

Antimicrobial effect of edible mixed herbal extracts on oral microorganisms

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Research Article

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Abstract

Background The oral cavity is inhabited by pathogenic bacteria, the growth of which is inhibited by synthetic oral drugs composed of antibiotics or chemical compounds. However, upon long-term use, the negative side effects of these drugs outweigh their benefits. Natural antimicrobial substances that do not elicit negative side effects may serve as effective synthetic agent substitutes for long-term use. Therefore, we evaluated the effects of edible mixed herbal extracts, comprising *Schizonepeta tenuifolia* Briq., *Mentha piperascens*, *Acanthopanax sessiliflous* Seem., and *Glycyrrhiza uralensis*, on oral pathogenic bacteria.

Methods The yield of each herbal extract was as follows: 5% *S. tenuifolia*, 10.94% *M. piperascens*, 5.47% *A. sessiliflous*, and 10.66% *G. uralensis*. For each extract, antimicrobial tests, morphological analysis using scanning electron microscopy, microbial surface hydrophobicity measurements, and oral malodor reduction tests, were performed.

Results The mixed herbal extracts significantly inhibit the growth of *Streptococcus mutans*, *Enterococcus faecalis*, *Candida albicans*, and *Porphyromonas gingivalis* compared to growth in the control group. Scanning electron microscopy results further reveal altered cellular morphology in the groups treated with mixed herbal extracts. Additionally, the hydrophobicity assay results show that the mixed herbal extracts reduce the oral adhesion of bacteria. Levels of volatile sulfur compounds, as the main components of oral malodor, are also reduced by the mixed herbal extracts.

Conclusions The edible mixed herbal extracts effectively eliminate oral pathogens and may be useful for improving oral health.

Background

The human oral cavity is inhabited by more than 600 different bacterial species, those that constitute disease-causing pathogens can induce gastrointestinal dysfunction and even tooth loss [1]. Indeed, dental caries and periodontal diseases are the most common chronic oral diseases. However, oral pathogens also induce dental pulp disease and fungal infections [2] and can cause both oral and systemic diseases [3].

To prevent, or suppress, the progression of oral diseases caused by bacteria, the causal pathogen must be eliminated, which is typically achieved with mouthrinse or antibiotics. Currently used antimicrobial agents such as chlorhexidine, erythromycin, tetracycline, and penicillin exert harmful effects on the human body upon long-term use along with various side effects, including microbial drug resistance [4]. To overcome these limitations, natural herbal extracts with high pharmacological efficacy and low toxicity have recently been applied to inhibit oral pathogens and prevent oral diseases [5, 6]. For instance, flavanone and flavanol, isolated from natural herbal extracts, can inhibit the glucosyltransferase that causes dental caries [7], while the natural extracts of mulberry [8], green tea [9], and cardinal [10] inhibit oral pathogens.

In this study, we prepared mixed herbal extracts of *Schizonepeta tenuifolia* Briq. (STB), *Mentha piperascens* (MP), *Acanthopanax sessiliflorus* Seem. (AS), and *Glycyrrhiza uralensis* (GU), as these four herbs have similar antimicrobial and anti-inflammatory effects. STB is an annual plant in the Lamiaceae family; the whole plant, commonly known as catnip, is primarily used for medicinal purposes due to its antioxidant, anti-inflammatory, antiviral [11], hemostatic, and antimicrobial [12] effects. MP is a perennial herb in the Lamiaceae family with known anticancer [13], anti-allergic [14], antioxidant [15], and analgesic effects [16]. ASS is a deciduous shrub in the Araliaceae family, the roots and barks of which, as well as those of plants in the same genus, are used as herbs. Chiisanoside is among the main components and has anti-inflammatory, anti-hepatotoxic, anti-diabetic, and antiviral effects [17] with positive therapeutic effects on diabetes, cancer, and rheumatoid arthritis [18]. Chiisanoside also exerts potent antioxidant effects by reducing DNA damage caused by oxidative stress or protein glycation [19]. GU is a perennial herb in the Fabaceae family; its roots and rhizomes are used as herbal medicines with various pharmacological effects including detoxification, antiulcer, anti-inflammation, antispasmodic, and antitussive activities, as well as anti-hepatotoxicity effects [20]. Moreover, GU contains licopyranocoumarin and licoaryl coumarin which have antiviral effects, as well as glycyrol and glycoumarin which exert antimicrobial effects [21].

To overcome the limitations of conventional chemical mouthrinse, the current study seeks to determine whether edible natural extracts can be used as substitutes for the chemical components of mouthrinse. To this end their antimicrobial effects against oral pathogens are assessed herein.

