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Synergistic Antibacterial Activity of *Capsella bursa-pastoris* and *Glycyrrhiza glabra* Against Oral Pathogens

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Background: Oral infections and dental caries are still considered as serious public health problem and inflict a costly burden to health care services around the world especially in developing countries.
Objectives: In the present study, we evaluated the antibacterial activity of *Capsella bursa-pastoris* alone and also combined with *Glycyrrhiza glabra* against *Streptococcus mutans, S. sanguis, Actinomyces viscosus, Enterococcus faecalis* as oral pathogens.
Materials and Methods: The antimicrobial activities of an ethanol extract of *C. bursa-pastoris* alone and in combination with G. glabra were *in vitro* tested against six reference strains of oral pathogenic bacteria. The antimicrobial activities of the extracts were examined using disc diffusion method and the minimum inhibitory concentration (MIC) determined by both broth and Agar dilution methods and minimum bactericidal concentration (MBC) by broth dilution methods.
Results: In this study, *C. bursa-pastoris* extract showed good antibacterial activity against six bacteria in using in of the mentioned methods. No strain in this study showed resistance against this extract. Antibacterial activity of mixed extract including *C .bursa-pastoris* and *G. glabra* was evaluated and showed that mixed extract was more effective against all bacteria than any of the cases alone that indicate the synergistic effect between these two extracts.
Conclusions: *C. bursa-pastoris* and its mixture with *G. glabra* are suggested as appropriate candidates to control dental caries and endodontic infections.

Keywords: Antibacterial Activity; *Capsella bursa-pastoris*; *Glycyrrhiza glabra*; Oral Pathogen

1. Background

Despite the developments in various field of medicine, oral infections, dental caries and periodontal diseases are still considered as serious public health problems and inflict a large burden to health care services around the world especially in developing countries (1-3). Bacterial plaque or biofilms accumulated on teeth surfaces that are composed of native oral microbiota, are the primary etiological agents for oral diseases which may resulted in teeth loss if left untreated (4, 5). On the other hand, development of resistance against antibiotics and antiseptics is a growing cause of concern which limited the preventive measurement. Therefore, there is a permanent need to search for new antimicrobial agents (6).

Over the last decade, plant antimicrobial activity has been studied in different regions of the world including Iran (2, 7, 8). *Capsella. bursa-pastoris* (L.) Medik., *Brassicaceae*, commonly known as shepherd's purse, is a wild plant with several useful medicinal properties such as anti-bleeding, anticancer, antithrombin, wound-healing, antioxidant agent and antibacterial effects. Although there are some studies on antimicrobial activity of *C. bursa-pastoris* but there is no research about its activity against oral pathogens (7, 9). The main use of its antimicrobial effect is urinary inflammations treatment (9). This plant has high nutritional value that can be eaten raw or cooked. This plant is grown in many provinces of Iran, such as Khorasan, Golestan, Mazandaran, Gilan, Azerbaijan, Isfahan, Fars, Tehran. *Glycyrrhiza. glabra* is one of the important medicinal plants grows in the various part of the world that is used for medicinal purposes. Root of this plant has several useful pharmalogical properties such as anti inflammatory, antiviral, antimicrobial, anticancer activities, immunomodulatory, hepato protective and cardio protective effects (2).

2. Objectives

In the present study, we evaluated the antibacterial ac-

Implication for health policy/practice/research/medical education:
C. bursa-pastoris is suggested as an appropriate candidate to control dental caries and endodontic infections.

tivity of *C. bursa-pastoris* and its mixture with *G. glabra* against oral pathogens.

3. Materials and Methods

3.1. Plant Material

3.1.1. Source, Collection and Identification

Total parts of *C. bursa-pastoris* and roots of *G. glabra* were collected from Garineh, a village near Neyshabour, Iran, in summer 2011. A voucher specimen was prepared and deposited at Research Institute of Plant Sciences Herbarium, Ferdowsi University of Mashhad, Iran.

