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Research Paper
Experimental Therapy

Antimicrobial activity of Chinese medicine herbs against common bacteria in oral biofilm. A pilot study

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Abstract. Twenty traditional Chinese medicines (TCM) were evaluated for their antimicrobial activity against four common oral bacteria. TCMs were tested for sensitivity against *Streptococcus mitis*, *Streptococcus sanguis*, *Streptococcus mutans* and *Porphyromonas gingivalis*. Aliquots of suspension of each bacterial species were inoculated onto a horse blood agar plate with TCMs soaked separately on 6 mm paper disks. The plates were incubated for 48 h anaerobically and the mean diameters of growth inhibition of three different areas obtained. 0.2% (w/v) chlorhexidine was used as a positive control. Broth microdilution assay was used to determine minimum inhibitory concentration and minimum bactericidal concentration. *Fructus armeniaca mume* was effective against all four bacteria. Thirteen TCMs demonstrated antimicrobial activity against *Porphyromonas gingivalis*, including *Cortex magnoliae officinalis*, *Cortex phellodendri*, *Flos caryophylli*, *Flos lonicerae japonicae*, *Fructus armeniaca mume*, *Fructus forsythiae suspensae*, *Herba cum radice violae yedoensis*, *Herba menthae haplocalycis*, *Pericarpium granati*, *Radix et rhizoma rhei*, *Radix gentianae*, *Ramulus cinnamomi cassia* and *Rhizoma cimicifugae*. *Cortex phellodendri* showed antimicrobial activity against *Streptococcus mutans*, while *Radix et rhizoma rhei* was effective against *Streptococcus mitis* and *Streptococcus sanguis*. *Fructus armeniaca mume* had inhibitory effects against *Streptococcus mitis*, *Streptococcus sanguis*, *Streptococcus mutans* and *Porphyromonas gingivalis* *in vitro*.

Key words: antimicrobial effect; Chinese medicine; bacteria; oral biofilm.

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The oral cavity contains a variety of bacteria that may cause dental caries, periodontal disease, postoperative infections after surgery or trauma or superinfections in immunocompromised patients. As new infections and bacterial resistance are con-

stantly emerging, the search for novel drugs or chemicals to combat oral infection is always warranted.

Oral bacteria usually aggregate to form a biofilm or plaque on tooth surfaces. Plaque formation involves initial colonization and

multiplication by pioneer species, followed by secondary colonization by other species and finally becoming a climax community¹. The ‘pioneer species’ of plaque are *Streptococcus oralis*, *Streptococcus mitis* and *Streptococcus sanguis*. Specific

bacteria are also closely related to specific dental diseases, for example, *Streptococcus mutans* and *Porphyromonas gingivalis* are associated with dental caries and periodontal disease, respectively¹¹.

Traditional Chinese medicines (TCM) have been used in China to treat infectious diseases for more than 4000 years. In the past decade, many new pathogenic microorganisms emerged and there were alterations to existing infections, such as Severe Acute Respiratory Syndrome (SARS), which was difficult to control and caused significant mortality. There is an urgent need to screen for natural products to identify a bank of antimicrobial materials that may be used against these infections, either alone or in combination. The use of natural products may decrease the opportunity for the development of drug resistance in microorganisms as they are surrounded by a group of antimicrobial chemicals acting together. In the oral cavity, a number of pathogenic organisms cause caries and periodontal diseases. Oral bacteria provide a good starting point for investigating the antimicrobial activities of TCM because the oral cavity is a strategic place for the development of infective disease and *in vivo* and clinical studies are easy to carry out. Unlike Western medicine, TCM works as a formula of herbs that is tailored to the individual patient and their specific condition. They are designed using one or two main ingredients that target the illness, with the addition of many other ingredients to adjust the formula to suit the patient's condition⁴. The advantage of using several TCMs together can also be considered a disadvantage because the treatment does not consist of a single drug. Some TCM formulae have been used effectively. One example is Realgar-Indigo naturalis for the treatment for promyelocytic leukaemia; a recent clinical trial showed a complete remission rate of 97%⁸. The mechanism of action has recently been investigated at the molecular, cellular and organism levels¹⁴. TCMs possess a variety of biological properties and can be developed to produce many effective drugs. Certain TCMs have antibacterial properties¹⁶. Few studies have been performed to screen these TCMs to see whether they are effective against bacteria forming an oral biofilm.

