The Journal of Medicinal Plants and By-Products

Original Article

The Inhibitory Effect of Alcoholic Extraction of *pimpinella anisum* against *candida albicans* Isolated from Gingivitis

Toleen Khalied Ibrahim[1], Widyan Alwan Khalaf[1] and Ali Ahmed Ali[2*]

[1] *Department of Field Crops- College of Agriculture-University of Samarra, Samarra, Iraq*
[2] *Department of Horticulture and Landscape Planning-College of Agriculture-University of Samarra, Samarra, Iraq*

*Corresponding Author: Email: ali.a@uosamarra.edu.iq

Article History: Received: 05 October 2024/Accepted in revised form: 07 November 2024

ABSTRACT

Anise, scientifically known as *Pimpinella anisum*, is a one-year herbaceous flowering plant commonly used as a spice, flavoring agent, and scent. Anise seeds have been extensively employed in traditional medicinal recipes throughout the Arab region, including Iraq. This study aimed to demonstrate the inhibitory effect of alcoholic extracts of *Pimpinella anisum* against *Candida albicans* isolated from patients with gingivitis. A cross-sectional study was conducted involving 120 samples from gingivitis patients aged 30 to 50 years, collected between September 2023 and July 2024. Samples were obtained using oral swabs from patients visiting a dental specialist center in Tikrit city and outpatient clinics, comprising both genders. Bacterial isolates were identified based on microscopic and macroscopic cultural characteristics, as well as biochemical tests. Additionally, a VITEK 2 compact system was employed for species-level identification. The results indicated that out of 120 samples, 80 tested positive for bacterial isolation while 40 tested negative. *Candida albicans* was isolated in 58.3% of the cases. The most common bacterial species causing infections were *S. mutans* (25%), followed by *Pseudomonas aeruginosa* (13%), *Staphylococcus aureus* (12%), and Proteus mirabilis and *Klebsiella pneumonia* (8%). The least common bacterial species isolated, accounting for 3%, included *Staphylococcus epidermidis*, *Acinetobacter lowffii*, and *S. pneumoniae*. In conclusion, *Pimpinella anisum* exhibits an inhibitory effect against *Candida albicans* and may serve as an alternative medicine in dentistry, potentially acting as an adjunct to conventional therapy for oral candidiasis.

Keywords: *Pimpinella anisum*, *Candida albicans*, Gingivitis, Alcoholic extraction

INTRODUCTION

For centuries, traditional medicinal herbs were used for nutrition and healing, playing an vital function in preserving human fitness and improving their life [1-3]. Aromatic herbs, like anise seeds, have a rich history of use in both traditional and modern medicine, including the pharmaceutical industry [4, 5]. Anise, scientifically referred to as *Pimpinella anisum*, is a type of herbaceous plant that completes its lifecycle within a 12 months. It is native to the Mediterranean place and is grown mainly for its fruits and seeds [6]. According to reports, the homeland of this species is Iran, India, and Turkey [7]. Anise seeds are used as a migraine reliever and possess carminative, aromatic, antiseptic, and diuretic properties in traditional medicine. Additionally, anise has strong antibacterial, antifungal, antioxidant, and antidiabetic characteristics [8].

Gingivitis and periodontitis are two conditions categorized under the broader term periodontal disease. This term encompasses a variety of conditions that impact the supporting tissues of the teeth [9]. Typically, one of the first indications of gingivitis is bleeding gums, which is a common symptom of the disorder [10]. If left untreated, gingivitis can progress to gingivitis, a condition characterized by deterioration of the attachment of the gums and alveolar bone, eventually leading to tooth loss. Gingivitis can be treated with antibiotics [11]. In recent years, there has been a growing fascination with infections caused by the opportunistic fungi Candida. The significance of candidiasis has increased, partly due to the rise in HIV infections and the broader use of immunosuppressive therapy. Identifying the specific Candida strains responsible for infections is crucial, as different isolates of Candida species vary significantly in their ability to cause disease and their susceptibility to antifungal drugs [12]. The goal of this research was to study inhibitory effect of *Pimpinella anisum* against *Candida albicans* isolated from patients with gingivitis

MATERIAL AND METHODS

Fundamental Study Plant Samples

The *Pimpinella anisum* seeds were obtained from local markets. The seeds were washed with distilled water to remove dust and microorganisms. They were then placed in a controlled environment at a temperature of 22-26°C, with continuous stirring to ensure proper drying and prevent rotting. Once dry, the seeds were crushed, milled, weighed, and stored in opaque glass containers. Each container was labeled with a piece of paper indicating the name of the plant and the specific plant part, and then stored in the laboratory until the extraction process was conducted.