3.1.2. Preparation of Extract

Different parts of *C. bursa-pastoris* (250 g) and roots of *G. glabra* (250 g) were dried at 25˚C and then powdered using a mechanical grinder separately. Each extraction was prepared using ethanol (80%, v/v) (Merck, Germany) for a period of 72 hours without using any heating procedure. The final volume of the filtrated mass was removed using a rotary vacuum evaporator (Heidolphlaborota 4000, Germany) at 40˚C to produce the concentrated extract, which was frozen and freeze-dried until the next use (2, 10). For preparation of mixed extract, equal amounts(2 mL) of the each extract (100 mg/mL) was thoroughly mixed in a sterile tube. So the concentration of each extract was 50 mg/mL in the mixed extract.

3.2. Antibacterial Activity

3.2.1. Microbial Strains

The microorganisms used in this study included *Streptococcus mutans* (PTCC 1683), *S. sanguis* (PTCC 1449), *Actinomyces viscosus* (PTCC 1202), *Enterococcus faecalis* (ATCC 29212) as oral pathogens and *Staphylococcus aureus* (ATCC 25923) and *Escherichia coli* (ATCC 29922) were used as controls. The bacterial strains were cultured in brain heart infusion (BHI) (Difco, MI, USA) under anaerobic condition in an anaerobic jar with Anaerocult A (Merk SA (Pty) Ltd), at 37˚C for 72 hours and subculturing was performed twice a week. The organisms suspensions were prepared by picking colonies from appropriately incubated agar cultures to sterile broth, to match a McFarland 0.5 turbidity standard (approximately 1.5 x 108 CFU/mL) (11).

3.3. Disk Diffusion and Well Diffusion Methods

The microbial growth inhibitory potential of the each extract was determined using the agar disk diffusion method as described by Clinical Laboratory Standards Institute (CLSI) (1, 12). *C. bursa-pastoris* and mixed extracts were diluted to produce concentrations ranging from 100 to 3.125 mg/mL and chlorhexidine 0.2% mouthwash (Shahr Daru, Tehran, Iran) with concentrations ranging from 0.0625 to 2 mg/mL and distilled water were used as positive and negative controls, respectively. Twenty microlitre of the plant extracts and chlorhexidine concentration were transferred onto sterile filter papers (6.4 mm diameter). Each Mueller-Hinton agar (Difco, USA) (with 5% sheep blood) was uniformly seeded by means of sterile swab dipped in the suspension and streaked on the agar plate surface. The plates were then incubated at 37°C for 48 hours anaerobically. All tests were performed in triplicate and inhibition zones were measured (12).

The agar-well diffusion method was performed as prescribed by National Committee for Clinical Laboratory Standards (NCCLS). Wells of 5 mm in diameter were punched in the Mueller-Hinton agar (with 5% sheep blood) using a sterile cork-borer about 2 cm apart. Approximately 20 μL of the extracts were filled with the extracts respectively. The rest of the process was followed as mentioned previously (13).

3.3.1. Determination of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

3.3.1.1. Macro Broth Dilution Method

The MIC of the extracts were determined according to methods described by CLSI 2006. *C. bursa-pastoris* and mixed extracts were diluted in different concentrations ranging from 100 to 0.78 mg/mL in Mueller-Hinton broth. In each dilution tubes, 0.1 mL of the bacterial inoculums was seeded. Control tubes with no bacterial inoculation were simultaneously maintained. Tubes were incubated anaerobically at 37°C for 24 hours. The lowest concentration of the extracts that produced no visible bacterial growth (turbidity) were recorded as the MIC (2, 14). To estimate the MIC of the extracts more precisely and for confirmation of the results, a more precise concentration in agar dilution method was used.

3.3.1.2. Agar Dilution Method

Agar dilution assay was used to test the susceptibility of the microorganisms to the *C. bursa-pastoris* and mixed extracts at different concentrations, as recommended by the CLSI. Serial dilutions of the extracts were prepared in plates according to the standard procedure. After solidification, the plates were incubated at 37°C for 2 hours in order to dry the agar surface. The assay plates were estimated to contain 50, 35, 30, 25, 20, 15, 12.5, 10, 5, 6.25, 3.125, 2.5 and 1.25 mg/mL of active extracts. Inoculants were applied to agar surfaces in 1 μL spots, giving approximately 1.5 x 10^5 CFU per spot. Plates lacking added extract were inoculated as viability controls and uninoculated media were also included to confirm sterility. All plates were inverted and incubated appropriately for 48 to 72 hours in an anaerobic condition. The MIC was considered as the

lowest concentration of extract which have a marked inhibition effect on microorganism growth as compared to the growth control. This extract was tested in triplicate vs. each organism (three separate inoculums preparations on three different days) (15).