Material And Methods

Herbal extracts

Prior to this study, STB, MP, AS, and GU, which were predicted to have antimicrobial effects, were purchased and evaluated in antimicrobial tests. Each herbal material was finely ground, and 100 g was mixed with 10× 70% ethanol. The mixture was shaken at 60 °C for 12 h. The supernatant was isolated by filtering through filter paper and then concentrated using a vacuum evaporator (R-114; BÜCHI, Flawil, Switzerland). The concentrate of each herbal extract was dehydrated using a freeze-dryer (FD, Ilshin Lab, Yangju, Korea) at -92 °C and then dissolved in dimethyl sulfoxide (Sigma-Aldrich, St. Louis, MO, USA) for subsequent use. The yield of each herbal extract was as follows: 5% STB, 10.94% MP, 5.47% ASS, and 10.66% GU. Table 1 presents the concentration of each herbal extract, based on which combinations of STB, MP, ASS, and GU extracts were prepared.

Table 1
Composition of mixed herbal extracts used in the experiments

Scientific name	Plant part	Concentration (mg/mL)
<i>Schizonepeta tenuifolia</i>	Above-ground	0.5
<i>Mentha piperascens</i>	Leaf	1.5
<i>Acanthopanax sessiliflous</i> Seem.	Bark	1.5
<i>Glycyrrhiza uralensis</i>	Root	2

Test Strains And Culture Conditions

Table 2 shows the microbial strains and respective culture conditions. All strains were obtained from the Korean Collection for Type Cultures of the Korea Research Institute of Bioscience and Biotechnology (Daejeon, Korea). The strains were subcultured at least three times prior to use.

Table 2
Four microorganisms and culture requirements

Strains	Straining property	Culture requirement	Media
<i>Streptococcus mutans</i> (KCTC3065)	Gram-positive	Facultative anaerobic	Brain heart infusion/agar
<i>Enterococcus faecalis</i> (KCTC2011)	Gram-positive	Facultative anaerobic	Brain heart infusion/agar
<i>Candida albicans</i> (KCTC7965)	Gram-positive	Aerobic	YM broth/agar
<i>Porphyromonas gingivalis</i> (KCTC5352)	Gram-negative	Anaerobic	Tryptic soy broth/agar
KCTC, Korean Collection for Type Cultures			

Antimicrobial Test On Mixed Herbal Extracts

To determine the viable cell count of the oral microbial strains, each test strain was cultured at 5×10^4 colony-forming units (CFU)/mL; a mixture of 100 μ L of bacteria and 900 μ L of mixed herbal extracts was prepared and cultured for 6 h. The cultured bacteria were spread on an agar dish and cultured for 24 h in a 37 °C incubator, after which the viable cell count was performed. To determine statistical significance, each measurement was performed in triplicate. The rate of microbial growth inhibition was calculated using the following equation:

$$\text{Rate of inhibition (\%)} = \frac{\text{Count of control group} - \text{Count of experimental group}}{\text{Count of control group}} \times 100$$

Morphological Examination Of Oral Pathogens Via Scanning Electron Microscopy

Bacteria in the control and experimental groups were inoculated into liquid media at 5×10^5 CFU/mL. In the experimental group, the mixed herbal extracts were diluted to 6 mg/mL and added to liquid media. The control and experimental groups were cultured at 37 °C for 24 h, after which the bacteria were transferred to a 1.5-mL tube for centrifugation at 3,000 rpm for 5 min. The supernatant was removed, and the pellet was washed with distilled water and centrifuged at 5,000 rpm for 5 min. The supernatant was removed, and the pellet was fixed in 2.5% glutaraldehyde solution (0.1 M sodium cacodylate buffer, pH 7.2, 4°C) for 1 h, followed by centrifugation. Subsequently, 300 µL of 1% osmium tetroxide was added to the supernatant; a drop was then placed on a cover slide for 1 h fixation. The fixed bacteria were dehydrated by successively increasing the ethanol concentration to 50%, 70%, 80%, 95%, and 100%. After freeze-drying and gold-coating, images of each sample were acquired using scanning electron microscopy.

Microbial Surface Hydrophobicity

The effects of the mixed herbal extracts on the surface hydrophobicity of each microbial strain were measured. The absorbance of each strain was set to 0.3 for OD₅₅₀. To a 15-mL round tube, 2 mL of microbial suspension was added along with an equal amount of *n*-hexadecane (Sigma-Aldrich), and the sample was mixed for 1 min with a vortex mixer. The mixed herbal extracts were left to stand at room temperature for 30 min, after which the supernatant was removed and the absorbance at OD₅₅₀ was measured using a spectrophotometer (Multiskan Go, Thermo Fisher Scientific, Waltham, MA, USA). Cell surface hydrophobicity was calculated using the following equation: %HP (hydrophobic) = [OD (initial) - OD (expt)] × 100/OD (initial). All measurements were performed in triplicate for statistical analysis.

Oral Malodor Reduction

Oral chroma (CHM-1, Nissha FIS, Inc., Osaka, Japan) was used to measure the reduction in oral malodor. For the control group, liquid media was placed in a 15-mL tube, into which 5×10^5 CFU/mL bacteria was inoculated. The experimental group was prepared in the same manner as the control group, however, 6 mg/mL of mixed herbal extracts was added. The control and experimental groups were cultured for 6 h. A syringe was then inserted into the tube to collect the gas from the cultured bacteria. The gas was applied to the oral chroma, and volatile sulfur compound (VSC) concentrations were measured. All measurements were performed in triplicate.