All TCMs consist of natural products, some of which are cheap and easy to obtain, although others come from endangered animal and plant species. If any TCMs are effective in inhibiting the bacteria commonly found in the oral biofilm, they might reduce the oral biofilm and aid in the prevention of dental caries and

periodontal disease. They could have an extensive impact on the prevention of oral disease and improve oral health.

Existing antimicrobial mouth rinses, such as chlorhexidine and Listerine[®], are inedible and may be harmful if ingested. There is a need to search for less harmful agents that can produce a similar antimicrobial effect.

Chlorhexidine gluconate is one of the most common conventional oral antimicrobial agents. It is effective against a broad spectrum of bacteria, yet it is artificial and inedible. Owing to overuse of antimicrobial agents, some bacteria start to develop resistance towards them. It is therefore necessary to develop new antimicrobial agents to replace or enrich the existing choice of oral antimicrobial agents. The potential advantage of using TCM rather than artificial oral antimicrobial agents is that they are natural substances and some are edible. It may also allow the combination of different TCM rather than a single oral antimicrobial agent, which lowers the chance of cross-resistance in bacteria. Further research is needed to confirm this. A number of TCM herbs are already used in oral healthcare products, such as toothpaste, to improve oral health, which shows that TCM has great potential to be developed as a product for day-to-day use.

In this study, the authors investigate the antimicrobial activity of some TCMs that have been used to treat symptoms related to infection (e.g. fever, inflammation, cough) on several bacteria found in the oral biofilm to identify potential agents to control oral infections and diseases.

Methods

Organism and culture condition

Type cultures of *S. mitis* (ATCC 15914), *S. sanguis* (ATCC 10556), *S. mutans* (ATCC 35668) and *P. gingivalis* (ATCC 33277) from the Oral BioScience Laboratories of the Faculty of Dentistry, the University of Hong Kong, were used in the study¹⁶. Frozen isolates were thawed and their identity reconfirmed using standard methodology. They were inoculated onto horse blood agar (HBA) and incubated for 3 days at 37 °C. The bacteria cultures were harvested and suspended in phosphate buffered saline at a concentration of 1×10^6 cells/ml (0.5 MacFarland Standard Units) for sensitivity studies.

Identification and preparation of TCM extracts

TCM were purchased from local Chinese medicine store and were identified mor-

phologically, histologically and chemically using standard Chinese herbal identification procedures³.

Voucher specimens including the identification and classification of all the TCMs to be tested were stored in the Hard Tissue Laboratory, University of Hong Kong. TCM extracts were prepared using standard protocols for aqueous extraction⁵. Briefly, 4 ml distilled water was added to 10 g of TCM powder. The mixture was boiled under stirring on a hot plate for 4 h. Distilled water was added occasionally to prevent drying. At the end of boiling, distilled water was added to make up the volume of the mixture to 4 ml. The mixture was cooled, centrifuged and filtered. This produced 2.5 g/ml of one TCM extract.

The 20 TCM drugs chosen for the study were *Cortex magnoliae officinalis* (hou po), *Cortex phellodendri* (huang bai), *Flos caryophylli* (ding xiang), *Flos chrysanthemi morifolii* (ju hua), *Flos lonicerae japonicae* (jin yin hua), *Fructus armeniaca mume* (wu mei), *Fructus forsythiae suspensae* (lian qiao), *Herba cum radice asari* (xi xin), *Herba cum radice houttuyniae cordatae* (yu xing cao), *Herba cum radice violae yedoensis* (zi hua di ding), *Herba ephedrae* (ma huang), *Herba portulacae oleraceae* (ma chi xian), *Herba menthae haplocalycis* (bo he), *Pericarpium granati* (shi liu pi), *Radix et rhizoma rhei* (da huang), *Radix gentianae* (long dan cao), *Radix isatidis* (ban lan gan), *Ramulus cinnamomi cassia* (gui zhi), *Rhizoma cimicifugae* (sheng ma) and *Semen raphani* (lai fu zi). Chlorhexidine gluconate, a common oral antiseptic, at a concentration of 0.2% (w/v) was used as a positive control for all experiments.