4. Results

In vitro antibacterial activity of *C. bursa-pastoris* extract and the mixture of the *C. bursa-pastoris* and *G. glabra* extracts and also their potency were quantitatively and qualitatively assessed by determining the inhibition zone diameter and MIC as given in Tables 1 -6. The analysis of *C. bursa-pastoris* extract showed positive inhibitory activity against six bacteria, in all methods. No strain in this study showed resistance to this extract. Results of antibacterial activity of these plants by agar diffusion method against six bacteria are shown in Tables 1 - 4. The inhibitory zone significantly increased in a dose dependent manner.

4.1. C. bursa-pastoris Extract

In agar dilution method MIC for *S. aureus*, *A. viscosus* , *E. faecalis* and *S. sanguis* were 15 mg/mL and for *E. coli* was 35 mg/mL. MIC for *S. mutans* was 12.5 mg/mL. *E. coli* demonstrated the greatest resistance to *C. bursa-pastoris* and appeared to be the most resistant bacterium (Table 5). The results of broth dilution method which are shown in Table 7 are consistent with the findings of the agar dilution method (Table 5).

4.2. Mixed Extract

In agar dilution method MIC for *S. aureus* , *A. viscosus* , *E. faecalis* and *S. sanguis* were 12.5 mg/mL and this amount for *E. coli* was 20 mg/mL. MIC for *S. mutans* was 10 mg/mL. *E. coli* demonstrated the greatest resistance to mixed extract and appeared to be the most resistant bacterium against *C. bursa-pastoris* extract (Table 5). In broth dilution method MIC for all of the bacteria were 12.5 mg/mL except *E. coli* that was 25 mg/mL (Table 6)

4.3. Chlorhexidine

For these microorganisms, MIC of chlorhexidine mouthwash in agar and broth dilution method was 0.0625 mg/mL except for *E. coli* that was 0.125 mg/mL (Tables 8, 9 and 10).

Table 1. Antimicrobial Activity of *C. bursa-pastoris* Against Oral Microorganisms and Controls Inhibition Zones in Millimeter by Disk Diffusion Method

Plant Extract[a]	Concentration, mg/mL	*S. mutans*	*S. sanguis*	*A. viscosus*	*E. faecalis*	*S. aureus*	*E. coli*
C. bursa-pastoris	100	25.4 ± 0.8	21.6 ± 0.5	22.8 ± 0.4	17.8 ± 0.3	26.7 ± 0.3	23.6 ± 0.5
	50	23 ± 0.0	20.3 ± 0.5	18.4 ± 0.4	16.3 ± 0.5	23.4 ± 0.4	20.8 ± 0.7
	25	20 ± 0.0	17.6 ± 0.3	14 ± 0.5	14.3 ± 0.5	21.6 ± 0.5	18 ± 0.0
	12.5	18.1 ± 0.2	15 ± 0.0	9.4 ± 0.0	11 ± 0.0	19.3 ± 0.5	14.7 ± 0.3
	6.25	16 ± 0.3	12.1 ± 0.2	8.2 ± 0.5	9 ± 0.0	15.3 ± 0.5	12.7 ± 0.3
	3.125	10.7 ± 1	8.2 ± 0.5	-[b]	8.2 ± 0.2	9.6 ± 0.3	11 ± 0.0
Negative Control		-[b]	-[b]	-[b]	-[b]	-[b]	-[b]

[a] These results showed that antibacterial activity of this extract was significantly greater than negative control (P value less than 0.05).
[b] No inhibition zone

Table 2. Antimicrobial Activity of Mixed Extract Against Oral Microorganisms and Controls Inhibition Zones in Millimeter by Disk Diffusion Method.

