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Synthesis of Herbal Solution Based on Thymus Kotschyanus as an Antimicrobial Agent Against Enterococcus Faecalis and Streptococcus Mutans Isolated from the Periapical Cases and Comparison of its Antimicrobial Effect with Antibiotics and Chlorhexidine

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Thymus kotschyanus, antimicrobial effects, Enterococcus faecalis, Streptococcus mutans, Periapical infections, Chlorhexidine

Abstract

Background: Thymus kotschyanus is an natural essential oil with the boost antimicrobial effects. The present research was aimed to assess the antimicrobial effects of T. kotschyanus essential oil, chlorhexidine 0.2% mouthwash and antibiotic agents against Enterococcus faecalis and Streptococcus mutans isolated from the periapical cases in vitro condition.

Methods: Aerial parts of the T. kotschyanus were prepared and used for essential oil extraction. Antimicrobial effects were examined using the disk diffusion and MIC and MBC determination.

Results: The diameter of the growth inhibition zones had ranges between 4.17±0.19 to 11.90±0.24 mm. The highest diameter of the growth inhibition zone was recorded for E. faecalis treated with T. kotschyanus (10 mg/ml), while the lowest was found for S. mutans treated with T. kotschyanus (2.5 mg/ml). Statistically significant differences were not obtained between some concentration of the T. kotschyanus and chlorhexidine, ampicillin, amoxicillin, and metronidazole antibiotic agents (P >0.05). The MIC values had ranges between 2.5 to 5 mg/ml. The MBC values ranges between 5 to 10 mg/ml. The lowest MIC was identified for E. faecalis treated by T. kotschyanus essentiall oil (2.5 mg/ml).

Conclusion: Overall, in our study it was shown that the essential oil of thyme T. kotschyanus at a concentration of 10mg/ml.It has effective antimicrobial effects on clinical isolates of S. mutans and E. faecalis. However, the antimicrobial effects of T. kotschyanus essential oil were similar to chlorhexidine 0.2% and antibiotics such as ampicillin, amoxicillin, and metronidazole. But note It is important that the essential oil is natural and does not have any chemicals or side effects, which can be recommended as a mouthwash, especially for periapical infection patients

1. Introduction

Apical periodontitis infection is more common among adults (1). In most cases, apical periodontitis infections occur in teeth that have already had their roots filled (2). If apical periodontitis is not treated, the infection progresses and intraradicular infection, extraradicular infection, and the creation of oral cysts are associated (3). Microorganisms, especially bacteria, play an important role in the development of periapical ulcers, the consequences of which are the destruction of tooth bone tissue (4). Bone resorption is a progressive process carried out by osteoclasts. Bacteria can cause tooth decay, periodontal infection and damage to the dental pulp through tooth surfaces (5). Bacteria that have the ability to colonize in the root canal of the tooth cause pulp necrosis and inflammation in the tooth bone (6). Colonization of bacteria in different areas of the tooth depends on the degree of echogenicity of the bacteria in dealing with the defense system or antibiotic resistance of the bacteria. In case of stable colonization of bacteria in