Preparation of Alcoholic Extraction

The alcoholic extraction method described in the study involved mixing 100 g of plant powder with 500 ml of 80% ethyl alcohol, resulting in a weight-to-volume ratio of 5:1. The mixture was heated to a boiling temperature of 100 °C, then removed from the heat source before adding the plant powder. It was subsequently placed in a vibrating incubator at 37 °C for 24 hours. After incubation, the mixture was filtered through multiple layers of medical gauze and transferred to glass dishes lined with thermal nylon to preserve the quantity of the extract. The extract was then dried in an electric oven at 38 °C for three days, confirmed to be dry, and stored in dark bottles with tight lids in the refrigerator at 4 °C until needed.

Specimens Collection

A cross-sectional study analyzed 120 samples from patients aged 30 to 50 with gingivitis between September 2023 and July 2024. The samples were collected using oral swabs from patients of both genders attending a dental specialist center and outpatient clinics in Tikrit city. Bacterial isolates were identified based on their microscopic and macroscopic cultural characteristics, as well as biochemical tests. To ensure species-level identification, the VITKE2 compact system was utilized in addition to evaluating the biochemical characteristics of the isolates. Samples were collected after a dental examination conducted in the periodontal department.

Gingival Crevicular Fluid (GCF) Specimens

After removing the supragingival calculus with a sickle scaler, the dental pockets were prepared for collecting gingival crevicular fluid (GCF). The teeth were isolated with cotton rolls to prevent contamination from saliva and blood. Following the scaling, pocket depth was evaluated using a periodontal probe. A sterile absorbent paper point measuring 30-45 mm was then used to collect the GCF sample. Two paper points were inserted into a periodontal pocket with a depth of 3-8 mm and left in place for 30 seconds. After this duration, the paper points were carefully removed with sterile tweezers and placed in a sterilized Eppendorf tube containing 5 ml of thioglycolate broth. This broth serves as a reducing and transportation medium for anaerobic bacteria. The bacteria were subsequently cultured on blood agar and incubated in a 5% CO2 environment at a temperature of 35-37°C for up to 48 hours.

Swap from Mouth

Please take a mouth swab to isolate aerobic bacteria, using transport media (Amies) to keep it moist until it reaches the college laboratory. The swab should be cultured aerobically on Blood Agar, MacConkey Agar, Mannitol Salt Agar, and Sabouraud Dextrose Agar (SDA). Incubate the cultures at 37°C for 24 to 48 hours. Additionally, chocolate agar should also be used for incubation.

Biofilms Formation Assay

To assess biofilm formation, we utilized pre-sterilized 96-well polystyrene microplates, following the modified method outlined in (12). Yeast was introduced into a tube containing 2 ml of YPD broth (yeast peptone dextrose broth) with 1% glucose using a loop, and the mixture was incubated at 37°C for 24 hours. After incubation, 200 µl of the resulting solution was transferred to each well of the microplate. The microplate was then sealed with lids and incubated at 37°C for another 24 hours. Following this incubation, the medium in the wells was extracted and rinsed twice with sterile phosphate buffer (PBS). The wells were then turned upside down to remove excess liquid and allowed to dry. A crystal violet solution (0.1 g/100 ml) was added to each well for staining, and the plates were incubated for 20 minutes. After incubation, the plates were rinsed twice with PBS, turned upside down to dry, and left to air dry.Next, a 200 µl volume of an acetone:ethanol mixture (20:80 v/v) was added to

each well. After approximately 10 minutes, the absorbance results were measured at a wavelength of 450 nm using an ELISA reader. The tests were conducted in triplicate, and the means were calculated. The optical density (OD) of each strain was then compared with the average absorbance of the negative controls (ODnc), which represented the mean without any inoculation. Biofilm formation was classified as follows: no biofilm production (ODs = ODnc), poor biofilm production (ODnc < ODsP ≥ 2ODnc), moderate biofilm production (ODnc ≤ ODs ≤ 4ODnc), and vigorous biofilm production (ODs > 4ODnc).