Data analysis

All experiments were repeated three times, and the results were analyzed using SPSS Statistics 24.0 software (SPSS, Inc., Chicago, IL, USA). The significance of variation between the control and experimental groups was determined using Mann–Whitney U test to compare the pre- and post-treatment of bacterial cells with the herbal extracts. A $p < 0.05$ was considered statistically significant.

Results

Inhibitory effects of mixed herbal extracts on oral pathogens

To determine the antimicrobial effects of the mixed herbal extracts containing STB, MP, AS, and GU on each oral pathogen strain, the bacteria were treated with mixed herbal extracts and cultured. The colonies were counted, and growth inhibitory effects were measured. Treatment with the mixed herbal extracts significantly decreased the colony-forming ability of *Streptococcus mutans*, *Enterococcus faecalis*, *Candida albicans*, and *Porphyromonas gingivalis* (Fig. 1; $p < 0.01$). Compared to those in the control group, the counts of mixed herb-treated *S. mutans*, *E. faecalis*, *C. albicans*, and *P. gingivalis* were reduced by 3.94 log, 4.01 log, 4.94 log and 4.79 log, respectively (Fig. 2).

Morphological Examination Of Oral Pathogens

The surfaces of each bacterial test strain, with or without the mixed herb treatment, were examined using scanning electron microscopy (Fig. 3). All strains cultured with the mixed herbal extracts exhibited clearly visible patterns of damage with an altered cellular morphology due to destruction of certain cell walls and membranes. Non-treated strains displayed a normal cellular morphology.

Bacterial Surface Hydrophobicity

The hydrophobicity of each test strain is shown in Fig. 4. Compared to that in the control group, each bacterial test strain significantly decreased hydrophobicity as the treatment time with mixed herbal extracts increased ($p < 0.05$). After 10 min of treatment with the mixed herbal extracts, the hydrophobicity decreased by 42.65% in *S. mutans*, 27.94% in *E. faecalis*, 80.08% in *C. albicans*, and 77.86% in *P. gingivalis*.

Measurement Of Vscs

The effect of oral malodor reduction was determined by measuring VSCs, which are the main components of oral malodor (Table 3). After treatment with the mixed herbal extracts, VSCs significantly

decreased by 91.25% in *S. mutans*, 97.03% in *E. faecalis*, 88.99% in *C. albicans*, and 100% in *P. gingivalis* compared to those in the control group ($p < 0.05$).

Table 3
Effects of mixed herbal extracts on the production of volatile sulfur compounds

	<i>S. mutans</i>	<i>E. faecalis</i>	<i>C. albicans</i>	<i>P. gingivalis</i>
Control	1244.33 ± 22.24	109.00 ± 20.60**	644.00 ± 28.00	47.00 ± 13.01
Mixed herbal extracts	109.00 ± 20.60**	38.33 ± 10.80**	71.00 ± 12.53**	0.00 ± 0.00**
* $p < 0.05$, ** $p < 0.001$				

Discussion

Various microbial species are found in the oral cavity, and the growth of pathogenic bacteria can induce oral diseases. Notably, oral diseases such as dental caries, periodontal disease, periapical lesions, and oral candidiasis are the main cause of tooth loss and oral malodor, for which effective management methods are urgently needed. As the most basic approach, mouthrinse containing chemical agents is used to eliminate bacteria or inhibit microbial activity. In a previous study, the use of mouthrinse containing antimicrobial and anticaries agents decreased dental plaque by approximately 61% [22]. In other studies, dental plaque formation was inhibited, and gingivitis was prevented by mouthrinse containing eucalyptol and thymol as the main components or mouthrinse containing cetylpyridinium chloride, both of which exhibit outstanding antimicrobial effects on oral pathogens [23, 24]. However, antibiotics as the main components of mouthrinse can have harmful physiological effects and induce adverse side effects, from antibiotic-resistance to superinfection [25]. Long-term use of mouthrinse can also discolor the teeth or soft tissue and alter the sense of taste [26]. In addition, the chemical agents in mouthrinse can eliminate the normal microbial flora along with pathogens in the oral cavity. Thus, recent studies have focused on using natural substances as substitutes for synthetic chemical agents to selectively eliminate pathogenic bacteria.