Plant Extract[a]	Concentration, mg/mL	*S. mutans*	*S. sanguis*	*A. viscosus*	*E. faecalis*	*S. aureus*	*E. coli*
Mixed extract	100	29.2 ± 0.5	26.6 ± 0.5	26 ± 0.0	21.2 ± 0.5	29.7 ± 0.3	28.7 ± 0.5
	50	27.4 ± 0.4	24.1 ± 0.2	23.2 ± 0.5	19.3 ± 0.5	28 ± 1	23 ± 0.0
	25	25 ± 0.0	21.8 ± 0.3	18 ± 1	17 ± 0.0	25.6 ± 0.5	21 ± 0.0
	12.5	22.6 ± 0.5	20.4 ± 0.4	14 ± 0.5	14.2 ± 0.5	22.2 ± 0.5	18 ± 1
	6.25	20.8 ± 0.3	16.8 ± 0.7	10.2 ± 0.5	13.1 ± 0.2	17.8 ± 0.3	15.2 ± 0.5
	3.125	14 ± 0.0	12 ± 0.0	8 ± 0.0	10 ± 0.0	13.4 ± 0.8	14 ± 0.0
Negative Control		-[b]	-[b]	-[b]	-[b]	-[b]	-[b]

[a] These results showed that antibacterial activity of this extract was significantly greater than negative control (P value less than 0.05).
[b] No inhibition zone

Table 3. Antimicrobial Activity of *C. bursa-pastoris* Against Oral Microorganisms and Controls Inhibition Zones in Millimeter by Well Diffusion Method.

Plant extract[a]	Concentration, mg/mL	*S. mutans*	*S. sanguis*	*A. viscosus*	*E. faecalis*	*S. aureus*	*E. coli*
C. bursa-pastoris	100	26.3 ± 0.5	20.8 ± 0.3	23.9 ± 0.5	22.1 ± 0.2	27 ± 1	25 ± 1
	50	23.3 ± 0.5	19.2 ± 0.2	18.8 ± 0.3	17 ± 0.0	22.3 ± 0.5	20.3 ± 0.4
	25	19.4 ± 0.5	17.8 ± 0.3	14 ± 0.0	14 ± 0.0	17.6 ± 0.5	18 ± 0.0
	12.5	16.3 ± 0.5	13.6 ± 0.5	11 ± 0.0	11 ± 0.3	15 ± 0.0	14.2 ± 0.2
	6.25	14 ± 0.0	9.8 ± 0.3	8.4 ± 0.0	9.3 ± 0.5	13 ± 0.0	11 ± 0.0
	3.125	10 ± 0.0	7.4 ± 0.5	-[b]	7.4 ± 0.5	10 ± 0.0	9.4 ± 0.3
Negative Control		-[b]	-[b]	-[b]	-[b]	-[b]	-[b]

[a] The results obtained by above mentioned method confirmed that antibacterial activity of this extract was significantly greater than negative control (P value less than 0.05).
[b] No inhibition zone

Table 4. Antimicrobial Activity of Mixed Extract Against Oral Microorganisms and Controls Inhibition Zones in Millimeter Using Well Diffusion Method

Plant Extract[a]	Concentration, mg/mL	*S. mutans*	*S. sanguis*	*A. viscosus*	*E. faecalis*	*S. aureus*	*E. coli*
Mixed extract	100	30.8 ± 0.3	27.1 ± 0.2	26.8 ± 0.3	22 ± 0.0	30 ± 1	29.4 ± 0.5
	50	27 ± 0.0	25 ± 1	23 ± 0.0	20.1 ± 0.2	26.4 ± 0.0	24 ± 0.0
	25	25.4 ± 0.5	23.7 ± 0.3	18.1 ± 0.2	17.2 ± 0.2	21.8 ± 0.3	21.8 ± 0.3
	12.5	20 ± 0.0	19.2 ± 0.2	14.4 ± 0.5	15 ± 1	17.8 ±0.3	17.3 ± 0.3
	6.25	16.4 ± 0.5	14 ± 0.0	12.8 ± 0.3	13. 3± 0.5	15 ± 0.0	13 ± 1
	3.125	13 ± 0.0	11 ± 0.0	12 ± 1	11.2 ± 0.2	11.2 ± 0.2	11.6 ± 0.5
Negative Control		-[b]	-[b]	-[b]	-[b]	-[b]	-[b]