Congo Red Agar Assay

The medium was prepared using the method outlined by Freeman et al [13]. Brain-Heart infusion broth (37 g), sucrose (50 mg), agar (15 mg), congo red (0.8 mg), and distilled water (900 ml). All components, except for congo red, were dissolved in 900 ml of distilled water and sterilized by autoclaving at 121°C for 15 minutes. After sterilization, the mixture was cooled to 55°C. Meanwhile, congo red was prepared separately by dissolving 0.8 g in 100 ml of distilled water and sterilizing it by autoclaving at 121°C for 15 minutes. This solution was then added to the cooled medium before being dispensed into sterile Petri dishes.

Statistical Analysis

Statistical analysis was conducted using either Pearson's chi-square test or Fisher's exact test, depending on the characteristics of the contingency table and the qualitative variables involved. Results were presented as mean ± standard deviation and percentages, with a p-value of less than 0.05 considered statistically significant.

RESULTS

The results are presented in Table 1 and Figure 1 for bacterial and fungal isolation. The VITEK 2 compact system was used to accurately diagnose all bacterial isolates. Out of 100 samples, 80 samples showed positive isolation for bacteria and candida, while 40 samples showed negative bacterial isolation. The most commonly isolated species causing bacterial infections were *S. mutans* (25%), followed by *Pseudomonas aeruginosa* (13%), *Staphylococcus aureus* (12%), Proteus mirabilis and *Klebsiella pneumoniae* (8%). The least commonly isolated bacterial species was *Staph. epidermidis*, *Acinetobacter lowifii*, and *S. pneumonia*, each representing 3% of the isolates. Additionally, 43.75% of the isolates were positive for *candida albicans.*

Table 1 Number and percentage of bacterial species isolated from gingivitis

Bacterial isolates	n	%
Streptococcus mutants	27	33.75
Pseudomonase aeruginosa	20	25
Staphalococcus aureus	11	13.75
Proteuse mirabilis(12)	15	18.75
Klebsiella pneumonia(35)	12	15
Staph. epidermis	6	7.5
Acinetobacter lowifii	3	3.75
S. pneumonia	12	15
Candida albicans	35	43.75
p-value	0.02	

In Table 2 and Figure 2 and 3, the results show that out of 35 *Candida albicans* samples, 19 (54.3%) displayed a strong biofilm when tested using the MTP method. Conversely, 16 (45.7%) samples showed a strong biofilm when tested using the CRA approach.

Table 2 Biofilm formation by *Candida albicans*

Number of isolation	MTP			CRA		
	Negative	Moderate	Strong	Negative	Moderate	Strong
Candida albicans (35)	5(14.3%)	11(31.4%)	19(54.3%)	7(20%)	12(34.3%)	16(45.7%)

*MTP: microtiter plate

*CRA: Congo red agar

The results presented in Figure 1 and Figure 2 indicate that *C. albicans* exhibited the largest inhibitory zone diameter at a concentration of 100%, with a diameter of 23. At concentrations of 75%, 50%, and 25%, the inhibitory zone diameters were 20.19, 18.32, and 10.32, respectively.

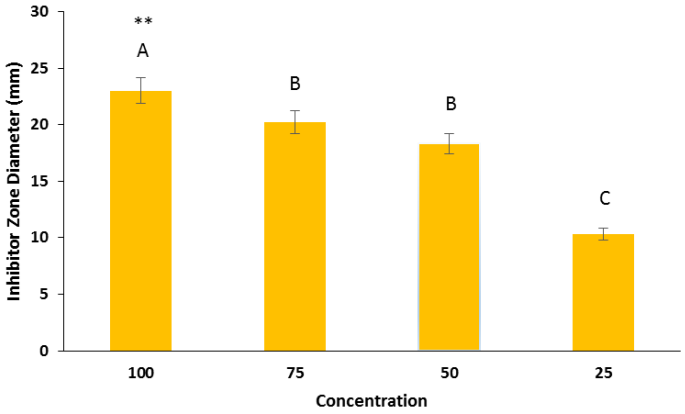

Fig. 1 The inhibitory effect of *Pimpinella anisum* against *C. albicans* with different concentration