In this study, the effects of a natural herbal extract mixture of STB, MP, AS, and GU on oral bacteria were determined to develop a mouthrinse comprised of natural substances. All herbal extracts used in the experiment are known to be edible, so it can be used as a gargle, but it can also be consumed. In particular, STB [27] and GU [28] have been reported to inhibit *Helicobacter pylori*, a major cause of gastric cancer and gastritis. The herbs in this extract mixture exhibit anti-inflammatory and antimicrobial effects; however, an overdose of GU can elicit negative effects, such as severe hypertension, hypokalemia, and hypermetabolism. Hence, GU must be used with caution [29]. Nevertheless, approximately 20% of GU is glycyrrhizin [30], which exhibits a mineralocorticoid effect to degrade nicotine and alcohol, thereby acting as a detoxifying agent [29]. The recommended daily dose of GU for adults is 0.7 g, although in patients with neurological disease, a dose of ≥ 0.1 g/mL can have negative effects [31]. Accordingly, in preparing

the mixed herbal extracts, only a small amount of the raw GU material was added to ensure a minimal dose without side effects.

The experimental results showed that *S. mutans*, which causes dental caries, and *E. faecalis*, which is a causative agent of dental pulp disease, were reduced by ≥ 3 log CFU/mL. Additionally, *C. albicans*, a fungal strain that infects the oral mucosa, and *P. gingivalis*, which causes periodontal disease, were reduced by ≥ 4 log CFU/mL. These results indicate that the mixed herbal extracts had stronger effects on gram-positive fungi and gram-negative bacteria than on gram-positive bacteria.

Morphological examination of each microbial strain after treatment with the mixed herbal extracts showed that some oral microorganisms exhibited a distorted pattern of damage. Particularly, *C. albicans*, showed an overall damaged morphology. Similarly, Sangetha et al [32]. treated *C. albicans* with *Cassia spectabilis* leaf extract for 24 h and found that the extract damaged the microbial surface. We obtained identical results after 24 h treatment with the mixed herbal extracts, verifying the antimicrobial effects of the extracts that likely caused morphological damage to the oral pathogens.

The hydrophobicity of cells is closely associated with microbial adhesion, and microbial strains with a high level of hydrophobicity can form biofilms. Many studies have reported that chlorohexidine, which is widely used as a component of mouthrinse, induces microbial adhesion. However, in one study, treatment of *S. mutans* with a high concentration of chlorohexidine decreased microbial adhesion, whereas biofilm formation was increased in another study [33, 34]. We found that treatment with the mixed herbal extracts reduced microbial adhesion, indicating that the extracts reduced the hydrophobicity of oral pathogens. Further studies are needed to investigate the mechanism underlying the effects of the mixed herbal extracts on microbial cell hydrophobicity.

Oral malodor, also known as halitosis, is a disease that can significantly impact quality of life. It is caused by oral, physiological, or pathological factors, with oral factors considered to be the main cause [35]. The main factors leading to oral malodor caused by bacteria are VSCs such as hydrogen sulfide, methyl mercaptan, and dimethyl sulfide. Indeed, the level of VSCs increases in patients with oral diseases such as dental caries [36]. Several studies have reported that reducing the level of anaerobic oral bacteria results in a decrease in the level of VSCs [37, 38]. We also demonstrated that the mixed herbal extracts reduced oral malodor by suppressing VSCs. Treatment with the mixed herbal extracts inhibited the growth of *P. gingivalis*, the causal bacteria of periodontal disease, by 100%, indicating that the extracts substantially reduced oral malodor.

We verified the antimicrobial effects of mixed herbal extracts on strains causing oral disease. However, as mixed extracts were used, the specific component responsible for the antimicrobial effects remains unclear. Thus, further studies are needed to isolate and examine the individual effects of the edible mixed herbal extract components.

Conclusions

We examined the antimicrobial effects of mixed herbal extracts on oral pathogens and found that the abundances of the four evaluated strains of oral pathogens were reduced by $\geq 99.99\%$. Additionally, treatment with the mixed herbal extracts damaged certain oral pathogen cells with *Candida albicans* exhibiting the greatest morphological damage. The four oral pathogen strains also showed decreased hydrophobicity, with *C. albicans* exhibiting the greatest decrease. Treatment with the mixed herbal extracts also reduced malodor caused by *S. mutans*, *E. faecalis*, *C. albicans*, and *P. gingivalis*. In particular, *P. gingivalis* was reduced by 100%. Our results suggest that the mixed herbal extracts exert antimicrobial effects against *S. mutans*, *E. faecalis*, *C. albicans*, and *P. gingivalis*, via altering their structural morphology. Based on these effects and the reduction in oral malodor, the edible mixed herbal extracts may represent an effective antimicrobial agent against oral disease pathogens.

Declarations

Ethics Approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

All data generated or analysed during this study are included in this published article.

Competing interests

The authors declare that they have no conflicts of interest.

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Authors' contributions

Contributions Conceptualization, SEY and BBRC; methodology, SEY and SHN; analysis, BBRC; writing—original draft preparation, SEY and SHN; writing—review and editing, BBRC and GCK; supervision, GCK. All authors read and approved the final manuscript.

