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Phenotypic Investigation of the Antimicrobial Effect of Organic and Hydro-Alcoholic Extracts of *Boswellia serrata* on Oral Microbiota

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A B S T R A C T

Objectives: Considering the emergence of resistant microbes and side effects of chemical drugs, in this study, the inhibitory effect of organic and hydro-alcoholic extracts of *Boswellia serrata* (*B. serrata*) on some oral microbiota was investigated.

Materials and Methods: In this experimental study, standard strains of *Candida albicans* (*C. albicans*; PTCC 5027), *Candida glabrata* (*C. glabrata*; PTCC 5295), *Candida krusei* (*C. krusei*; PTCC 5297), and *Streptococcus mutans* (*S. mutans*; PTCC 1688) were collected from the Iranian Research Organization for Science and Technology (IROST). Then, the minimum inhibitory concentration (MIC) of organic and hydro-alcoholic extracts of *B. serrata* was determined based on the CLSI protocol and using the micro-dilution method. The contents of each well were subcultured in Müller-Hinton agar (*Candida* species) and blood agar (*S. mutans*). The lowest concentration with no growth was considered as the minimum fungicidal concentration (MFC) or bactericidal concentration (MBC). Statistical analyses were performed using Mann-Whitney test.

Results: Hydro-alcoholic extract of *B. serrata* at the concentration of 50 mg/ml inhibited the growth of *C. albicans* and *S. mutans*. It also inhibited the growth of *C. krusei* and *C. glabrata* at the concentration of 100 mg/ml. Organic extract of *B. serrata* at the concentration of 200 mg/ml only inhibited the growth of *C. glabrata*.

Conclusion: Hydro-alcoholic extract of *B. serrata* had a greater inhibitory effect on *C. albicans* and *S. mutans* compared to the organic extract.

Keywords: *Candida albicans*; *Candida glabrata*; *Streptococcus mutans*; *Boswellia serrata*; Minimum Inhibitory Concentration

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INTRODUCTION

Fungal diseases had been rarely the cause of death in the past, but nowadays, due to the increase of immunocompromised patients, they have become a major life-threatening factor [1,2].

There are about 300 microorganism species in the oral cavity, 20 of which are Candida species. Among them, *Candida albicans* (*C. albicans*) is the most common type that can cause local or systemic infection, which if not treated, it can be the cause of mortality in patients [1-4].

Candida glabrata (*C. glabrata*) is the most common type of non-albicans Candida in neutropenic patients, which is often resistant to fluconazole [2]. Biofilm formation in urinary catheters, dental devices, and prostheses is another feature of the pathogenicity of this fungus [2]. *Candida krusei* (*C. krusei*) is a nosocomial agent primarily found in patients with immune deficiency and leukemia [5]. Mortality rates by non-albicans species have been reported to be ranging from 35% to 65% [4]. The lowest mortality rate has been associated with *Candida parapsilosis* (*C. parapsilosis*), whereas the highest rate has been associated with *Candida tropicalis* (*C. tropicalis*) and *C. glabrata* (40-70%). Other species, such as *C. krusei*, have an overall mortality rate similar to that of *C. albicans* (20-40%) [4]. Most fungal infections are treated with antifungal agents such as nystatin, amphotericin B, and azole compounds with complications such as the bad taste of nystatin, renal and hepatic toxicity with amphotericin B, and different effects of teratogens in the azole group [6].

Streptococcus mutans (*S. mutans*), is a Gram-positive, facultative anaerobic coccus that is another member of the human oral cavity microbiota. These bacteria are the most important cause of tooth decay. So far, many substances have been recommended to combat these bacteria, among them, chlorhexidine mouthwashes have shown the highest efficacy; however, long-term use of this mouthwash causes complications in the mouth [7-11].