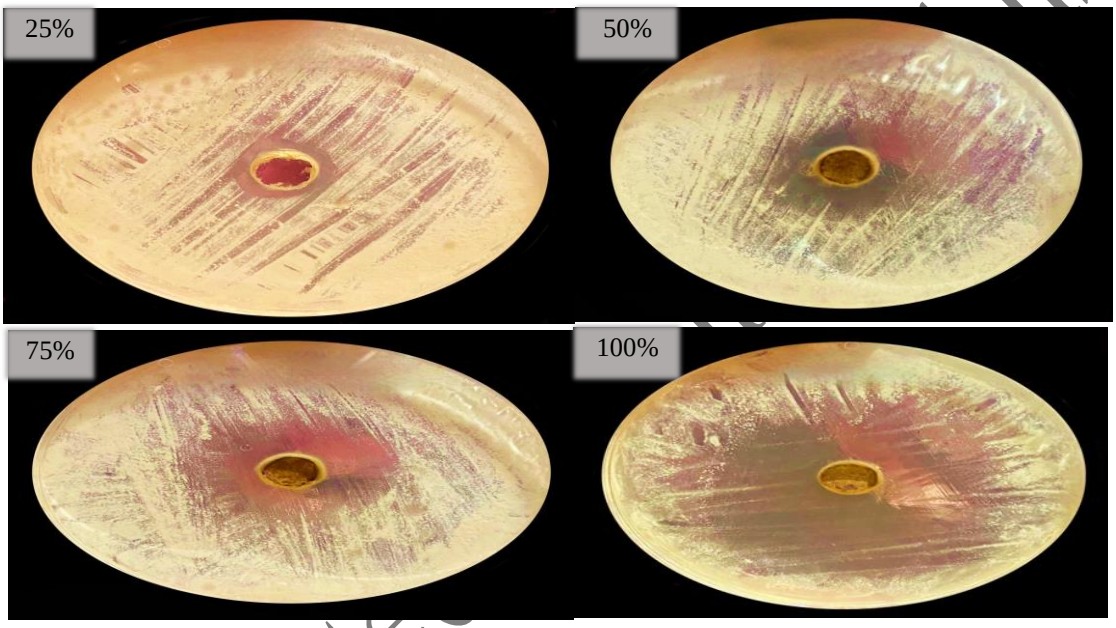

Fig. 2 The inhibitory effect of alcoholic extraction of *Pimpinella anisum* against *C. albicans*

DISCUSSION

The research findings indicate that a high percentage of *Candida albicans* was isolated from gingivitis patients, and 75% were isolated from the oral cavity of patients with periodontitis [14]. *Candida albicans* can act as a pathogenic microbe in patients with weakened immune systems, leading to the development of oral candidiasis and dental caries. This microorganism can cause each superficial and invasive infections, especially in people with weakened immune structures [15]. The transition of Candida spp. from an innocuous commensal organism to a pathogenic one is attributed to its virulent characteristics. These include its capacity to adhere to host tissue, invade, form biofilms, and secrete extracellular hydrolytic enzymes [16].

Studies show that for gingivitis, *S.mutans* is more common than other bacteria. According to references [17], the most frequently encountered vector species was *S. mutans* (18%), followed by *S. mitis* (13%) and S. aureus (12%) .*Staphylococcus aureus*, *Klebsiella pneumonia*, and *Acinetobacter lowifii* accounted for 3.57%, 10.72%, and 7.14% of the isolates, respectively [18]. This finding contradicts the results of (18), which showed that S. aureus was the predominant bacteria. Oral streptococci species, such as *S. mutans* and *Streptococcus mitis*, significantly impact the development of plaque above the gum line and dental cavities [19].

An alcoholic extraction of *Pimpinella anisum* has shown an inhibitory effect against *Candida albicans* at concentrations of 100%, more than 75%, 50%, and 25%. Other studies suggest potent antifungal activity against various microorganisms, including *Candida albicans*, with inhibition zones ranging from 10 to 26 mm, compared

to Clotrimazole using the agar diffusion method [20]. Basheer et al, mentioned that *Pimpinella anisum* contains a significant amount of anethole, which is an effective antifungal substance [21]. The inhibitory efficiency of the alcoholic extract of Pimpinella anisum on *C. albicans* yeast cells is due to the presence of multiple effective compounds within *Pimpinella anisum*, such as p-coumaric acid, catechin, oleuropein, gallic acid, caffeic acid, and others, which increase its effectiveness against various microorganisms [21]. Different mechanisms explain the effectiveness of the alcoholic extract of *Pimpinella anisum* in inhibiting *Candida albicans*, consisting of its potential to penetrate biofilms, exchange cell wall permeability, and adjust gene expression [22]. *Pimpinella anisum* is likewise characterized by using its antifungal and anti-biofilm results. The alcoholic extract of *Pimpinella anisum* has a greater inhibitory impact than the aqueous extract because of the natural compounds that dissolve higher in alcohol than in water [23]. Additionally, anise seeds are rich in chemical compounds that possess high-quality antioxidant, antibacterial, and antibiofilm properties [24]. These properties are due to the high concentration of primary ingredients, which possess harmful mechanisms that impact the entire bacterial cell. These mechanisms include disrupting cell walls, damaging membranes and proteins, and impairing the proton transfer system [25].

