

Original Article

The Effectiveness of *Nigella Sativa* Alcoholic Extract on the Inhibition of *Candida Albicans* Colonization and Formation of Plaque on Acrylic Denture Plates: an *In Vitro* Study

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KEY WORDS

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ABSTRACT

Statement of the Problem: Due to growing concerns on complications of chemical drugs, the use of herbal extracts has been considered as denture cleaning solutions.

Purpose: The aim of this study was to evaluate the *in-vitro* effects of *Nigella sativa* on the cleansing of the formation of *Candida albicans* plaque on the acrylic resin pieces.

Materials and Method: In this experimental study, 30 pieces of acrylic resin were contaminated by *Candida albicans* suspension. Then, the acrylic pieces were randomly divided into six groups and treated with 0.2, 0.4, 20, and 200 mg/ml of *Nigella sativa*, 100,000 units of nystatin (positive control), and distilled water (negative control) for 8 hours. At the end of the exposure period of the drugs, the rinse solution from acrylic pieces was cultured in Sabouraud Dextrose Agar and the average of the colonies from each group was compared.

Results: The average number of colonies obtained at concentrations of 0.2, 0.4, 20, and 200 mg/ml of *Nigella sativa* were 122.6, 117.8, 73.4, and 14.4 colonies, respectively, as compared to distilled water (141.6) and nystatin (0) that had a significant difference ($p < 0.001$).

Conclusion: *Nigella sativa* extract at definite concentration is capable of clearing dental prosthesis, but compared to nystatin, it is weaker. However, due to the indirect immunoregulatory effects of *Nigella sativa*, it is suggested that other studies be conducted to investigate the therapeutic properties of *Nigella sativa* from the aspects of antimicrobial, anti-inflammatory, and oral ulcer healing in candida oral lesions.

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Introduction

Candida, as natural flora, lives in the oral cavity of 30 to 75% of healthy people [1-2]. Several host factors including oral health conditions, diet and prosthesis characteristics along with candida virulence factors such as protection against host defense mechanisms, adhesion ability to host tissue, production of proteolytic enzymes,

and phenotype switching [3] are related to the rate of colonization and pathogenesis of candida [4-7]. Oral candidiasis is one of the most common clinical forms of candida infection, which is closely related to the use of denture [8-9]. In various studies, the important role of candida in dental stomatitis has been shown [6, 10-11]. *Candida albicans* (*C.albicans*) is considered as the most

common cause of oral candidiasis (70-80%) and other candida species such as *Candida glabrata*, *Candida tropicalis*, *Candida parapsilosis*, *Candida krusei*, and *Candida dubliniensis* are ranked next in terms of medical importance [12-13]. *Candida* species have the ability to form biofilms on the surfaces of medical devices [11]. Dental plaque is one of the first known biofilm samples in medicine, which is formed through the heterogeneous community of microorganisms that is responsible for many oral infections and the formation of biofilm is known as the first and most important stage in the pathogenesis of dental stomatitis [14]. Despite many efforts, it is very difficult to prevent joining microorganisms to dental prostheses. In comparison with planktonic counterpart cells, sessile cells are often much more resistant to various antifungal drugs [14-15], and the formation of biofilms reduces the effectiveness of dental detergent solutions [16-18].

Various studies have shown that hygiene and cleansing of microbial plaque are necessary to prevent dental stomatitis [6, 19-23]. Tooth brushing is one of the most common methods for hygiene control of denture. However, because of the movement constraints of the elderly, the use of chemical methods is an appropriate alternative [14, 24]. This method is based on the use of commercial or household cleaners [25]. Some cleaners are not customer-friendly due to their high cost, damage to dentures, or side effects such as oral mucosal sensitivities [25]. A denture cleaner should be biocompatible, good antimicrobial properties and without damage to the denture structure and could be used efficiently, simply, and cost-effectively [26]. Therefore, the demand for new antimicrobial agents, especially herbal extracts has increased [27]. In recent decades, the evaluation of the antifungal activity of herbal extracts against candida has increased dramatically, as about 258 plant species from 94 families have been studied in recent researches [26, 28]. Some of these compounds have the acceptable antimicrobial properties. They can be used as good alternatives for synthetic drugs [26, 29-30]. Among this herbal extracts, *Nigella sativa* (*N. Sativa*) is commonly used in the Middle East, particularly in Iran [28].

