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Original Article

A Comparison of Antibacterial Effects of Licorice Root Ethanolic Extract, Chlorhexidine, and Doxycycline on Fusobacterium nucleatum: An in Vitro Study

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Abstract

Background and Aim: Antibiotics and mouthwashes which are used to prevent and treat periodontal diseases have side effects such as antibiotic resistance, mouth burning, and xerostomia. With the advancement of technology, plants have been considered as alternative antibacterial agents. Licorice plant with different species has been used in traditional medicine to treat gastritis and respiratory diseases. Considering the properties of licorice in traditional medicine, this study aimed to investigate the antibacterial properties of licorice root extract with different concentrations compared with Chlorhexidine mouthwash and Doxycycline antibiotic on *Fusobacterium nucleatum* in vitro.

Materials and Methods: After the preparation of 0.5 McFarland bacterial suspension, the plant and its ethanolic extract, and six extract dilutions were also prepared. Agar disk diffusion and broth microdilution tests were carried out against *Fusobacterium nucleatum* ATCC 2558. The antibacterial effect of Chlorhexidine mouthwash 0.2% and Doxycycline 100 mg antibiotic were also determined to be compared with licorice extract. The data were analyzed using Mann-Whitney and Kruskal-Wallis statistical tests in spss26 software at a significance level of 5%.

Results: All the six concentrations had significant antibacterial effects compared with each other, chlorhexidine and doxycycline (P-value<0.05). The inhibitory concentrations of extract, MIC50, MIC70, and, MIC90 were related to 12/5, 50, and 200 mg/ml, respectively. The inhibitory percentages of chlorhexidine and doxycycline were 67.6% and 88.7%, respectively.

Conclusion: Licorice ethanolic extract exhibited an excellent antimicrobial effect (MIC=6.25mg/ml), so that in concentrations higher than 25, a greater antimicrobial effect was observed than in chlorhexidine (P<0.05).

Keywords: Antibacterial agents, Fusobacterium nucleatum, Glycyrrhiza glabra extract

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Introduction

Various substances such as mouthwashes, toothpaste, and antibiotics are used to maintain oral and dental hygiene, and consequently prevent and/or treat periodontal diseases. Chlorhexidine mouthwash has been introduced as the gold standard. However, it has various side effects such as causing tooth discoloration, changing the sense of taste, mouth burning, and xerostomia (1-3). Periodontal diseases are among the most common multifactorial polymicrobial infectious diseases that cause progressive destruction in the periodontal ligament and alveolar bone, with an increase in probing depth (4-7). Gram-negative and anaerobic bacteria such as Actinomyces, *Prevotella intermedia*, *Porphyromonas gingivalis*, and *Fusobacterium nucleatum* play effective roles in periodontitis (8,9). *Fusobacterium nucleatum*, an anaerobic, spindle-shaped gram-negative rod is a common component of the subgingival flora in periodontitis and periodontal abscess. In the subgingival ecosystem it can facilitate coaggregation between different species (10). Today, due to side effects of chemical drugs, plants have been considered as alternative antibacterial remedies. The licorice plant, with the scientific name, Glycyrrhiza glabraL., is one of the perennial herbaceous plants with different species such as typical, glandolifera, vilosa, and uralensis. Licorice plants are distributed in southern Europe, Iran, Russia, Afghanistan, Syria, Pakistan, and India. In Iran, it is abundantly found in almost all regions of the country's northeast, west, and center. Licorice root has different compounds such as sugars, flavonoids, sterols, amino acids, and saponins. Glycyrrhizin acid, the main saponin with mineralocorticoid activity, is used in the treatment of rheumatism, inflammations, and Addison's disease. Moreover, this plant has been used in traditional medicine of Asia and Europe to treat gastritis, respiratory infections, and peptic ulcers (11-17).

