human oral cavity, while other species, such as *C. glabrata*, *C. tropicalis* and *C. dubliniensis*, are less frequently found (1). The reported prevalence of *Candida* in normal healthy adults varies considerably among population groups, ranging from 6% to 55.4%, with a median of 34.4% (2). Infection due to *Candida* species has increased dramatically in recent years.

***Corresponding author:** Dr. Hojjatollah Shokri, Faculty of Veterinary Medicine, Amol University of Special Modern Technologies, Imam Khomeini Street, 24th Aftab, Amol, Iran. Phone: +98 11 44271057, Fax: +98 11 44271055., Email: hshokri@ausmt.ac.ir

This is due to the increase in the number of individuals with impaired immunity, especially those wearing the complete dentures, resulting in the conversion of the normal commensal to an infection causing pathogen. Various factors enhance development and progression of the disease. The prosthesis acts as a focus and trauma from the denture will facilitate infection (3). *Candida* colonization on dentures can be affected by the species of colonizing *Candida*, personal hygiene, such as denture removal at night, denture cleanser use, smoking, and denture characteristics including vertical dimensions, material integrity and fit (4). Stomatitis has been reported in more than 60% of denture wearers, and although it is typically asymptomatic, it is associated with leukoplakia, pseudomembrane (thrush) formation, erythema and angular cheilitis (5).

The difficulties associated with the management of oral *Candida* infections necessitate the discovery of new antifungal agents in order to widen the spectrum of activity against *Candida*. Plant-derived natural products may offer potential leads to new compounds which could act on these fungi. Since plants produce a variety of compounds with antimicrobial properties, it is expected that screening programs for some under-represented targets, such as antifungal activity, may yield candidate compounds for developing new antifungal drugs (6). We assessed the prevalence of denture stomatitis in complete denture wearers and its association with particular species of colonizing *Candida*. In addition, the present study was the first study of the antifungal activity of three herbal mixtures containing *Nigella sativa, Foeniculum vulgare and Camellia sinensis* against *Candida* strains using punch-hole test in an attempt to contribute to the use of these plant extracts as alternative products for fungal control.

Materials and Methods

Patient selection

Ninety-three completely edentulous individuals wearing complete denture prosthesis were randomly selected from 3 hygiene centers of Lorestan province, Iran at least for the past one year (from February 2014 to February 2015). Information relating to individual demographics including age, gender, drug use history, smoking habits, stomatitis symptoms, in addition to factors relating to denture wear, such as how long respective patients had used dentures, cleaning methods used, daily use frequency and vertical dimensions were collected in questionnaire forms by study dentists. All participants had complete dentures for both the mandible and the maxilla. Patient's consent to participate in the study was obtained. Ethical clearance was obtained from the ethical committee of Shahed University, Iran (Ethic No: 1025). Inclusion criteria were as follows: 1. Age groups more than 20 years and male or female individuals, 2. individuals wearing complete denture prosthesis, 3. individuals wearing the prosthesis for more than a year, 4. individuals not on any antifungal medication, 5. individuals who are willing to participate in the study. Exclusion criteria were as follows: 1. Age less than 20 years, 2. individuals not wearing complete dentures and wearing partial dentures, 3. individuals wearing the prosthesis for less than a year, 4. individuals on antifungal agents, 5. individuals who are not ready to participate in the study.

Sample collection

After examination of the oral cavity, denture specimens were collected by swabbing the oral mucosa or lesions (if present), as well as internal denture surfaces after individuals rinsed their mouths with tap water. Samples were inoculated onto Sabouraud dextrose agar plates (SDA) (Merck Co., Darmstadt, Germany) containing 0.01 g chloramphenicol (Fluka, Steinheim, Switzerland). Plates were incubated at 37°C for one week and examined frequently for developing colonies. The results were scored based on the number of colonies identified and respective colonies were then subcultured and purified on SDA and Chromagar *Candida* (CHROMagar, Paris, France) plates. Germ tube and beta-glycosidase testes were carried out to differentiate *C. albicans* from *C. dubliniensis*. Isolates were stored at -20°C until further analyzed.

Plant materials

Aerial parts and seeds of *N. sativa*, *F. vulgare* and *C. sinensis* were purchased from a local market in the region of Delfan, Lorestan province, Iran. The materials were thoroughly washed and dried in the shade at room temperature for 24 hours. Details of the plants and voucher botanic specimens were given in Table 1.