Agar diffusion assay

The standard agar diffusion assay for sensitivity testing^{9,10} was performed according to a standard protocol designed by SAMARANAYAKE et al.¹². Briefly, 20 µl aliquots of suspension of each bacterial species were inoculated on an HBA plate using a glass rod, then 6 mm diameter paper disks soaked in 10 µl of each of the TCM extracts at a concentration of 2.5 g/ml were placed concentrically on the HBA plate. Disks soaked in 10 µl of 0.2% (w/v) chlorhexidine were used as positive controls and were also placed in the HBA plates. The HBA plates were incubated for 48 h anaerobically at 37 °C. Naked eye measurements of the growth inhibition zone, if any, were evaluated using calibrated computer software. The diameters of growth inhibition in three different

Table 1. Sensitivity of bacteria to the TCM tested.

	<i>S. mutans</i>	<i>S. mitis</i>	<i>S. sanguis</i>	<i>P. gingivalis</i>
<i>Cortex magnoliae officinalis</i> (hou po)	–	–	–	4.3 (±0.2)*
<i>Cortex phellodendri</i> (huang bai)	8.0 (±0.1)*	–	P	38.3 (±0.3)*
<i>Flos caryophylli</i> (ding xiang)	–	–	–	9.8 (±0)*
<i>Flos chrysanthemi morifolii</i> (ju hua)	–	–	P	P
<i>Flos lonicerae japonicae</i> (jin yin hua)	–	–	P	5.5 (±0.4)*
<i>Fructus armeniaca mume</i> (wu mei)	9.0 (±0)*	13.1(±0.1)*	11.7 (±0)*	10.6 (±0.1)*
<i>Fructus forsythiae suspensae</i> (lian qiao)	–	–	–	7.4 (±0.1)*
<i>Herba cum radice asari</i> (xi xin)	–	–	–	–
<i>Herba cum radice houttuyniae cordatae</i> (yu xing cao)	–	–	P	–
<i>Herba cum radice violae yedoensis</i> (zi hua di ding)	–	–	–	9.0 (±0)*
<i>Herba ephedrae</i> (ma huang)	–	–	P	P
<i>Herba portulacae oleraceae</i> (ma chi xian)	–	–	–	–
<i>Herba menthae haplocalycis</i> (bo he)	–	–	–	2.3 (±0.3)*
<i>Pericarpium granati</i> (shi liu pi)	–	–	–	12.9 (±0)*
<i>Radix et rhizoma rhei</i> (da huang)	–	4.4 (±0)*	3.6(±0)*	31.7 (±0.1)*
<i>Radix gentianae</i> (longdancao)	–	–	–	11.4 (±0.1)*
<i>Radix isatidis</i> (ban lan gan)	–	–	–	–
<i>Ramulus cinnamomi cassiae</i> (gui zhi)	–	–	–	8.5 (±0)*
<i>Rhizoma cimicifugae</i> (sheng ma)	–	–	–	13.0 (±0.2)*
<i>Semen raphani</i> (lai fu zi)	–	–	–	–
Control	12.1 (±0)*	7.4 (±0.1)*	9.1 (±0)*	23.7 (±0.1)*

(P) Stands for partial inhibitory effect.

(–) Indicates for no inhibitory effect.

Values in parenthesis indicate standard deviation

* Indicate significant differences ($p < 0.05$).

areas were measured and the mean diameter of growth inhibition was calculated for each organism. The experiment was repeated on three separate occasions.

Broth microdilution susceptibility test

The TCMs, *Rhizoma coptidis* and *Galla chinensis*, which showed potent antimicrobial activity against the four tested bacteria in the agar diffusion assay screening test, were selected for minimum inhibitory concentration (MIC) determination using the standard broth microdilution assay^{2,6,7}.

In brief, bacteria were cultured anaerobically in HBA for 3–7 days at 37 °C. The bacterial cultures were harvested and suspended in an appropriate medium (depending on the species) at a concentration of 1×10^6 cells/ml (0.5 MacFarland Standard Units). Broth microdilution assay was performed in polystyrene, 96-well microtiter plates as previously described¹³. Each plate included a positive control (bacteria without the drug) and negative control (medium only) and serial two-fold dilution of the drug. *P. gingivalis* suspensions were prepared in 'P. gingivalis broth' whereas other bacterial species were prepared in brain heart infusion broth. Bacterial inoculums were prepared as described previously and 100 µl of 1×10^6 cells/ml bacterial suspensions were added to each well. Subsequently, microtiter plates were incubated for 48 h at 37 °C in an anaerobic chamber. Visual inspection was used to determine the MIC.