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the tooth, it becomes very difficult to heal the periapical lesions. Bacterial invasion of the dental pulp leads to periapical abscess. The position of the pulp is inside the hard shell of the tooth, which consists of dentin and enamel. This hard coating around the pulp can protect it from infections caused by microbes in the mouth. With tooth decay, this external coating is lost and creates cavities that can expose the dental pulp to microbes (6). One of the important bacteria related to tooth root infection Enterococcus faecalis. Streptococcus mutans prefers to eat simple sugars. When you give them sugar, they eat it and produce acid. These acids change the pH of the mouth. This pH imbalance can lead to the leaching of calcium from your teeth. S. mutans the main cause is tooth decay (7,8). *S.mutans* creates an acidic and low-oxygen environment in the lesion. This causes tooth decay and attracts selected species of lactobacilli from food or oral flora (9). *S.mutans* is a Gram-positive and facultatively anaerobic cocci and survives well in anaerobic environments such as dental cavities and is the main cause of tooth decay (10). Tooth decay is caused by pathogenic bacteria. They break down sugars and carbohydrates in food and turn them into acids that destroy tooth enamel and cause tooth decay (11). Additionally, tooth decay is the most common cause of pulp infection. In the beginning of tooth decay, inflammation occurs in the sub odontoblastic region. Infectious products, such as lipopolysaccharides and lipoteichoic acid, invade the periapical region and lead to stimulation of the host's immune responses, which leads to bone destruction caused by apical periodontitis (12). Enterococcus faecalis is a gram-positive, facultative anaerobic coccus that is commonly found as a commensal of the human gut, but can colonize transiently in the oral cavity and cause periodontitis and persistent root canal infections (13,14). Recently, *E. faecalis* and *S.mutans* are resistant to many antibiotics, including ampicillin, amoxicillin, and metronidazole (15). These drugs are often used to treat dental infections (16). Thyme plant with many therapeutic properties is used as a reliever of rheumatic, joint and muscle pain, antibacterial and antifungal properties, treatment of body infections and prevention of inflammation and chronic diseases in traditional Iranian medicine. Recent studies have shown that thyme has properties It is very strong antibacterial (17). The important species of thyme include *Thymus vulgaris*, *Thymus daenensis, Thymus schimperi, Thymus zygis* and *Thymus kotschyanus* (18). Today, with the increase of antibiotic resistance among bacteria, the use of plants with medicinal properties to treat antibiotic-resistant bacteria has increased (19). In traditional medicine, different types of thyme are used as antibacterial, antiviral, and antacid drugs (20). Two of the most effective phenols in thyme oil are carvacrol and thymol, which have antioxidant properties (21). Chlorhexidine is one of the widely used mouthwashes used in dentistry to treat bacterial infections of the mouth and mouth ulcers. Chlorhexidine is effective on a wide range of Gram-positive and negative bacteria, as well as some fungi and some viruses. Chlorhexidine overdose has side effects that include mouth inflammation, urticaria, skin inflammation, dizziness, etc. Therefore, use Herbal mouthwashes can be a good substitute for chlorhexidine (22). Therefore, the purpose of the study, antimicrobial effect of *T. kotschyanus* against *E. faecalis* and *S. mutans* isolated from the peri-epical cases and comparison of its antimicrobial effect with antibiotics and chlorhexidine.

2. Materials and Methods

Extraction of the essential oil of T. kotschyanus

To obtain the essential oil of *T. kotschyanus*, the aerial parts of *T. kotschyanus* were obtained from the research farm of Isfahan city. The seeds of *T. kotschyanus* were completely dried. Then the plant is ground and the essential oil was extracted by ethanol distillation using a Cloninger machine. The essential oil was passed through a 0.45 micrometer microfilter using sodium sulfate and stored in a dark glass container at a temperature of 4 degrees Celsius and away from Sunlight was preserved. The prepared treatments included 0.2% chlorhexidine and *T. kotschyanus* essential oil with certain concentrations.

Bacterial isolation

Bacteria were isolated from the periapical cases who reffered to the hospital for routine check-up (Figure 1). Swab sample was used for this purpose. The collected samples were cultured on a blood agar medium incubated aerobically in a jar with a gas pack (Oxoid, England) at 37˚C for 24 h. The colonies were then sub -cultured and purified on Mitis Salivarius Agar (MSA) (HIMedia, India) for 24 h under anaerobic conditions at 37˚C. Isolates were identified based on many characteristics such as colony

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morphology on MSA, Gram stain, catalase test, growth in 6.5% NaCl, growth in 45˚C, and optochin sensitivity. Furthermore, the isolates were identified by Vitek 2 system using Vitek 2 Gram -positive identification card (GP) (bioMerieux, France).

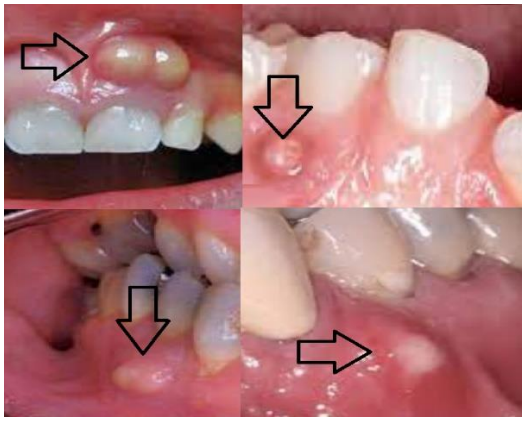

Figure 1. Some cases of periapical lesions, abcess, and infections included in the study.

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Investigation of antibiotic resistance

To prepare bacterial suspension, fresh bacterial colonies were transferred to Mueller Hinton Broth culture medium (Merck, Germany). The turbidity of the microbial suspension is based on the 0.5 McFarland standard and the absorption was measured at a wavelength of 630 nm (23, 24). Then, 8 different concentrations of essential oil (20, 10, 5 and 2.5 microliters) were used in the disk. The final concentration of essential oil by adding dimethyl sulfoxide included 20 microliters of each concentration. Dimethyl sulfoxide was used as a negative control in measuring the growth halo diameter and Minimum inhibitory concentration (MIC) and minimum bacterial concentration (MBC) were evaluated (24).