There are few studies about the antimicrobial effects of this plant and its comparison with chlorhexidine and doxycycline on periopathogenic bacteria. For example, in a clinical trial on 104 patients, Jain *et al*. investigated the efficacy of licorice mouthwash versus 0.2% Chlorhexidine mouthwash. The results showed that both types of mouthwash were effective in improving the condition of plaque. However, Chlorhexidine has shown a higher degree of efficacy in the clinic. Licorice mouthwash also reduced bleeding on probing (18). In 2020, Sidhu *et al*. showed that polysaccharides in licorice could prevent Porphyromonas gingivalis from attaching to bacterial plaque. Moreover, it was effective in preventing the early stages of infection in children (19). Thus, due to the properties of licorice in traditional medicine and the lack of sufficient studies on periopathogens, we decided to investigate the antibacterial effects of licorice root extract with different concentrations compared with the Chlorhexidine mouthwash and Doxycycline antibiotic on *Fusobacterium nucleatum* in vitro.

Materials and Methods

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Preparation of the Licorice Root Ethanolic Extract

The licorice plant (Glycyrrhiza glabraL.) was obtained from the agricultural land of Ardestan, Iran, and the authenticity of the plant was confirmed by the Agricultural Organization.

Plant extraction was done in the Pharmacy Faculty, Isfahan University of Medical Sciences. The dried powder of licorice root (200 gr) was poured into a glass desiccator, and 1000 ml of 70% ethanol was added to it. Then, it was soaked for 7 days, and extraction was done using the maceration method. Extraction was repeated 3 times. The obtained extract was filtered with filter paper, and the final extract was concentrated in a rotary device. The average of three repetitions of the test was considered as the dry weight of the extract(33.21gr) (20, 21).

The amount of 200 mg of the dried extract was dissolved in 1 ml of DMSO solvent. This concentration (200 mg/ml) was used as the main dilution of the extract (or 100% dilution). Subsequently, using the culture medium, subsequent dilutions of the extract were also created.

Preparation of Standard Bacterial Suspension

The *Fusobacterium nucleatum* (ATCC 2558) was purchased from the Faculty of Pharmacy, Isfahan University of Medical Sciences. For growth and initial

preparation, blood agar culture medium containing 5 mg/ml -hemin and 10 µg/ml vitamin K and an anaerobic incubator (N2, 80%; H2, 10%; CO2, 10%) were used.

To perform the antimicrobial tests, a standard suspension of bacteria equivalent to the turbidity of half McFarland's suspension was prepared in physiological serum, containing 1.5×10^8 CFU/ml with optical absorption between 0.08 to 0.13 at 625 nm.

Investigation of Antimicrobial Activities

1. Disc Diffusion Method on Agar

From each of these 6 prepared dilutions of the extract (100, 50, 25, 12.5, 6.25, 3.125), 20 µl were inoculated into empty sterile discs. The discs were placed at 37˚C until they were completely dried and prepared for disc placement. The standard suspension of bacteria (100 µl) was inoculated on Muller Hinton agar medium containing 5% blood, and the prepared discs were placed on the inoculated culture medium at a suitable distance and were incubated for 24 hours in a CO2 incubator at 37°C. Then, the plates were examined for the presence of a growth inhibition halo. The diameter of the growth inhibition halos around the discs was measured by a millimeter ruler. To compare the antimicrobial properties of the extracts with antibacterial substances, this test was also performed with 0.2% Chlorhexidine and 100mg Doxycycline antibiotic.

2. Microdilution Broth Method (dilution in liquid culture medium)

The sensitivity of *Fusobacterium nucleatum* to the licorice root extract was also investigated using the microdilution broth method in BHI broth in 96-well microtiter plates. 100 µl of culture medium, 100 µl of each of six dilutions (3.125, 6.25, 12.5, 25, 50, 100%) of the extract along with 20 µl of bacterial suspension, equivalent to 0.5 McFarland were added to the wells of each column of the microtiter plate. The positive control wells (wells of the first row of the plate) contained only the culture medium (100 µl), bacterial suspension (20 µl), and 100 µl of physiological serum instead of the extract. The negative control wells (wells of the last row of the plate) contained only culture medium (100 µl), sterile physiological serum instead of bacterial suspension (20 µl), and 100 microliters of the extract.