Preparation of essential oil from *Foeniculum vulgare*

The seeds of *F. vulgare* were completely immersed in water and hydro-distilled in a full glass Clevenger-type apparatus giving greenish-yellow oil. The extraction was carried out for 2 hours. When the condensed materials cooled down, the water and essential oil were separated. The oil was decanted to be used as essential oil. To improve its recovery, the essential oil was taken up in *n*-pentane (Merck Co., Darmstadt, Germany), dried over anhydrous sodium sulphate (Merck Co., Darmstadt, Germany) until

Table 1. Some characteristics of the tested plants

Scientific name	Voucher No.	Family	Local name	Major constituent
Camellia sinensis	3000280	Theaceae	Green tea	Catechin, Epicatechin, Gallocatechin
Foeniculum vulgare	1242	Apiaceae	Raziyaneh	*Trans*-anthole, Limonene, Fenchone
Nigella sativa	1084	Ranuculaceae	Siyah daneh	Thymoquinone, Thymohydroquinone, Thymol

Source: Reference 20.

the last traces of water were removed and stored in sealed brown vial at 4°C until further analysis (7).

Plant extracts

(A) Preparation of alcoholic extract from *Nigella sativa*

Seeds (100 g) were washed, dried and crushed into coarse powder with an electric mill. The powder was exhaustively extracted with 96% ethanol at room temperature for 48 hours. The mixture was subsequently filtered and concentrated under vacuum at 55°C. The residue was suspended in saline solution (8).

(B) Preparation of aqueous extract from *Camellia sinensis*

The aerial part of plant (100 g) was cleaned and dried in shadow and powdered using a mechanical grinder. The powder (100 g) was added to 400 mL hot water, boiled for 15 minutes and filtered through a Whatman paper (No. 42). The filtrate was evaporated to dryness under reduced pressure to obtain a viscous residue. The residue was suspended in normal saline (8). It is necessary to mention that the plant extract solutions were sterile.

(C) Preparation of herbal mixtures

Three different herbal mixtures were prepared with different concentrations as follows: No. 1: *N. sativa* (20 µL) + *F. vulgare* (5 µL) + *C. sinensis* (5 µL); No. 2: *N. sativa* (15 µL) + *F. vulgare* (10 µL) + *C. sinensis* (5 µL); No. 3: *N. sativa* (10 µL) + *F. vulgare* (15 µL) + *C. sinensis* (5 µL) (9).

Yeast inoculum preparation

Various *Candida* species isolated from oral samples were selected. *Candida* isolates were inoculated onto SDA broth and grown overnight on a rotary shaker at room temperature. Then, cells were washed three times with sterile distilled water. The count of yeasts was adjusted to yield 5×10^6 CFU/mL using the standard 0.5 McFarland counting method.

Susceptibility testing

Anti-*Candida* activities of the plant essential oil and extracts were assayed against *Candida* species using punch-hole method (10). Briefly, 100 µL of yeast inoculum (5×10^6 cells/mL) was uniformly spread onto SDA (Merck Co., Darmstadt, Germany) using a bent glass rod. Then, three wells of 6 mm diameter were punched by a borer into the SDA medium and filled with 30 µL of 2-fold serial dilutions of essential oil dissolved in 5% dimethyl sulfoxide (DMSO), extracts and their mixtures. Plates were incubated for 48 hours at 35°C. Anti-*Candida* activity was determined by measuring the zone of inhibition. Experiments were carried out 3 times.

Statistical analysis

Quantitative data were analyzed using the analysis of variance (ANOVA) and independent sample *t* test. All data were analyzed using SPSS (SPSS Inc., Chicago, IL, USA) version 15.0 software. A P value < 0.05 was considered to be significant.

Results and Discussion

Denture-related stomatitis has a multifactorial etiology that is associated with denture use, and disease presentation is affected by both endogenous and exogenous factors (4). A critical risk factor, however, is colonization of the oral mucosa by *Candida* species (54%-74%) and their resistant to some standard antifungal drugs. The activity of plant extracts against different organisms has been studied for many years. In this idea, several Chinese, African and Asian plant extracts have been evaluated for their antimicrobial and antifungal activities (6). *N. sativa*, *F. vulgare* and *C. sinensis* are commonly used in Middle East, especially in Iran. The present study was conducted for the first time in order to investigate the antifungal activities of each of these plants and their mixtures against *Candida* isolates from individuals wearing complete dentures.

Of the 93 individuals with complete dentures selected for this study, 76 (81.7%) were female and 17 (18.3%) were male. Most of the subjects were between 50 to 59 years of age. Minimum age was 38 years and maximum age was 85 years. The average age of the subjects was 58.4 years (Table 2). Following examination of the oral cavity, 53 (46.5%) cases showed erythema as the clinical sign of denture stomatitis. The oral cavities of all denture wearers (100%) were colonized with yeasts. Cultures of the oral mucosa and dentures of these subjects yielded 93 fungal colonies. Based on standard mycological methods, the most frequently isolated species was *C. albicans* with 45 isolates (48.4%), followed by *C. tropicalis* with 14 (15%), *C. krusei* with 9 (9.7%), *C. glabrata* with 6 (6.5%), *C. dubliniensis* with 4 (4.3%) and *Candida* spp. with 15 (16.1%). In accordance with many authors (11,12), we also found *C. albicans* as the most frequent species of *Candida* in individuals wearing dentures, especially in old persons. Higher prevalence of *C. albicans* might be attributed to the formation of biofilms on different surfaces of oral mucosa and dentures. In addition, rough surfaces of dentures in old subjects provide a larger surface area and a more sheltered environment for the development of plaque in vivo, since these surfaces are less able to be cleansed of micro-organisms. This was confirmed by Edgerton et al (13), who reported that *C. albicans* selectively adsorbs salivary mucins, statherin and proline-rich-proteins, facilitating its adherence to saliva coated acrylic resins. In addition, our data support this observation, similar to previous reports that *C. tropicalis* was the second predominant isolate recoverable from the oral mucosa