Boswellia serrata (*B. serrata*) belongs to the Burseraceae family and Spindales order. The other name of this plant in English is Frankincense [11]. *B. serrata* has anti-inflammatory, anti-angiogenesis, anti-apoptosis, anti-tumor, anti-cancer, and anti-oxidant properties and regulates the immune system [12-22]. Oral prescription of this plant during breast-feeding increases the baby's memory power [23]. According to previous studies, *B. serrata* improves shortness of breath, bronchitis, and asthma attacks [24-28]. In addition, it has an anti-ulcer effect on the gastrointestinal system and it can lower blood sugar [24-28]. Due to the presence of acetyl-11-keto-β-boswellic acid (AKBA) in *B. serrata*, it has an anti-bacterial effect on oral pathogens and prevents bacterial biofilm formation [29,30]. This substance is used as a decay reducer agent in chewing gums [31]. The effects of this substance on some bacteria and fungi have been examined in different studies but its effect on *S. mutans* and Candida species, which are the most common oral microorganisms, has not been evaluated [32-36].

In a study by Schillaci et al [30], *B. serrata* essential oil prevented *C. albicans*, *Staphylococcus aureus* (*S. aureus*), and *Staphylococcus epidermidis* (*S. epidermidis*) biofilm formation [30]. Camarda et al [35] analyzed the antimicrobial effect of *B. serrata* essential oil on *C. tropicalis* and *C. albicans* and some Gram-negative and Gram-positive bacteria. Their results showed the antifungal effect of *B. serrata* with no antibacterial effect on tested Gram-positive and Gram-negative bacteria [35]. In contrast, in a study by Hasson et al [36], none of the organic and alcoholic extracts of *B. serrata* had efficacy against *C. albicans*. Therefore, performing more experiments on the effect of *B. serrata* on different types of Candida species is necessary.

In this study, the inhibitory effect of organic and hydro-alcoholic extracts of the Iranian *B. serrata* plant, which can dissolve both polar and non-polar substances, was evaluated against *C. albicans*, *C. krusei*, *C. glabrata*, and *S. mutans*, as different members of the normal flora of the oral cavity.

MATERIALS AND METHODS

B. serrata resin was prepared from Iran's pharmaceuticals market and scientifically identified at the Faculty of Pharmacy of Shahid Beheshti University of Medical Sciences, Tehran, Iran.

Preparation of B. serrata organic extract:

Soaking method in an organic solvent was used for extraction. For this purpose, 100 g of *B. serrata* powder was weighed accurately on a digital balance (GF300, Tokyo, Japan) in the pharmacognosy laboratory. Then, 2000 ml of propylene glycol was added as a solvent to an Erlenmeyer flask. The Erlenmeyer flask was completely covered with a piece of aluminum sheet and placed for 10 days in the dark. The flask was incubated for 24 hours on a shaker (Heidolph Unimax 2010, Schwabach, Germany) operating at 90 revolutions per minute (rpm). The solutions were purified using filter papers and Buchner funnel. To dry the extract, the purified solution was placed in a crystallizer container closed with a piece of porous aluminum sheet and kept at room temperature to dry.

Preparation of hydro-alcoholic extract of B. serrata:

For hydro-alcoholic extraction, a specified volume of *B. serrata* was weighed as described previously, and a certain volume of 96% hydro-alcoholic solvent was added to it. The resultant solutions were then placed on the shaker with low intensity for 48 hours. The solution was purified using Buchner funnel and dried. To eliminate the antibacterial effect of alcohol, the hydro-alcoholic extract container was left open for alcohol evaporation.

Microbial strains:

Standard microbial strains used in this study include *C. krusei* [Persian Type Culture Collection (PTCC) 5297], *C. albicans* (PTCC 5027), *C. glabrata* (PTCC 5295), and *S. mutans* (PTCC 1688), which were purchased from the Iranian Research Organization for Science and Technology (IROST).

Micro-dilution method for determining the minimum inhibitory concentration (MIC):

At first, *S. mutans* and Candida species were cultured on blood agar and Müller-Hinton agar media, respectively, to achieve fresh colonies. According to the CLSI, fungal and bacterial suspensions were prepared with turbidity equivalent to 0.5 McFarland's turbidity [1.5×10^8 colony-forming units (CFUs)/ml]. The optical density (OD) of 0.5 McFarland's suspension at a 625-nm wavelength was 0.08-0.1 [37]. Eight dilution series (200, 100, 50, 25, 12.5, 6.25, 2.125, and 1.56 mg/ml) were prepared. To provide these dilutions, eight capped sterile microplates, which contained 100 µl of Müller-Hinton broth, were prepared. Then, the organic extract of *B. serrata* was diluted using 2% dimethyl sulfoxide (DMSO; Cenavisa Co., Reus, Spain) solution to 200 mg/ml, and 100 µl of this concentration was added to the first well, and serial dilutions were prepared. Then, 10 µl of fungal and bacterial suspensions (1:20 dilutions) was added based on the CLSI protocol. The microplates were incubated for 24 hours at 37°C. The first clear well with no turbidity was considered as the MIC.