N. Sativa is an annual flowering plant in the family of Ranunculaceae, also known as black seed and black cumin [31]. It has more than 100 different chemical compounds, which contain many sources of essential

fatty acids and often have medical use. Various active compounds such as thymoquinone, thymohydroquinone, dithymoquinone, thymol, carvacrol, nigellidine, and nigellicine are isolated from *N. sativa* [32]. Several biological activities have recently been described, such as antioxidant, anti-inflammatory, anti-cancer, antimicrobial, and immune-stimulant properties [32-35]. Various studies have suggested different uses of *N. sativa* extract in dentistry and its properties in periodontal diseases, dental caries, and oral ulcers have been considered [36-38]. Several studies have been carried out to evaluate the antifungal activity of *N. sativa*. Each of these studies had different goals or methods [26, 39-40].

Increasing concern about the side effects of chemical drugs and the ineffectiveness of some of the drugs in long-term use have led to more attention to herbal extracts as an alternative or complementary treatment. The aim of this study was to investigate the inhibitory effect of *N. sativa* extract on preventing the binding and growth of *C. albicans* on acrylic resin pieces in laboratory conditions.

Materials and Method

Manufacturing acrylic pieces

In order to make acrylic pieces, a thin layer of wax with the thickness of about 1.5 mm was prepared for flasking and after the wax removal step; it was replaced by acrylic resin (Bayer, Liechtenstein). The acrylic resin was then divided into similar pieces with the size of 10× 10× 1.5mm. Finally, all pieces were autoclaved (121°C, 20 min, 15 pounds). To avoid dehydration, it was stored in sterilized distilled water at 4°C.

Preparation of *N. sativa* alcoholic extract

In this study, extraction was carried out using soaking method. First, 200 g *N. sativa* was thoroughly ground using an electric mill and *N. sativa* powder was mixed with a 1000 ml of 80% ethanol inside a decanter (Simax, Germany). During the extraction period, the decanter was covered with aluminium foil to prevent unwanted chemical interactions of the plant components resulting from exposure to light. The extraction procedure was carried out by shaking or repeatedly stirring for three days at room temperature. Every 24 hours, the liquid inside the decanter was passed through Whatman filter paper No. 4 and again, 1000 ml of 80% ethanol was added to the *N. sativa* powder in the decanter. After

three stages of extraction, 2400 ml of *N. sativa* extract was obtained. It was then used to remove solvent from the rotary device (IKA, Germany) at 70°C. Finally, 200 mg of the extract was collected using this method and it was sterilized by a 0.45 µm filter. It was kept at 4°C until use.

Preparation of microbial suspension and contamination of acrylic pieces

C. albicans (ATCC 10231) was inoculated in Sabouraud Dextrose Broth (Pronadisa, Spain). Then, a concentration of 2×10^7 CFU/ml of yeast suspension was prepared using a hemocytometer lam. In order to form an experimental biofilm, all acrylic pieces were immersed in yeast suspension and incubated for 72 h at 37°C in 100 rpm shaking condition. Then, all resin fragments were rinsed three times with sterile distilled water for 5 minutes at 100 rpm, to remove unbound yeasts.

Exposure to test and control groups

All the contaminated resin pieces were randomly divided into six equal groups and were immersed in different drug solutions. The first four groups consisted of different concentrations of *N. sativa* solution (200, 20, 0.4, and 0.2mg/ml) and the next two groups contained 100000 units of nystatin (positive control) and sterile distilled water (negative control). Then, all the samples were stored in a shaker incubator (100 rpm) at 37°C for 8 hours. At the end of the incubation period, acrylic pieces were rinsed three times with sterile distilled water. Finally, by adding 1 ml sterile distilled water on each piece and sonication with sonicator device (Hielscher, Germany), 100 µl of the sonicated rinse solution of each piece was cultured separately on the Sabouraud Dextrose Agar medium and was kept at 37°C for 24 to 48 hours. Finally, by counting the number of yeast colonies isolated from each resin piece, the average number of yeast cells after exposure to drug solutions was estimated.