Preparation of MIC and MBC using disc diffusion method

To determine the diameter of the halo of non-growth for MIC and MBC of bacteria, the disc diffusion method was used (26-29). After the bacterial culture, sterile blanck discs with a thickness of 6 mm containing the concentrations of essential oils were used on the culture medium, then the culture medium was kept for16-24 h and incubated at a temperature of 37°C. A caliper was used to measure the diameter of the growth halos (30). To compare the effect of essential oils, 0.2% chlorhexidine mouthwash was used by disking method. MIC of essential oil was determined by microbroth dilution method. For each treatment in a 96-well microplate, 1 to 8 corresponding to dilutions of 20 to 0.16 microliters of essential oil were considered. Then it was incubated for 16-24 hours at 37 °C. After incubation, the amount of optical absorption of the microplate wells was read in an ELISA reader at a wavelength of 630 nm. In addition, the created turbidity was checked by visual examination of the wells. The lowest dilution of the tested substance was considered as MIC and the next well was considered as MBC. Each experiment was repeated three times (31,32).

Statistical analysis

Statistical analysis was performed using Minitab statistical software version 16 and comparison of means was performed using Duncan's test at 1% statistical level. The data obtained from the experiment were entered into the Excel program for statistical analysis. P value < 0.05 is considered a significant level (33-345).

3. Results

Disk diffusion

Table 1 demonstrations the growth inhibition zone of *T. kotschyanus* against *E. faecalis* and *S. mutans* isolated from the peri-epical cases and comparison of

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its antimicrobial effect with antibiotics and chlorhexidine. The diameter of the growth inhibitory zones was between 4.17±0.19 and 11.90±0.24 mm. The most growth inhibitory zones were obtained in *E. faecalis* bacteria treated with *T. kotschyanus* 10mg/ml, while the lowest and most zones were obtained. Growth inhibition in *S. mutans* bacteria treated with *T. kotschyanus* 2.5mg/ml was obtained. Statistically, there was no significant difference between the other dilutions of T. *kotschyanus* and the antibiotic agent's chlorhexidine, ampicillin, amoxicillin and metronidazole.

Table 1. The growth inhibition zone of *T. kotschyanus* against *E. faecalis* and *S. mutans* isolated from the peri-epical cases and comparison of its antimicrobial effcet with antibiotics and chlorhexidine.

Concentrations		**zone (mm)**	
		S. mutans	***E. faecalis***
T. kotschyanus	20	10.88±0.31 [a]	11.90±0.24 [a]
	10	7.21±0.14 [b]	8.29±0.27 [b]
	5	5.33±0.26 [c]	7.15±0.09 [c]
	2.5	4.17±0.19 [d]	6.30±0.41 [d]
Chlorhexidine 0.2%		9.75±0.42 [a]	10.55±0.18 [a]
Ampicillin		10.20±1.00 [a]	11.28±0.64 [a]
Amoxicillin		9.28±0.11 [a]	10.25±0.36 [a]
Metronidazole		9.12±0.34 [a]	10.55±0.63 [a]

*Dissimilar shows significant differences about P <0.05.

MIC and MBC results

In figures 2 and 3, MIC and MBC concentrations of T. kotschyanus and chlorhexidine are compared. MIC values were between 2.5 and 5 mg/ml. MBC values were between 5 and 10 mg/ml. The lowest MIC was detected for E. faecalis treated with *T. kotschyanus* essential oil (2.5 mg/ml).

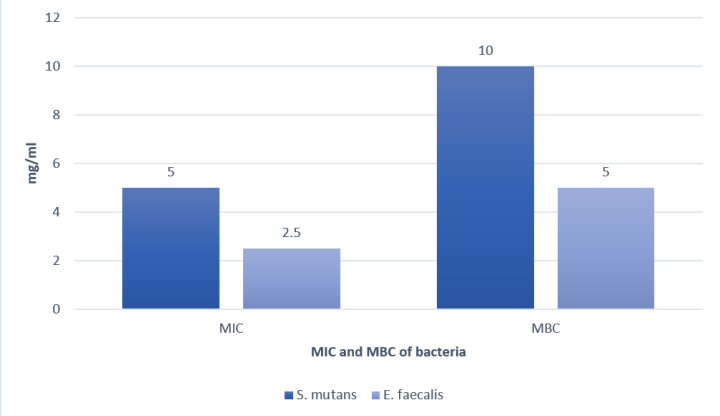

Figure 2. MIC and MBC concentrations of *T. kotschyanus* essential oil in contradiction of bacteria.