Moreover, 0.2% Chlorhexidine and 100 mg Doxycycline antibiotic were used for comparison. In this way, in each group, there were 100 µl of each of these substances along with 100 µl of culture medium and 20 µl of the bacterial suspension. The contents of each well were mixed for 2 minutes, and the optical densities of each well were measured at 620 nm by an ELISA Reader (Dana brand, made in Iran) equipped with a shaker, after 18 hours of incubation in anaerobic condition. This experiment was performed in three separate replications.

Finally, the average optical density of positive control wells and the wells under the influence of antimicrobial compounds were compared, and then the inhibitory percentage of each was determined.

The percentage of bacterial growth inhibition in the presence of different concentrations was calculated as follows:

$$X=100-(OD_T \times 100)/ODc)$$

OD_T: the average optical density of the wells of each group

OD_C: the average optical density of positive control wells

Finally, the concentrations of MIC[1]50, MIC70, and MIC90 were also determined as the concentrations in which more than 50%, 70%, and 90% of the bacterial growth are inhibited, respectively.

Statistical Analysis

Data analysis was carried out at two descriptive and inferential levels. At the descriptive level, the mean, standard deviation, and statistical charts were used, and at the inferential level, due to the non-normality of the data distribution, non-parametric statistical methods, including the Kruskal-Wallis and Mann-Whitney tests, were used. The data were analyzed in SPSS 26 software at a significance level of 5%. GraphPad Prism 8 software was also used to draw the graphs.

Results and Discussion

In this study, licorice root ethanolic extract in 6 concentrations, 0.2% Chlorhexidine, and Doxycycline (100 mg) antibiotic were used. The antibacterial effects of the mentioned substances on *Fusobacterium*

[1] . Minimum inhibitory concentrations

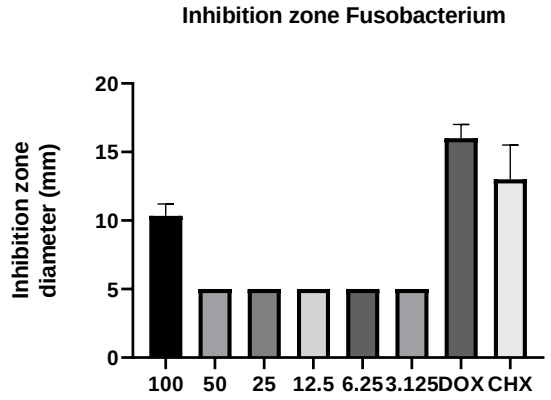

Figure 1. Growth Inhibition Halo Diameter (mm) of *Fusobacterium nucleatum* in the Presence of Different Dilutions of Licorice Root Ethanolic Extract, Doxycycline (DOX) and Chlorhexidine (CHX).

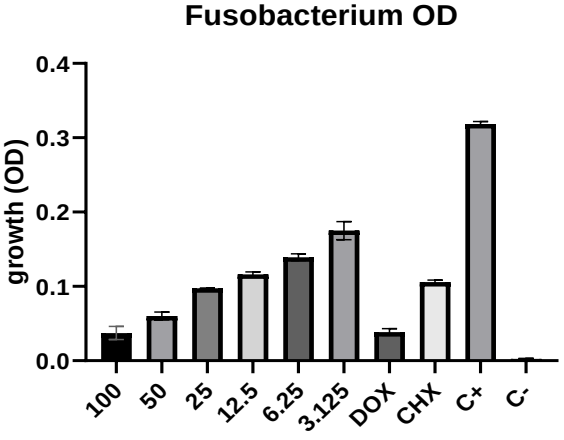

Figure 2. Optical Density, an Indicator of *Fusobacterium nucleatum* Growth in the Presence of Different Dilutions of Licorice Root Ethanolic Extract, Doxycycline (DOX), and Chlorhexidine (CHX).