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References

1. Aas JA, Paster BJ, Stokes LN, Olsen I, Dewhirst FE. Defining the normal microbial flora of the oral cavity. *J C Microbiol* 2005;43(11):5721–32.
2. Jeon HD, An KS, Park CW, Lee HS, Kim SG. Relationship between adherence of *Candida albicans* to human buccal epithelial cells in vitro and their virulence. *J Hanyang Med Rev* 1987;7:855–76.
3. Kim CS, Choi YK. Survey of adults` perceptions of the association between chronic diseases and oral health. *J Dent Hyg Sci* 2017;17:12–9.
4. Katsura H, Tsukiyama RI, Suzuki A, Kobayashi M. In vitro antimicrobial activities of bakuchiol against oral microorganism. *Antimicrob Agents Chemother* 2001;45:3009–13.
5. Lee SS, Zhang W, Li Y. The antimicrobial potential of 14 natural herbal dentifrices: Results of an in vitro diffusion method study. *J Am Dent Assoc* 2004;135:1133–41.
6. Estafan D, Gultz J, Kaim JM, et al. Clinical efficiency of herbal tooth paste. *J Clin Periodontol* 1998;9:31–3.
7. Namba T, Tsunetzuka M, Hattori M, Koda S. Dental caries prevention by traditional Chinese medicines. *Planta Medica* 1982;44:100–3.
8. Park KM, You JS, Lee HY, Beak NI, Hwang JK. Kuwanon G: antimicrobial agents from the root bark of *Morus alba* against oral pathogens. *J Ethnopharmacol* 2003;84:181–5.
9. Bae KH, Ji JM, Park KL. The anticariogenic component from *Cinnamoni* cortex against a cariogenic bacterium, *Streptococcus mutans* OMZ 176. *Arch Pharm Res* 1991;15:239–41.
10. Takasago K. Inhibitor of cariogenic bacteria. Japan patent. 1990;25890.
11. Chen SG, Cheng ML, Chen KH, et al. Antiviral activities of *Schizonepeta tenuifolia* Briq. against enterovirus 71 in vitro and in vivo. *Sci Rep* 2017;7:935.
12. Fung D, Lau CB. *Schizonepeta tenuifolia*: chemistry, pharmacology, and clinical applications. *J Clin Pharmacol* 2002;42:30–6.
13. Kim SM, Cho YS, Park S. Cytotoxicity of methanol extracts of edible herbs against L1210 cells with the changes of antioxidant enzymes activities. *Korea J Pharmacog* 2002;33:376–83.
14. Inoue T, Sugimoto Y, Masuda H, Kamei C. Antiallergic effect of flavonoid glycosides obtained from *Mentha piperita*. *L Biol Pharm Bull* 2002;25:256–9.
15. Dragland S, Senoo H, Wake K, Holte K, Blomhoff R. Several culinary and medicinal herbs are important sources of dietary antioxidants. *J Nutr* 2003;133:128–90.
16. Gobel H, Schmidt G, Soyka D. Effect of peppermint and eucalyptus oil preparations on neurophysiological and experimental algometric headache parameters. *Cephalalgia* 1994;14:228–34.
17. Bae EA, Yook CS, Oh OJ, Chang SY, Nohara T, Kim DH. Metabolism of chiisanoside from *Acanthopanax divaricatus* var. *Albeofructus* by human intestinal bacteria and its relation to some biological activities. *Biol Pharm Bull* 2001;5:582–5.