Table 2. Frequency distribution of gender and hygiene centers in relation to age

	Gender (No., %)		Hygiene center (No., %)		
	Female	Male	Norabad	Dehkabod	Golam
Age (y)					
20-40	4(4.3)	0(0)	0(0)	4(4.3)	0(0)
41-60	43(46.2)	7(7.5)	1(1.1)	29(31.2)	15(16.1)
≥ 61	29(31.2)	10(10.8)	4(4.3)	25(32.2)	15(16.1)
Total	76 (81.7)	17(18.3)	5(5.4)	58(62.4)	30(32.2)

of denture wearers (14). The high relatively prevalence of *C. tropicalis* might be attributed to its relative surface free energy value, since hydrophobic micro-organisms seem to be more adherent to acrylic surfaces. Recently, Vanden Abbeele et al (15) and Zomorodian et al (12) reported that *C. glabrata* was the second most prevalent species in healthy denture wearers.

The present study was also conducted to investigate the antifungal activity of the essential oil of *F. vulgare*, the alcoholic extract of *N. sativa* and the aqueous extract of *C. sinensis* against 39 different *Candida* species, such as *C. albicans*, *C. tropicalis*, *C. krusei*, *C. glabrata*, *C. dubliniensis* and *Candida* spp. The results obtained using the punch-hole method was reported as inhibition zones in Table 3. With the exception of the aqueous extract of *C. sinensis*, other plants showed remarkable antifungal activity against almost all of the tested *Candida* strains. Among them, the best anti-*Candida* activity was found for alcoholic extract of *N. sativa* (mean value: 12.3 mm), followed by essential oil of *F. vulgare* (mean value: 7.9 mm). The zones of inhibition ranged from 8 to 15.5 mm for *N. sativa* and 4 to 11.8 mm for *F. vulgare*. For *C. sinensis*, the fresh aqueous extract showed no antifungal activity against all tested *Candida* isolates (zone of inhibition: 0 mm). The alcoholic extract of *N. sativa* had the highest antifungal effect against *C. krusei* (mean value: 15.5 mm), while the lowest activity of the same plant extract was demonstrated against *C. albicans* (mean value: 8 mm), representing significant difference between *C. krusei* and *C. albicans* ($P < 0.05$). Furthermore, the highest and lowest activities of the essential oil of *F. vulgare* were related to *C. tropicalis* (mean value: 11.8 mm) and *C. krusei* (mean value: 4 mm), respectively, representing significant difference between *C. tropicalis* and *C. krusei* ($P < 0.05$). According to the literature, the investigation of natural products activity against *Candida* species increased significantly in the last 10 years, with the investigation of approximately 258 plant species from 94 families (16). Our findings in the present study showed the high anti-*Candida* potential of *N. sativa* as a natural source for the production of new antifungal drug. In line with this finding, several investigators revealed that *N. sativa* seeds and its active components significantly were able to inhibit the growth of various *Candida* species in models of in vitro and in vivo (17). As reported by Taha et al (18), thymoquinone (0.1 mg/mL) was the most potent active component of *N. sativa* against *Candida* species, followed by thymohydroquinone (0.5 mg/mL) and thymol (0.5 mg/mL). In a study conducted by Khosravi et al (19), the main changes of *N. sativa*-treated fungal cells were observed in the cell wall, plasma membrane and membranous organelles; in particular, in the nuclei and mitochondria. The essential oil of *F. vulgare* was the second most effective of tested plants, indicating the moderate activity on various *Candida* species. These recorded activities are in accordance with Naeini et al (20) and Park and Seong (21), who reported that *F. vulgare* has antifungal effect on *C. albicans*. Many studies have been carried out on chemical composition of *F. vulgare* essential. More than 80% of the essential oil components of *F. vulgare* were composed of trans-anethole, representing the main inhibitory effect on fungi (3,22). Interestingly, trans-anethole may act as a synergizing agent, increasing the effectiveness of some other phytochemicals against fungi, especially *Candida* species. For example, Himejima and Kubo reported that anethole synergized the antifungal activity of polygodial isolated from various plant sources against *C. albicans* (24). They demonstrated that the antifungal activity of polygodial against *C. albicans* was increased 32-fold by anethole. For this reason, we selected this herbal plant in combination with *N. sativa* for preparing the herbal mouthwash.