After MIC determination, 20 µl of suspension from each well was inoculated in blood agar plates for 48 h for observation. The lowest drug concentration that yielded no growth was documented as minimum bactericidal concentration (MBC). For *P. gingivalis* all the procedures took the slow growth of the organism into account.

Results

TCMs that passed all three tests are classified as TCMs that have an inhibitory effect towards specific bacteria. Those that showed only a weak inhibitory effect in one or two tests, but not all, are classified as partially effective in the inhibition of specific bacteria.

Initial biofilm forming bacteria

The inhibition zone measured for chlorhexidine against *S. mitis* was 7.4 mm compared

with those of *Radix et rhizoma rhei* (da huang) and *Fructus armeniaca mume* (wu mei) which were 4.4 mm and 11.3 mm, respectively (Table 1; Figs. 1 and 5). The average inhibition zone diameter measured for chlorhexidine against *S. sanguis* was 9.1 mm, compared with 3.6 mm and 11.7 mm for *Radix et rhizoma rhei* (da huang) and *Fructus armeniaca mume* (wu mei), respectively (Table 1; Figs. 2 and 5).

Radix et rhizoma rhei (da huang) demonstrated a comparable effect to chlorhexidine against *S. mitis*, while *Fructus armeniaca mume* (wu mei) showed signs of a stronger effect than chlorhexidine (9.1 mm) against *S. mitis* and *S. sanguis*.

Caries causing bacteria

Two of 20 TCM extracts tested demonstrated consistent antimicrobial activity with zones of growth against *S. mutans*.

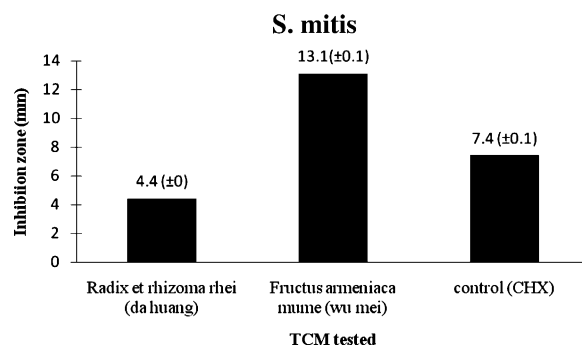


Fig. 1. Sensitivity of *S. mitis* to two out of 20 TCM tested.

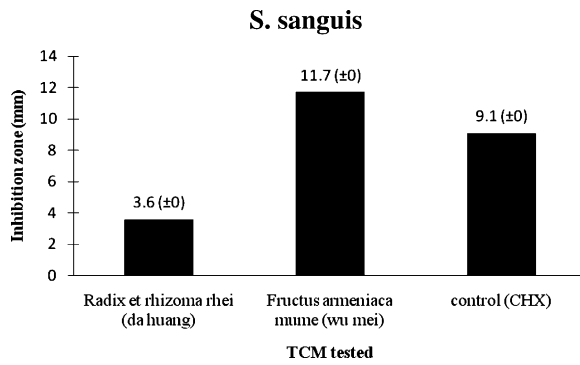


Fig. 2. Sensitivity of *S. sanguis* to two out of 20 TCM tested.

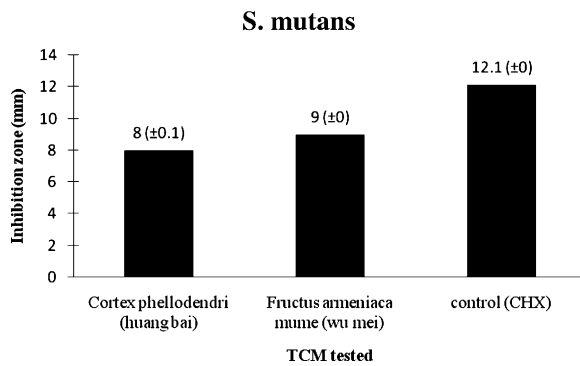


Fig. 3. Sensitivity of *S. mutans* to two out of 20 TCM tested.

The average inhibition zone diameter for *Cortex phellodendri* (huang bai) and *Fructus armeniaca mume* (wu mei) to *S. mutans*, was 8 mm and 9 mm, respectively (Table 1; Figs. 3 and 5).