This experiment was repeated three times, and four microplates were considered for the four microorganism species. Overall, twelve microplates were used for three Candida species and *S. mutans*. The contents of the well with no growth were cultured on Müller-Hinton agar, and after 24 hours of incubation, the lowest concentration with no growth was considered as the minimum bactericidal (MBC) or minimum fungicidal concentration (MFC).

Preparation of nystatin and chlorhexidine dilution series as a control:

Working concentration of nystatin readymade solution (Sigma-Aldrich, catalog number: N-3503) was prepared based on the manufacturer's protocol and using 5 mg/ml DMSO to make a clear yellow solution [37]. Final 50 mg/ml stock suspension in water was prepared and stored at -20°C. The primary concentration of chlorhexidine was 0.2%. Serial dilutions of 0.2% chlorhexidine solution and nystatin were prepared using the same method previously described. The statistical analyses were performed using Mann-Whitney test.

RESULTS

In this study, the inhibitory effects of organic and hydro-alcoholic extracts of *B. serrata* on Candida species and *S. mutans* were analyzed in comparison with nystatin and chlorhexidine. The hydro-alcoholic extract of *B. serrata* at the 50 mg/ml concentration was able to inhibit *C. albicans* and *S. mutans*. In addition, at the 100 mg/ml concentration, the growth of *C. krusei* and *C. glabrata* were inhibited (Table 1). At the 200 mg/ml concentration, the organic extract of *B. serrata* only inhibited *C. glabrata* (Table 1; Fig. 1).

Table 1: Minimum inhibitory concentration (MIC) and minimum bactericidal (MBC) and minimum fungicidal concentrations (MFC) of hydro-alcoholic extract (HAE) and organic extract (OE) of *Boswellia serrata* (*B. serrata*) against the studied species

HAE & OE of *B. serrata* (mg/ml)	*C. albicans*	*C. krusei*	*C. glabrata*	*S. mutans*
MIC (HAE)	50	100	100	50
MBC & MFC (HAE)	100	200	200	100
MIC (OE)	NE	200	NE	NE
MBC & MFC (OE)	NE	NE	NE	NE

NE: No Effect

At a 0.06 mg/ml concentration, nystatin inhibited the growth of *C. glabrata* and *C. krusei*. At the 0.03 mg/ml concentration, it inhibited the growth of *C. albicans* (Table 2).

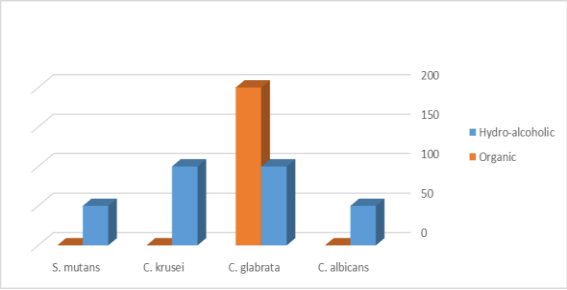

Fig. 1: Comparison of the effects of hydro-alcoholic and organic extracts (mg/ml) of *Boswellia serrata* (*B. serrata*) on the studied species

Chlorhexidine was able to inhibit the growth of *S. mutans* at a concentration of 0.003 mg/ml with a MIC and MBC of 0.003 and 0.006 mg/ml, respectively. However, it is routinely used as a 0.2% solution. The MICs of the organic extract of *B. serrata* compared to nystatin against the four strains were significantly different. The hydro-alcoholic extract of *B. serrata* showed a significant correlation with MIC reduction ($P<0.05$) but the organic extract did not show such correlation ($P>0.05$), except for *C. glabrata*.