Statistical analysis of data

After determining the number of colonies in each group, the statistical distribution of the data was evaluated using the Kolmogorov-Smirnov test. As regards the normal distribution of data, analysis of variance was used to compare the number of fungal colonies in each group. Since the difference between the levels of fungal colonies was significant among the groups, Post hoc test was used for pair-wised comparison of the test groups. All

analyses were performed using SPSS software and for all tests, p-value of ≤ 0.05 was considered for statistical significance.

Results

In this study, the effects of various concentrations of *N. sativa* extract on the prevention of bonding and growth of *C. albicans* on acrylic resin components were evaluated and the results were compared with 100000-unit nystatin solution (positive control). The results of this study showed that *N. sativa* at low concentrations (0.2 and 0.4 mg/ml) had low antifungal effect against *C. albicans*. However, by increasing the concentration of *N. sativa*, the number of candida colonies decreased significantly. The average number of colonies obtained from the different groups is shown in Table 1.

Since the difference between the levels of fungal colonies among six groups was significant, post hoc test was performed for pair-wised comparison of the groups. The results of the test showed that *N. sativa* at concentrations of 0.2 and 0.4 mg/ml was not statistically significant ($p= 0.706$). On the other hand, the difference between each pair-wised groups was statistically significant ($p= 0.001$).

Discussion

The results of this study show that *N. sativa* extract has antifungal effects at definite concentrations, but it is weaker than nystatin. Although, *N. sativa* has shown better antifungal effects than distilled water at low concentrations, but the antifungal effect in low concentrations is not reliable. In the study of Khan *et al.* [26], the effect of *N. sativa* essential oil and a number of commercial solutions on cleansing of acrylic denture pieces were investigated and no significant difference was found.

Table 1: One-way analysis of variance to compare the values of fungal colonies for different groups

Group	Mean ± Standard deviation	ANOVA
Nystatin 100000 unit	0	
Distilled water	141.6±8.38	
*Nigella sativa (0.2 mg/ml)	122.6±6.38	F=634.94
*Nigella sativa (0.4 mg/ml)	117.8±6.72	p< 0.001
Nigella sativa (20 mg/ml)	73.4±2.70	
Nigella sativa (200 mg/ml)	14.4±2.07	

* The results of the test showed that *N. sativa* at concentrations of 0.2 and 0.4 mg/ml was not statistically significant ($p= 0.706$). On the other hand, difference between the each pair-wised groups was statistically significant ($p= 0.001$).

nd between the Fittydent commercial solution and herbal extracts. However, it was reported that *thyme* has a better antifungal effect than *N. sativa*, though; the difference was not statistically significant. In the present study, *N. sativa* alcoholic extract was used instead of essential oil. In addition, in the study of Fareid *et al.* [39], the essential oils of cinnamon and cloves had the most inhibitory effective among the six plant extracts studied, while the cumin and *N. sativa* had the lowest inhibitory effect. The results of these studies are consistent with the present study. Although, Fareid *et al.* [39] evaluated the antifungal activity of essential oils using broth microdilution method, whereas, in the present study the effect of *N. sativa* alcoholic extract on the inhibition of *C. albicans* colonization and formation of plaque on acrylic denture pieces was investigated. On the contrary, Sitara *et al.* [40] reported that *N. sativa* essential oil has a strong antifungal activity at a concentration of 0.15% against *Aspergillus*, *Fusarium*, *Drechslera*, and *Alternaria*. In the study of Naeini *et al.* [28], the antifungal effects of three combined herbal extracts including *N. sativa*, *Foeniculum vulgare*, and *Camellia sinensis* against candida were evaluated using agar dilution method. The results of their study showed that the herbal mixture was active against candida, and the alcoholic extract of *N. sativa* showed the highest antifungal effect against candida species. However, *N. sativa* had the most antifungal activity against *Candida krusei* and the least effect against *C. albicans* [40]. Therefore, in different studies, the antifungal effect of *N. sativa* extract has been investigated and various results have been reported. However, due to the inconsistency of the study method, the type of microorganisms and also the difference in extraction methods and the origin of *N. sativa* from different geographical regions, reports with different degrees of consistency have been recorded. As noted earlier, there are several reasons for the inconsistency of the results in different studies. Therefore, finding the best extraction method with the highest fungicide power requires more comparative studies. In addition, the extracts obtained from different geographical areas do not have the same components and compounds and their germicide effect is not the same, therefore, in order to commercialize the herbal extracts, the active ingredient of the herbal extract should be determined and considered as a measure of its