Limitations of the Study

One limitation of our study was the small sample size of the participants. Future research should aim to include a larger number of individuals for more comprehensive investigation.

CONCLUSION

This study explores the use of easily accessible medicinal plants, including *Pimpinella anisum*, in dentistry. These plants can serve as alternative treatments and supplements to traditional therapies, particularly in developing countries where economic resources and access to oral healthcare facilities are limited. The research was conducted in a laboratory setting outside of a living organism. The exact duration of contact between the extracts and *C. albicans* in vivo is uncertain. Further research is recommended to isolate the potentially active compounds of the plants.

Authors' contribution

Conceptualization: Toleen Khalied Ibrahim
Data curation: Widyan Alwan Khalaf
Formal analysis: Ali Ahmed Ali
Funding acquisition: Toleen Khalied Ibrahim
Investigation: Toleen Khalied Ibrahim
Methodology: Widyan Alwan Khalaf
Project administration:), Ali Ahmed Ali
Resources: Toleen Khalied Ibrahim, Ali Ahmed Ali
Software: Ali Ahmed Ali
Supervision: Widyan Alwan Khalaf
Validation: Widyan Alwan Khalaf
Visualization: Toleen Khalied Ibrahim
Writing–original draft: Toleen Khalied Ibrahim, Widyan Alwan Khalaf
Writing–review & editing: Toleen Khalied Ibrahim, Ali Ahmed Ali

Ethical Considerations

This study was conducted based on the ethical standards stipulated in the Declaration of Helsinki. The Ethics Committee of University of Samarra approved this study Before taking the sample. The patient's informed written and verbal agreement was obtained, after the review and approval of the study protocol and subject's information by the local ethics committee according to the document number 1152. Ethical issues (including plagiarism, data fabrication, double publication) have been completely Observed by the authors.

Conflicts of Interest

The authors declare that they have no competing interests.