[a] The results obtained by above mentioned method confirmed that antibacterial activity of this extract was significantly greater than negative control (P value less than 0.05).
[b] No inhibition zone

Table 5. Mean MIC (mg/mL) Results of *C.bursa-pastoris*Extract, *C. bursa-pastoris* Extract and Mixed Extract on Oral Microorganisms and Controls Using Agar Dilution Method

	S. mutans	*S. sanguis*	*A. viscosus*	*E. faecalis*	*S. aureus*	*E. coli*
***C.bursa-pastoris* Extract**						
MIC	12.5	15	15	15	15	35
***C. bursa-pastoris* Extract**						
MIC	12.5	25	25	25	25	50
MBC	12.5	25	25	25	25	50
Mixed Extract						
MIC	10	12.5	12.5	12.5	12.5	20

Table 6. Mean MIC and MBC (mg/mL) Results of Mixed Extract on Oral Microorganisms and Controls using Broth Dilution Method

	S. mutans	*S. sanguis*	*A. viscosus*	*E. faecalis*	*S. aureus*	*E. coli*
MIC	12.5	12.5	12.5	12.5	12.5	25
MBC	12.5	12.5	12.5	12.5	12.5	25

Table 7. Antimicrobial Activity of the Chlorhexidine Against Oral Microorganisms and Controls Inhibition Zones in Millimeter Using Disk Diffusion Method.

Plant Extract	Concentration, mg/mL	*S. mutans*	*S. sanguis*	*A. viscosus*	*E. faecalis*	*S. aureus*	*E. coli*
Chlorhexidine	2	26.2 ± 0.2	17.2 ± 0.2	23 ± 0.0	25.2 ± 0.2	26 ± 1	24 ± 0.2
	1	22.2 ± 0.2	16 ± 0.0	17.7 ± 0.4	22.2 ± 0.1	23 ± 0.5	21.2 ± 0.0
	0.5	18 ± 0.0	15.2 ± 0.2	13.7 ± 1	17.4 ± 0.7	19 ± 0.0	18.4 ± 0.99
	0.25	14 ± 0.0	11.7 ± 0.99	11.7 ± 0.4	11 ± 0.0	15.4 ± 0.7	15 ± 0.0
	0.125	10 ± 0.0	10 ± 0.0	9.5 ± 0.7	8.2 ± 0.2	11.2 ± 0.2	12 ± 0.0
	0.625	8.5 ± 0.5	-	7.2 ± 0.2	6 ± 0.0	8 ± 1	-
Negative Control		-	-	-	-	-	-

Table 8. Antimicrobial Activity of the Chlorhexidine Against Oral Microorganisms and Controls Inhibition Zones in Millimeter Using Well Diffusion Method

Plant Extract	Concentration, mg/mL	*S. mutans*, Mean ± SD	*S. sanguis*, Mean ± SD	*A. viscosus*, Mean ± SD	*E. faecalis*, Mean ± SD	*S. aureus*, Mean ± SD	*E. coli*, Mean ± SD
Chlorhexidine	2	28.2 ± 0.2	20 ± 0.0	22.2 ± 0.2	26 ± 0.0	26.8 ± 0.2	25 ± 0.2
	1	23.9 ± 0.7	16.7 ± 0.4	18.2 ± 0.2	23.2 ± 0.1	24.6 ± 0.5	23 ± 0.0
	0.5	20.2 ± 0.2	14.2 ± 0.2	14.7 ± 0.4	18 ± 0.0	21 ± 0.0	22.2 ± 0.2
	0.25	14.4 ± 0.0	11.2 ± 0.2	12.5 ± 0.1	13 ± 0.2	16.7 ± 0.4	16.8 ± 0.0
	0.125	12 ± 0.0	9.7 ± 0.4	10.2 ± 0.2	10.8 ± 0.0	13.2 ± 0.2	12.8 ± 0.0
	0.625	9.4 ± 0.5	-	8 ± 0.0	-	-	-
Negative control		-	-	-	-	-	-

Table 9. Mean MIC (mg/mL) Results of Chlorhexidine Extract on Oral Microorganisms and Controls using Agar Dilution Method