efficacy. In this regard, thymoquinone, thymohydroquinone and thymol have been introduced as the active compounds of *N. sativa* against candida, respectively [27, 41-42].

One of the characteristics of *N. sativa* is its immune regulation effects [35]. Khan *et al.* [43] showed that *N. sativa* extract at 6.6 ml/kg for 3 days had an inhibitory effect on candida growth in rat and they reported that aqueous extract of *N. sativa* had no direct antifungal effect and its antifungal effect was induced by stimulating the immune system. Therefore, the aqueous extract of *N. sativa* has a lower germicide effect in *in-vitro* conditions, while the antifungal effect of *N. sativa* has been proven in animal model studies [43-44]. Hence, it is recommended that the *N. sativa* extract, as a mouthwash solution, should be evaluated from different aspects such as antimicrobial, anti-inflammatory and healing oral ulcers.

Another parameter that influences the germicidal strength of cleaning solutions is the biofilm structure. It is clearly shown that sessile cells in the biofilm structure have more resistance to antimicrobial drugs or disinfectants, since the drug concentrations required to sessile cells are 5 to 8 times higher than planktonic counterpart cells and the effective concentration is 30 to 2000 times more than the minimum inhibitory concentration (MIC) [15]. Therefore, infections associated with dental biofilm are difficult to treat because they are resistant to host defense and antimicrobial therapy [11]. Mechanism of drug resistance of the biofilm structure is the poor drug penetration into the biofilm matrix, phenotype switching, and slow growth of microorganisms [14]. Considering that, in this study, the anti-candida effects of *N. sativa* extract on the experimental biofilm formed on the surface of acrylic resin plates were evaluated. It was predictable to obtain weaker results as compared to other studies that evaluated the effectiveness of *N. sativa* extract on planktonic cells.

One of the limitations of this study was the creation of experimental biofilms in the *in-vitro* conditions. Although *in-vitro* studies are inexpensive and suitable for antifungal tests [10-11], but the presence of protein in the host *in-vivo* conditions facilitate the formation of biofilms [14]. On the other hand, the role of saliva in connecting of candida to dentures is complicated; saliva has a cleansing effect due to immunosuppressive agents

such as lysozyme, lactoferrin, histatin and IgA [11, 45]. In addition, proteins in the saliva facilitate the attachment of candida to denture [28]. On the other hand, candida biofilm is composed of a mixture of different microorganisms in the *in-vivo* conditions [16], that can interfere with the binding and drug sensitivity [27, 46].

Low cost and easy access are the most important factors for choosing a drug as an antiseptic for artificial teeth, especially in the elderly [32]. In Iran, a species of *N. sativa* grows naturally and it is cultivated in some places in abundance, therefore, the extract of this plant can be prepared easily and at a low cost.

New methods in biotechnology have introduced changes in plant extracts, for example, the development of lipid nanostructures in these compounds improves the antimicrobial activity of plant extracts, which reduces the effective dose and side effects [47]. These nanostructures reduce the minimum inhibitory concentration of plant extracts by up to 9 times [2]. Moreover, considering the indirect effects of *N. sativa* due to stimulation of the immune system and its anti-inflammatory properties, it is suggested that other studies should be conducted to investigate the therapeutic properties of *N. sativa* in candida oral lesions.

Conclusion

It can be concluded that *N. sativa* extract at high concentrations can clear dental prostheses plaques; however, the commercialization of this plant compound requires further studies to evaluate the uncertainties surrounding the determination of effective dosage, biocompatibility, and its potentially damaging effects on the structure of dentures.

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Conflict of Interest

The authors declare that there is no conflict of interests.

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