nucleatum were investigated with two qualitative tests, i.e. evaluation of the growth inhibition halo and microdilution broth. Based on the results of comparing six concentrations with each other and with Chlorhexidine as well as Doxycycline, all the licorice concentrations had significant antibacterial effects. The result of the *Fusobacterium nucleatum* growth inhibition halo test has been presented in Figure 1, the optical density test result has been shown in Figure 2, and finally, the percentage of growth and growth inhibition in the presence of antibacterial agents has been presented in Figure 3. The minimum bactericidal concentration (MBC) was not achieved in any of the

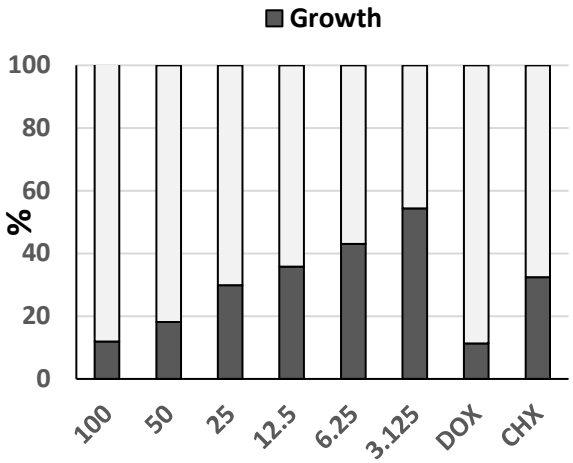

Figure 3. The Percentage of Growth and Non-Growth of *Fusobacterium nucleatum* in the Presence of Different Dilutions of Licorice Root Ethanolic Extract, Doxycycline (DOX), and Chlorhexidine (CHX).

licorice concentrations for this bacterium. The minimum inhibitory concentration was reported as MIC50, MIC70, and MIC90, or the minimum inhibitory concentrations of 50%, 70%, and 90% of the bacteria; which were 12.5, 50, and 200 mg/ml, respectively.

Mann-Whitney test was used to compare pairs of means, and Kruskal-Wallis test was used to compare several groups with another one. The results of the Mann-Whitney test for the comparison of pair groups for both components of optical density and growth inhibition halo showed that the P-value for optical density was equal to 0.001, and 0.002 for the growth inhibition halo component which is less than 0.05. Thus, the statistical null hypothesis (equality of means) is rejected.

Moreover, the results showed that there was a significant difference in the growth inhibition halo test between all the groups with a concentration of 200 mg/ml, except for chlorhexidine(P=0.346), and this difference regarding the optical density component was not significant for doxycycline (P=1).

Periodontal disease is the inflammation in tooth-supporting tissues, and multiple bacteria are involved in its pathogenesis, including *Fusobacterium nucleatum*, which is one of the etiological factors (22). To control and treat periodontal diseases, antibiotics such as Doxycycline and mouthwashes such as Chlorhexidine were used, and each of them had unfavorable side

effects. The use of Chlorhexidine, which is known as the gold standard; despite its high antimicrobial properties, can lead to changes in teeth color and the sense of taste in patients (23). Moreover, the widespread antibiotic resistance caused by the indiscriminate use of antibiotics has recently drawn attention to herbal medicines due to their fewer side effects and multiple properties. Essential oils and plant extracts have exhibited significant microbicidal and inhibitory effects against pathogenic agents, which can replace chemical substances (24, 25).

The aim of the present research was to investigate the antimicrobial effect of licorice root ethanolic extract at different concentrations of 6.25 to 200 mg/ml on the *Fusobacterium nucleatum* in vitro. Therefore, in this research, we used two methods, i.e. the disc diffusion method on agar, and comparing the growth by examining optical density with the microdilution broth method in the presence of different concentrations of licorice root ethanolic extract, 0.2% Chlorhexidine mouthwash (CHX) and Doxycycline (DOX) 100mg antibiotic.