Table 2: Minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) of nystatin against the Candida species

Nystatin (mg/ml)	*C. albicans*	*C. krusei*	*C. glabrata*
MIC	0.03	0.06	0.06
MFC	0.06	0.12	0.12

DISCUSSION

Candida is one of the common causes of infection, decreasing patients' quality of life. The emergence of Candida species resistant to common antifungal agents has been observed over the recent years, indicating the necessity of replacing alternative drugs. Due to the acceptable effects of herbal remedies and their fewer complications, they can be an adequate choice for replacing the commonly used chemical drugs [3].

In the current study, the inhibitory effects of hydro-alcoholic and organic extracts of *B. serrata*, nystatin, and chlorhexidine against Candida species and *S. mutans* was evaluated using the micro-dilution method. Based on the results, nystatin and chlorhexidine showed higher inhibitory effects on Candida species and *S. mutans* compared to different extracts of *B. serrata*, which is probably due to the use of pure nystatin and chlorhexidine in comparison with *B. serrata* impure extracts.

If *B. serrata* is fractionated and the effective ingredient is determined, a more precise comparison can be made with nystatin and chlorhexidine in future studies.

The higher inhibitory effect of the hydro-

alcoholic extract in comparison with organic extract of *B. serrata* can be attributed to its strong solubility [38]. Similarly, in the study by Hasson et al [36], the alcoholic extract was more effective than aqueous extract of *B. serrata*.

In a study by Raja et al [29], the antibacterial effect of *B. serrata* extract on oral cavity pathogens, such as *S. mutans* and *Actinomyces*, was analyzed. They showed that the alcoholic extract of *B. serrata* with the MIC of 2-4 µg/ml effectively reduced the growth of these bacteria [29]. Camarda et al [35] analyzed the effect of *B. serrata* essential oil on Gram-positive (*S. aureus* and *S. epidermidis*) and Gram-negative (*Escherichia coli* and *Pseudomonas aeruginosa*) bacteria as well as *C. tropicalis* and *C. albicans*. They showed that *B. serrata* essential oil with the MIC of 2.65-12.8 µg/ml effectively reduced the growth of *C. albicans* and *C. tropicalis* despite no inhibitory effects on the tested Gram-positive and Gram-negative bacteria [35].

Such a difference between the results of the current study and the cited study may be related to differences between the essential oil and the extract. Essential oils are more concentrated and rich than organic and hydro-alcoholic extracts [38]. Therefore, the higher MIC of organic and hydro-alcoholic herbal extracts, in comparison with essential oils, is justifiable. In addition, it must be noted that different climatic conditions, soil diversity, and harvest season may affect the amount of the effective ingredient of *B. serrata* in different regions of Iran and other parts of the world. In addition, differences in the evaluation methods and the age of the microbial culture may affect the MIC [37].

Sabra and Al-Masoudi [31] evaluated the effectiveness of *B. serrata* extracts in chewing gum on oral bacteria. The results of the mentioned study showed that after 1-5 hours of chewing gum with *B. serrata* extract, a significant reduction of such bacteria was detected. Consequently, the use of *B. serrata* extracts in chewing gum to reduce microbial concentration in the mouth is more effective than other medicines available in the market [31].

B. serrata has no toxic effect on human health and only a dose of more than 1 g/kg of it has shown toxicity in animal experiments [39]. Because of the low toxicity, there are probably no serious side effects in case of sudden swallowing of the mouthwash by children or disabled individuals [11-39].

The results of the current study, as well as the above-mentioned studies, show a new horizon to produce new effective products of *B. serrata* to combat the oral pathogenic microbes.

CONCLUSION

According to the results of the present study, hydro-alcoholic extract of *B. serrata* is more effective against *C. albicans*, *C. glabrata*, *C. krusei*, and *S. mutans* in comparison with the organic extract. Hydro-alcoholic extract of *B. serrata* was most effective against *C. albicans* and *S. mutans*. To achieve definitive results, fractionation of *B. serrata* extract is recommended to find the effective ingredient.

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CONFLICT OF INTEREST STATEMENT

None declared.

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