18. Fujikawa T, Yamaguchi A, Morita I, Takeda H, Nishibe S. Protective effects of *Acanthopanax senticosus* Harms from Hokkaido and its components on gastric ulcer in restrained cold water stressed rats. *Biol Pharm Bull* 1996;19:1227–30.
19. Ryu AR, Kim JH, Lee MY. Suppressive effect of *Acanthopanax sessiliflorus* extraction on the DNA and cell damage by dieldrin. *Kor J Medicinal Crop Sci* 2012;20:245–50.
20. Kim SH, Jung KK, Kang SY, et al. The effects of Jackyagkamcho-tang on follicular maturation and estrogen production in the immature rat. *Kor J Pharmacogn* 1997;28:104–11.
21. Tanaka Y, Kikuzaki H, Fukuda S, Nakatani N. Antimicrobial compounds of licorice against upper airway respiratory tract pathogens. *J Nutr Sci Vitaminol* 2001;47:270–3.
22. Baker PJ, Coburn RA, Genco RJ, Evans RT. The in vitro inhibition of microbial growth and plaque formation by surfactant drugs. *J Periodontal Res* 1978;13:474–85.
23. Allen DR, Davies R, Bradshaw B, et al. Efficacy of a mouthrinse containing 0.05% cetylpyridinium chloride for the control of plaque and gingivitis: A 6-month clinical study in adults. *Compend Contin Educ Dent* 1998;19:20–6.
24. Mandel ID. Chemotherapeutic agents for controlling plaque and gingivitis. *J Clin Periodontol* 1988;15:488–98.
25. Ukuizumi T, Nagamatsu H, Kojo T, Inoue H. Induction of salivary antibodies to inhibit *Candida albicans* adherence to human epithelial cells by tonsillar immunization in rabbits. *FEMS Immunol Med Microbiol* 2006;47:398–404.
26. James P, Worthington HV, Parnell C, et al. Chlorhexidine mouthrinse as an adjunctive treatment for gingival health. *Cochrane Database Syst Rev* 2017;31:3:CD008676.
27. Xueying Z, Mingwei Z. Review on Chemical Constituents of *Schizonepeta tenuifolia* Briq. and Their Pharmacological Effects. *Molecules* 2022; 27(16): 5249.
28. Toshio F, Ai M, Kiyoshi K, et al. Anti-*Helicobacter pylori* flavonoids from licorice extract. *Life Sci* 2002; 9;71(12):1449-63.
29. Asl MN, Hosseinzadeh H. Review of pharmacological effects of *Glycyrrhiza* sp. and its bioactive compounds. *Phytother Res* 2008;22:709–24.
30. Wen D, Liu Y, Li W, Liu H. Separation methods for antimicrobial and antirheumatism agents in plant medicines. *J Chromatogr B Analyt Technol Biomed Life Sci* 2004;812:101–17.
31. Størmer FC, Reistad R, Alexander J. Glycyrrhizic acid in liquorice—Evaluation of health hazard. *Food Chem Toxicol* 1993;31:303–12.
32. Sangetha S, Zuraini Z, Sasidharan S, Suryani S. Fungicidal effect and oral acute toxicity of *Cassia spectabilis* leaf extract. *Jpn J Med Mycol* 2008;49:299–304.
33. Meurman JH. Ultrastructure, growth, and adherence of *Streptococcus mutans* after treatment with chlorhexidine and fluoride. *Caries Res* 1988;22:283–7.
34. Dong L, Tong Z, Linghu D, et al. Effects of sub-minimum inhibitory concentrations of antimicrobial agents on *Streptococcus mutans* biofilm formation. *Int J Antimicrob Agents* 2012;39:390–5.

35. Quirynen M, Zhao H, van Steenberghe D. Review of the treatment strategies for oral malodour. Clin Oral Investig 2002;6:1–10.
36. Morita M, Wang HL. Relationship of sulcular sulfide level and oral malodor in subjects with periodontal disease. J Periodontol 2001;72:79–84.
37. Burton JP, Chilcott CN, Moore CJ, Speiser G, Tagg JR. A preliminary study of the effect of probiotic *Streptococcus salivarius* K12 on oral malodour parameters. J Appl Microbiol 2006;100:754–64.
38. Miyazaki H, Sakao S, Katoh Y, Takehara T. Correlation between volatile sulphur compounds and certain oral health measurements in the general population. J Periodontol 1995;66:679–84.

Figures

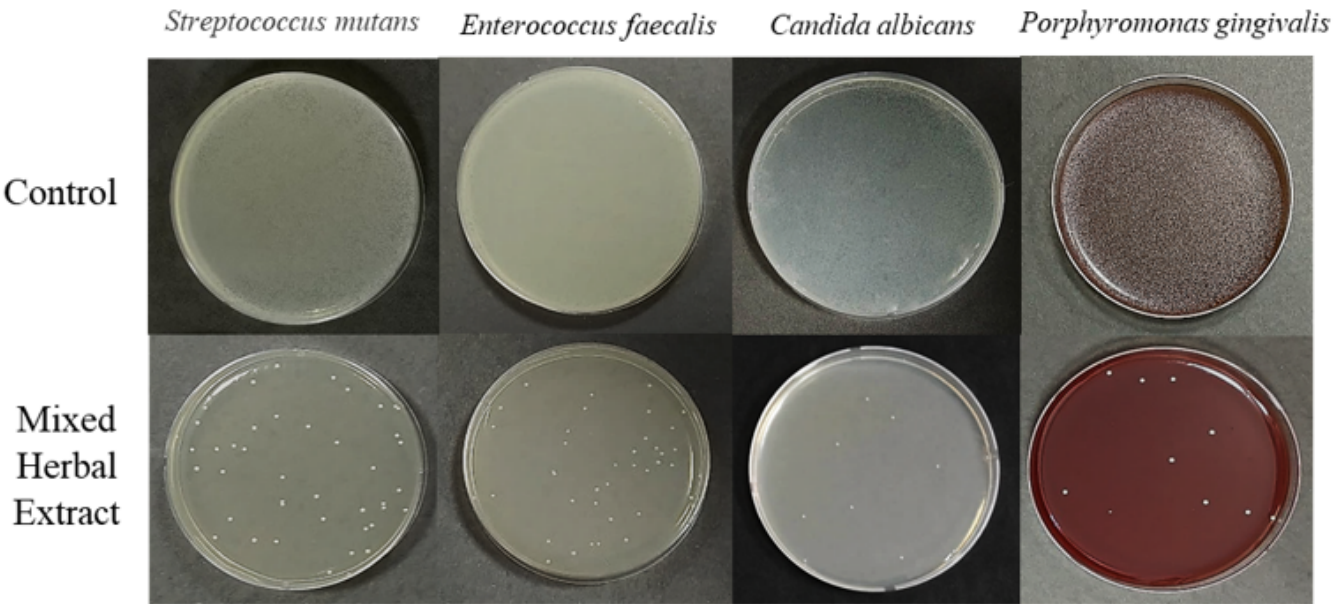


Figure 1

Colony-forming units (CFU) of oral pathogens following treatment with mixed herbal extracts (mixed herbal extracts: *Schizonepeta tenuifolia*, *Mentha piperascens*, *Acanthopanax sessiliflorus*, and *Glycyrrhiza uralensis*).