Funding/Support
Non-Funding

REFERENCES
1. Ammar E.E., Aioub A.A., Elesawy A.E., Karkour A.M., Mouhamed M.S., Amer A.A., El-Shershaby N.A. Algae as Bio-fertilizers: Between current situation and future prospective. Saudi J Biological Sci. 2022; 29(5): 3083-3096.
2. Tabin S., Gupta R.C., kamili A.N., parray J.A. Medical and medicinal importance of Rheum spp. collected from different altitudes of the Kashmir Himalayan range. Cell. Mol. Biomed. Rep. 2022; 2(3): 187-201.
3. Fooladvand Z., Fazeli-nasab B. Antibacterial activities of Stachys lavandulifolia Vahl. extract against eight bacteria. J Herbal Drugs (An Int J on Med Herbs). 2014; 5(1): 13-18.
4. AbouZid S.F., Mohamed A.A. Survey on medicinal plants and spices used in Beni-Sueif, Upper Egypt. J Ethnobiology and Ethnomedicine. 2011; 7: 1-6.
5. Ahani H., Attaran S. Therapeutic potential of Seabuckthorn (Hippophae rhamnoides L.) in medical sciences. Cell. Mol. Biomed. Rep. 2022; 2(1): 22-32.
6. Günay A., Gümüşçü A. The Effects of Different Fertilizer Applications on Yield and Quality of Anise (Pimpinella anisum L.). Int J Agric Forestry and Life Sci. 2023; 7(1): 6-9.
7. Mohammed H.N. Broiler performance response to anise seed powder supplementation. Iraqi J Veterinary Sci. 2019; 33(1): 131-135.
8. Rodríguez-Hernández A.A., Flores-Soria F.G., Patiño-Rodríguez O., Escobedo-Moratilla A. Sanitary Registries and Popular Medicinal Plants Used in Medicines and Herbal Remedies in Mexico (2001–2020): A Review and Potential Perspectives. Horticulturae. 2022; 8(5): 377.
9. Jelavić S., Bajić Ž., Filipčić I.Š., Čulina I.J., Filipčić I., Aurer A. Periodontal status and the efficacy of the first-line treatment of major depressive disorder. Clinical and Experimental Dental Res. 2022; 8(1): 366-373.
10. Umeizudike K.A., Iwuala S.O., Ozoh O.B., Ayanbadejo P.O., Fasanmade O.A. Association between periodontal diseases and systemic illnesses: A survey among internal medicine residents in Nigeria. The Saudi Dental J. 2016; 28(1): 24-30.
11. Sanz M., D'Aiuto F., Deanfield J., Fernandez-Avilés F. European workshop in periodontal health and cardiovascular disease—scientific evidence on the association between periodontal and cardiovascular diseases: a review of the literature. European Heart J Supplements. 2010; 12(suppl_B): B3-B12.
12. Byadarahally Raju S., Rajappa S. Isolation and identification of Candida from the oral cavity. Int Scholarly Res Notices. 2011; 2011(1): 487921.
13. Freeman D., Falkiner F., Keane C. New method for detecting slime production by coagulase negative staphylococci. J Clinical Pathology. 1989; 42(8): 872-874.
14. Jepsen K., Falk W., Brune F., Cosgarea R., Fimmers R., Bekeredjian-Ding I., Jepsen S. Prevalence and antibiotic susceptibility trends of selected enterobacteriaceae, enterococci, and candida albicans in the subgingival microbiota of German periodontitis patients: a retrospective surveillance study. Antibiotics. 2022; 11(3): 385.
15. Nualmalang R., Thanomsridetchai N., Teethaisong Y., Sukphopetch P., Tangwattanachuleeporn M. Identification of pathogenic and opportunistic yeasts in pigeon excreta by MALDI-TOF mass spectrometry and their prevalence in Chon Buri province, Thailand. Int J Environmental Res Public Health. 2023; 20(4): 3191.
16. Vila T., Sultan A.S., Montelongo-Jauregui D., Jabra-Rizk M.A. Oral candidiasis: A disease of opportunity. J fungi. 2020; 6(1): 15.
17. Jaseem R.M., Yasin L.Q. Isolation and identification of some predominant bacteria and assessment of TNF-α level in serum of patients with gingivitis. Tikrit J Pure Sci. 2023; 28(5): 1-11.
18. Periferakis A.-T., Periferakis A., Periferakis K., Caruntu A., Badarau I.A., Savulescu-Fiedler I., Scheau C., Caruntu C. Antimicrobial properties of capsaicin: available data and future research perspectives. Nutrients. 2023; 15(19): 4097.
19. Larsen T., Fiehn N.E. Dental biofilm infections–an update. Apmis. 2017; 125(4): 376-384.
20. Abd-Elhafeez E., Ramadan B.R., Abou-El-Hawa S.H., Rashwan M.R. Chemical Composition and Antimicrobial Activity of Anise and Fennel Essential Oils. Assiut J Agric Sci. 2023; 54(2): 127-140.
21. Basheer M., Abdelshakour A.K., Mohamed M.K., Elshobky A.M., Abdelmoneam A.S., Makeen F.E., Eissa Z.M., Hossny H.H., Elemam S.N., Mahfouz E.N. Mini review: Anise (Pimpinella anisum L.): phytohemical and Pharmacological activities. ERU Research J. 2023; 2(4): 723-733.
22. Dumitrescu E., Muselin F., Tîrziu E., Folescu M., Dumitrescu C.S., Orboi D.M., Cristina R.T. Pimpinella anisum L. essential oil a valuable antibacterial and antifungal alternative. Plants. 2023; 12(13): 2428.
23. Gülçın İ., Oktay M., Kıreççı E., Küfrevıoğlu Ö.İ. Screening of antioxidant and antimicrobial activities of anise (Pimpinella anisum L.) seed extracts. Food Chem. 2003; 83(3): 371-382.

24. Kosalec I., Pepeljnjak S., Kuštrak D. Antifungal activity of fluid extract and essential oil from anise fruits (Pimpinella anisum L., Apiaceae). Acta Pharmaceutica. 2005; 55(4): 377-385.
25. Macwan S.R., Dabhi B.K., Aparnathi K., Prajapati J. Essential oils of herbs and spices: their antimicrobial activity and application in preservation of food. Int J Current Microbiol Applied Sci. 2016; 5(5): 885-901.