The investigation of the diameter of the growth inhibition halo showed that inhibition was observed only in the presence of 200mg/ml of extract, CHX, and DOX. No statistically significant difference was observed between the effect of this concentration of the extract and Chlorhexidine (P-Value=0.376); while a significant difference was observed with regard to Doxycycline (P value=0.046). In other words, Doxycycline had the greatest antimicrobial effect. The next level was observed for Chlorhexidine, and the concentration of 200 mg/ml had an equivalent effect.

In the microdilution broth method, which was investigated in 96-well microtiter plates, all the extract concentrations had significant differences from the control group. According to the average of the variables, concentrations of 200 and 100 mg/ml, showed better antibacterial effects than Chlorhexidine, and based on statistical analysis this difference was significant ($p<0.05$). At concentrations of 50mg/ml, the effect of the extract was almost equal to Chlorhexidine (p value=0.05). 100 mg/ml concentration had almost the same performance as Doxycycline and did not show any statistically significant difference ($p>0.05$). However, in 50mg/ml concentration, the performance of Doxycycline was better and a significant difference was observed ($p<0.05$). The minimum microbicidal concentration was not achieved in any of the extract concentrations. The minimum inhibitory concentration was reported at about 50%, 70%, and 90%, with MIC50, MIC70, and MIC90 titles in 12.5, 50, and 200mg/ml, respectively (6.25, 25, and 100% dilutions) (Figure 3).

Ajagannanavar *et al*. investigated the antimicrobial effects of aqueous and alcoholic extracts of licorice compared with Chlorhexidine on *Lactobacillus acidophilus* and *Streptococcus mutans* bacteria. Contrary to the results of this research, the diameter of the growth inhibition halo in the presence of licorice alcoholic extract was greater than the aqueous extract and CHX. This difference is definitely due to the distinction in the intrinsic characteristics of the bacteria and the concentration of the alcoholic extract (250 compared with 200 mg/ml).

In the study conducted by Ajagannanavar, the alcoholic extract was effective, particularly for *Lactobacillus acidophilus* bacteria up to the concentration of 6.25 mg/ml which is consistent with the results of our study (26). Markus *et al*. (2020) examined three isoflavones from licorice (Glabridin, Licoricidin, and Licochalcone A) on endodontic pathogens, including *Fusobacterium nucleatum*, compared with Chlorhexidine. The results showed that the MIC levels for the mentioned compounds were equal to 12.5, 25 and 12.5 µg/mL, respectively, and for Chlorhexidine it was equal to 1.95 µg/mL; which shows that the effect of Chlorhexidine was greater than licorice compounds. In our study, in low concentrations, Chlorhexidine performed better. Hence, the results of this study are in line with those of our study (27). In a clinical trial on 104 patients, Jain *et al*. investigated the efficacy of licorice mouthwash versus 0.2% Chlorhexidine mouthwash. The results showed that both types of mouthwashes were effective in improving plaque condition. However, Chlorhexidine has shown better behavior in the clinic (18). In a clinical trial on 45 patients with chronic periodontitis, Mahmudpourmoteshakker et al. compared the effect of licorice tablets with the Doxycycline antibiotic and showed that the amount of PD, CAL, and BOP significantly decreased after treatment in all the three groups (7). Farhad *et al*. (2013) compared the therapeutic effects of Doxycycline and licorice on the level of MMP-8 in gingival crevice fluid

in patients with chronic periodontitis. They found that licorice could inhibit the matrix metalloproteinases production by host cells, and had a therapeutic effect similar to doxycycline (2).

Conclusion

The licorice root ethanolic extract in this research showed a beneficial antimicrobial effect on the periopathogenic bacterium *Fusobacterium nucleatum*. Thus, at the minimum concentration, (6.25 mg/ml) it showed 45.6% growth inhibition. Moreover, at a concentration of 200 mg/ml, an effect equal to Doxycycline and better than Chlorhexidine was observed.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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