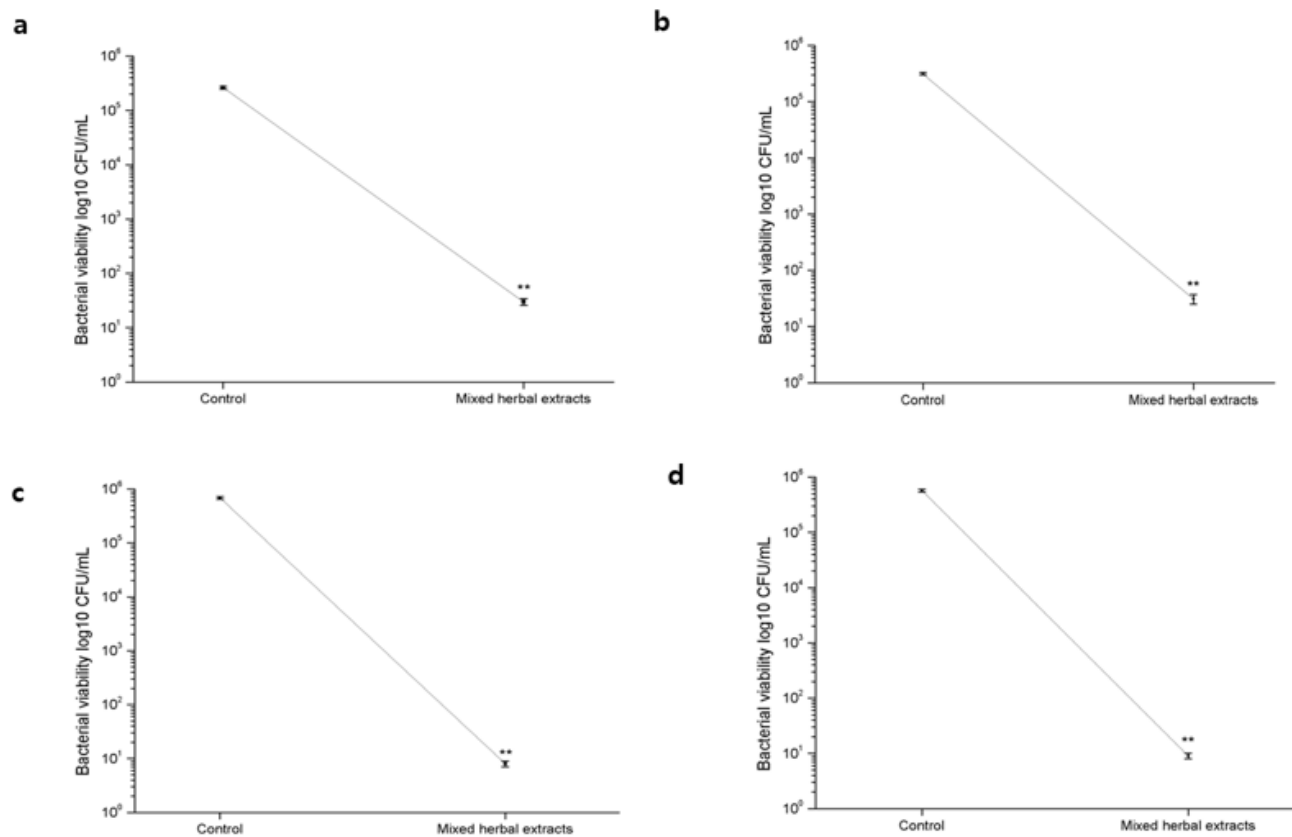


Figure 2

Growth inhibition of four microorganisms treated with mixed herbal extracts. a. *Streptococcus mutans*, b. *Enterococcus faecalis*, c. *Candida albicans*, and d. *Porphyromonas gingivalis*.