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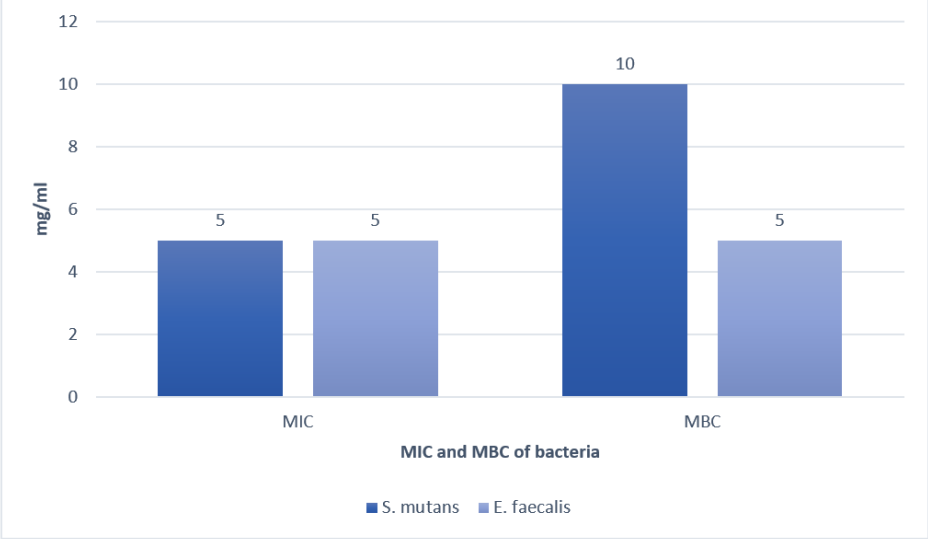

Figure 3. MIC and MBC concentrations of chlorhexidine in contradiction of bacteria.

4. Discussion

From a clinical view, it is important to assess the role of new methods in dental sciences (36-43). In this regard, several studies have been conducted to assess the microbial community of dental lesions and also suggested the use of natural essential oils as in treatment of microbial infections (44-56).

In the present study, antimicrobial effect of *T. kotschyanus* essential oil was tested against *S. mutans* and *E. faecalis* bacteria and then compared with ampicillin, amoxicillin, and metronidazole antibiotics and also chlorhexidine. Findings showed the higher antimicrobial effects of the *T. kotschyanus* essential oil against *E. faecalis*. Effective factors in the antimicrobial activities of herbal essential oils include the type of chemical compounds in essential oils, synergistic effects. It should be noted that the antibacterial mechanism of different essential oils is different from each other. In studies, it has been shown that herbal essential oils are effective against gram bacteria positive have more effect than gram-negative bacteria (57 ,58). The reason that gram-positive bacteria are sensitive to all kinds of plant killers can be related to the structure of the bacteria. In gram-negative bacteria, the presence of lipopolysaccharide (LPS) greatly reduces the penetration of hydrophobic compounds. The structure of LPS does not exist in gram-positive bacteria, and this causes the penetration of hydrophobic compounds into the bacteria. Of course, gram-positive bacteria have a strong peptidoglycan wall. Among the disadvantages of this layer, we can point out the low density against small molecules, which makes it easy for antibiotic compounds to penetrate through the bacteria (59). Also, gram positive bacteria can reduce the release of hydrophobic compounds of essential oils due to lipophilic acid lipophilic end units (60). In a previous study, the strong effects of *T. kotschyanus* essential oil on a variety of bacteria including *Staphylococcus aureus*, *Escherichia coli* (61), *Streptococcus*.spp (62), *Salmonella. typhimurium* and *Pseudomonas aeruginosa* (63) were shown. Interestingly, the effect of plant essential oils on bacterial strains with multidrug resistance (MDR) including *Acinetobacter baumannii, P. aeruginosa, and S. aureus* was shown (64). Edible nature of *T. kotschyanus* essential oil and its high antimicrobial effects, make it safe to use a dental antimicrobial agent, especially for the treatment of periapical cases.

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5. Conclusion

Overall, in our study it was shown that the essential oil of thyme *T. kotschyanus* at a concentration of 10mg/ml.It has effective antimicrobial effects on clinical isolates of *S. mutans* and *E. faecalis*. However, the antimicrobial effects of *T. kotschyanus* essential oil were similar to chlorhexidine 0.2% and antibiotics such as ampicillin, amoxicillin, and metronidazole. But note It is important that the essential oil is natural and does not have any

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chemicals or side effects, which can be recommended as a mouthwash, especially for periapical infection patients.

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