Green tea, *C. sinensis* is one of the most popular beverages and second to water in its popularity, with high daily consumption in Asia, especially in Iran. Several properties including antioxidant, anticaries, antibacterial, antiviral, antidiabetic, antimutagenic and antitumural properties are addressed for green tea. Its remedial effects are associated with the polyphenol contents comprising catechin, epicatechin and gallocatechin (25). In agreement with some previous studies (26), anti-*Candida* activity was not exhibited for *C. sinensis* in our study. Since *C. sinensis* has beneficial effects, such as flavoring agent, inhibition of the growth and cellular adherence of periodontal pathogens, and improvement of plaque induced gingivitis and inflammatory periodontal indices (27,28), we included it in the herbal mixtures despite its low antifungal activity.

In this study, we also assayed the anti-*Candida* activity of 3 herbal mixtures containing *N. sativa*, *F. vulgare* and *C. sinensis* at different concentrations. The diameters of inhibition zone were illustrated in Table 3. The results exhibited that all herbal mixtures were active against various tested *Candida* isolates, ranging from 7.8 to 15

Table 3. Antifungal susceptibility of *Nigella sativa*, *Foeniculum vulgare*, *Camellia sinensis* and their compound mouthwashes against various *Candida* strains isolated from individuals wearing dentures (mean, ranges)[a]

***Candida* isolate**	***N. sativa* (mm)**	***F. vulgare* (mm)**	**Mixture (mm)**		
			No. 1	No. 2	No. 3
Candida albicans (No. 12)	8(0-14)	5.8 (0-15)	7.8 (0-15)	7.6 (0-16)	7 (0-14)
C. dubliniensis (No. 4)	9.8 (0-15)	7 (0-11)	10.5 (0-23)	11 (0-25)	9.8 (0-21)
C. tropicalis (No. 6)	11.7 (8-16)	11.8 (5-20)	12 (0-26)	10.8 (0-21)	7.8 (0-16)
C. krusei (No. 4)	15.5 (14-17)	4 (0-7)	15 (13-19)	15.5 (13-18)	15 (12-18)
C. glabrata (No. 6)	14.5 (9-23)	10.4 (5-20)	14.2 (5-17)	15.7 (5-22)	13.2 (0-17)
Candida spp. (No. 6)	14.2 (0-25)	8.3 (0-15)	13.3 (0-18)	13 (0-19)	12.2 (0-16)

[a] *Camellia sinensis* showed no anti-*Candida* activity and its results were not illustrated in Table 3.

mm, 7.6 to 15.5 mm and 7 to 15 mm inhibition zones for herbal mixtures no. 1, 2 and 3, respectively. The highest inhibition zone was related to mixture no. 2 (mean value: 12.3 mm), followed by mixture no. 1 (mean value: 12.1 mm) and mixture no. 3 (mean value: 10.8 mm). Although lower concentrations of *N. sativa* along with higher concentrations of *F. vulgare* led to lower activity of herbal mixtures, but there were no significant differences in action of three herbal mixtures. The highest and lowest activities of the tested mixtures were seen against *C. krusei* and *C. albicans*, respectively. To our knowledge, there was a lack of sufficient evidence for antifungal effects of herbal mixtures. In the current study, the results were similar to previous studies (29,30), representing the inhibitory effects of *Salvadora persica* mixture and *Cuminum cyminum* mixture against *C. albicans* with an average inhibition zone diameter of 10.9 and 40 mm, respectively.

Conclusion

In conclusion, although *C. albicans* was the most frequently isolated species, our results also demonstrated a relatively high prevalence of *C. tropicalis*, *C. krusei* and *C. glabrata* in complete denture wearers. *N. sativa* and *F. vulgare* showed remarkable antifungal activity against various *Candida* species isolated from denture wearers. In addition, the results of this study indicated that all three herbal mixtures were active against all *Candida* isolates; especially *C. krusei* and *C. glabrata* which are intrinsically resistant to antifungal drugs. Clinical studies are needed to confirm the efficiency of in vivo application of these compounds.

Authors' contributions

AN and HS contributed to all the steps of experimental work, final approval of the study and final editing of the manuscript. SSS contributed to project management and final approval of the study. AD contributed to data analysis and manuscript draft preparation. AK and AA contributed to data collection, presenting patients for sampling and all the steps of experimental work.

Conflict of interests

The authors declare no competing interests.

Ethical considerations

Ethical issues (including plagiarism, misconduct, data fabrication, falsification, double publication or submission and redundancy) have been completely observed by the authors.

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