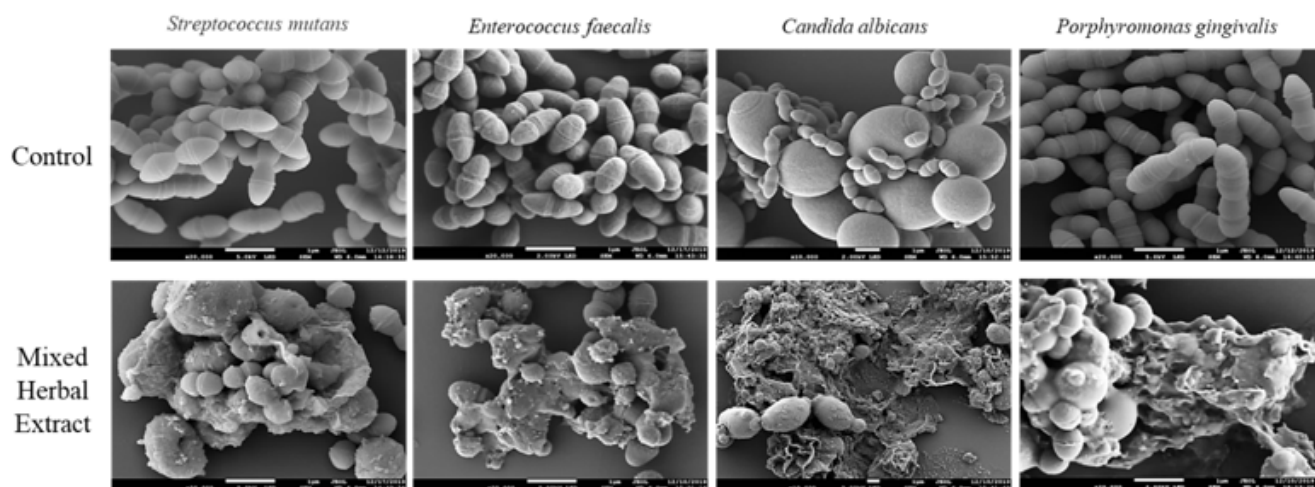


Figure 3

Scanning electron microscope images of four microorganisms.

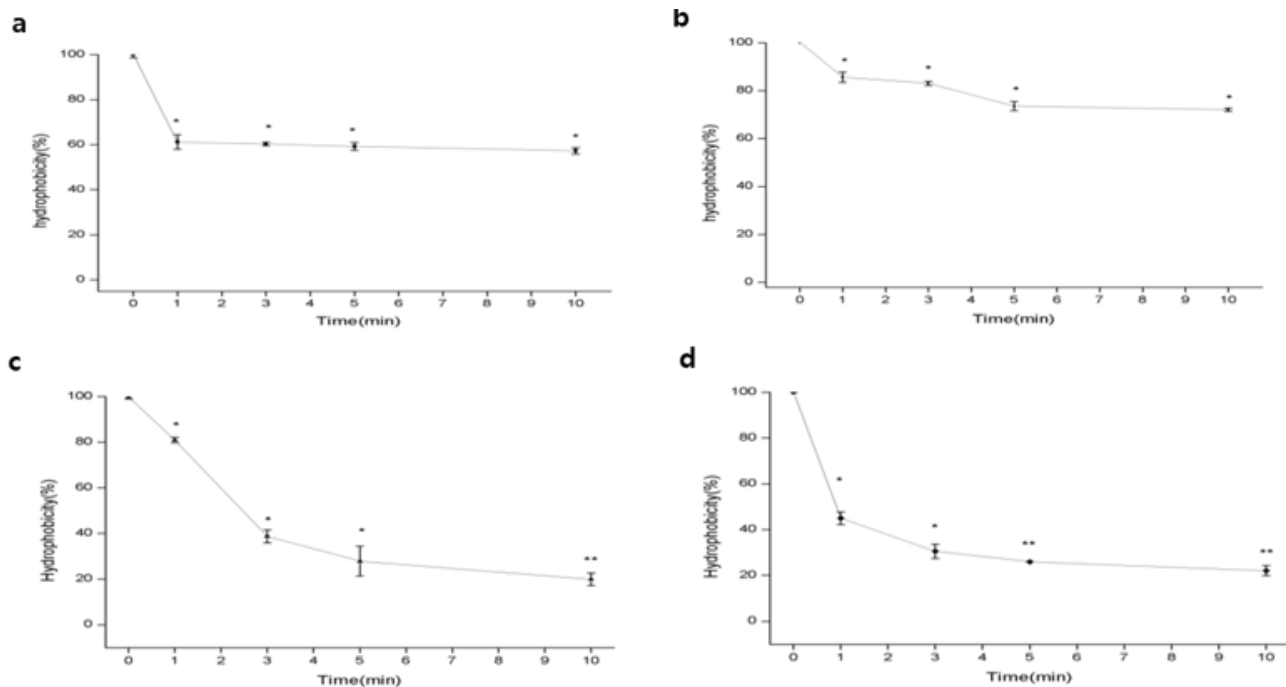


Figure 4

Effects of mixed herbal extracts on the hydrophobic property of four microorganisms. a. *Streptococcus mutans*, b. *Enterococcus faecalis*, c. *Candida albicans*, and d. *Porphyromonas gingivalis*.

(* $p < 0.05$, ** $p < 0.001$).