	S. mutans	*S. sanguis*	*A. viscosus*	*E. faecalis*	*S. aureus*	*E. coli*
MIC	0.0625	0.0625	0.0625	0.0625	0.0625	0.125

Table 10. Mean MIC and MBC (mg/mL) Results of Chlorhexidine Extract on Oral Microorganisms and Controls using Broth Dilution Method

	S. mutans	*S. sanguis*	*A. viscosus*	*E. faecalis*	*S. aureus*	*E. coli*
MIC, mg/mL	0.0625	0.0625	0.0625	0.0625	0.0625	0.125
MBC, mg/mL	0.125	0.125	0.0625	0.125	0.125	0.125

5. Discussion

Several antiseptic agents like Chlorhexidine have been used widely in dentistry to inhibit bacterial growth. However, these substances have several side effects. In addition, the development of antimicrobial resistant pathogens is a growing concern. Above mentioned reasons and many others explain the need of further researches and development of safe natural antimicrobial agents targeting specific oral pathogens of the hosts (6).

In recent decade, antimicrobial activity of plants in different areas of the world and Iran has also been studied (7, 8, 16). All these studies show that plant species with anti-microbial activity are very diverse around the world and also in Iran. For example, in one study the methanol extracts of 306 plants of 52 families obtained from Northeast of Iran, were tested for antimicrobial activity. Among 171 extracts with antimicrobial effects, 10 extracts had the highest activity (7). In the present study, we evaluated the antibacterial activity of two extracts from these plants including *C. bursa-pastoris* and its mixture with *G. glabra* against oral pathogens.

The ethanolic extract of *G. glabra* had effective MIC values against all oral bacteria especially *S. mutans*, *A. viscosus*, and *E. faecalis* and exhibited the highest MIC value against *E. coli*, so maybe antibacterial activity of *G. glabra* against gram positive bacteria was more than gram negative bacteria (2).

The antimicrobial activity of *C. bursa-pastoris* extract has been shown in some other studies but antibacterial effects of this plant against oral pathogens has not been

studied yet (9). The present study supports the idea that *C. bursa-pastoris* extract might be useful as an antibacterial agent against oral pathogens. The findings propose that *C. bursa-pastoris* can inhibit the growth of *S. mutans*, *A. viscosus*, *S. sanguis*, and *E. faecalis*. In this study, for the first time, antibacterial activity of *C. bursa-pastoris* against *S. mutans*, *A. viscosus* and *S. sanguis* was confirmed and was shown that the ethanolic extract of this plant revealed a promising MIC value against all oral bacteria especially *S. mutans*.

Although in some studies, it has been reported that *C. bursa-pastoris* extract has antibacterial activity against several bacteria such as *S. aureus*, *E. faecalis*, and *E. coli*, but a few studies have conducted on oral pathogens such as *A. viscosus* and *S. sanguis* (9). In one study, it was shown that Gram-positive bacteria were more susceptible than Gram-negative ones (9). Similarly, in the present study, ethanolic extract of *C. bursa-pastoris* exhibited a high MIC value against *E. coli*, so maybe antibacterial activity of *C. bursa-pastoris* against gram positive bacteria was more than gram negative bacteria which is similar to *G. glabra* extract (2). In another study, it has been reported that *C. bursa-pastoris* had no antimicrobial effect against *E. coli* (17).

In the present study, antibacterial activity of mixed extract including *C .bursa-pastoris* and *G. glabra* was evaluated and showed that the mixed extract was more effective against all bacteria than any single extracts that indicating the synergistic effect between these two extracts. The prevalence of oral infections, as one of the major problems in oral health, has caused increasing use of mouthwash products. Herbal mouthwashes, compared with chemical drugs, have fewer side effects and are more economical. This in-vitro study suggests *C. bursa-pastoris* and its mixture with *G. glabra* as a candidate may help us to control dental cavities and oral infections. The effects of this extract maybe more beneficial if it is incorporated in gum, toothpaste, mouthwash, and dental products to reduce plaque and dental caries.

Further studies are required for a better evaluation of this extract effectiveness if used as endodontic irrigants and In vivo clinical testing is essential to confirm the in-vitro effects (2).

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Authors' Contribution

None declared.

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