Periodontal disease causing bacteria

Thirteen of the 20 drugs demonstrated consistent antimicrobial activity with zones of growth inhibition ranging from

2 to 39 mm against *P. gingivalis*. The sequences of drug in ascending orders of effectiveness towards *P. gingivalis* were *Herba menthae haplocalycis* (bo he) (2.3 mm), *Cortex magnoliae officinalis* (hou po) (4.3 mm), *Flos lonicerae japonicae* (jin yin hua) (5.5 mm), *Fructus forsythiae suspensae* (lian qiao) (7.4 mm), *Ramulus cinnamomi cassia* (gui zhi) (8.5 mm), *Herba cum radice violae yedoensis* (zi hua di ding) (9.0 mm), *Flos caryophylli* (ding xiang) (9.8 mm), *Fructus armeniaca mume* (wu mei) (10.6 mm) and *Radix gentiana* (long dan cao) (11.4 mm), *Pericarpium granati* (shi liu pi) (12.9 mm), *Rhizoma cimicifugae* (sheng ma) (13.0 mm), *Radix et rhizoma rhei* (da huang) (31.7 mm) and *Cortex phellodendri* (huang bai) (38.3 mm).

Radix et rhizoma rhei (da huang) (31.7 mm) and *Cortex phellodendri* (huang bai) (38.3 mm) show a significant antimicrobial effect compared with chlorhexidine (23.7 mm) against *P. gingivalis* (Table 1; Figs. 4 and 5).

Partial effects

For the remaining TCMs, *Cortex phellodendri* (ding xiang), *Flos chrysanthemi morifolii* (ju hua), *Flos lonicerae japonicae* (jin yin hua), *Herba cum radice houttuyniae cordatae* (yu xing cao) and *Herba ephedrae* (ma huang) showed weak antimicrobial effects against *S. sanguis*. While *Flos chrysanthemi morifolii* (ju hua) and

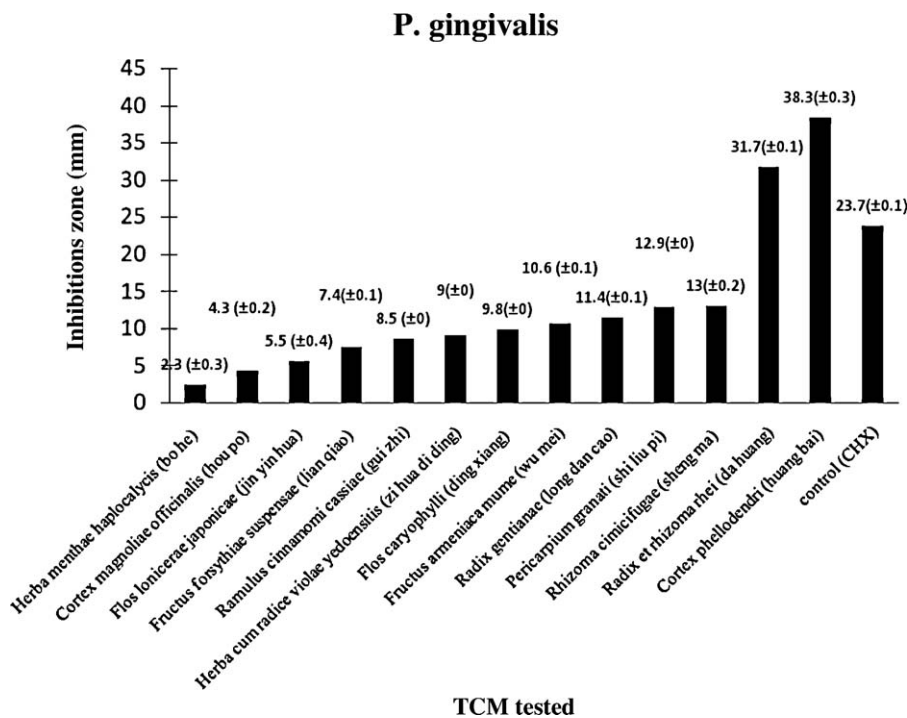


Fig. 4. Sensitivity of *P. gingivalis* to 13 out of 20 TCM tested.

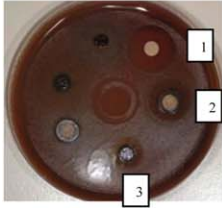
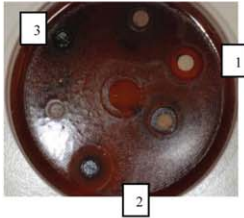
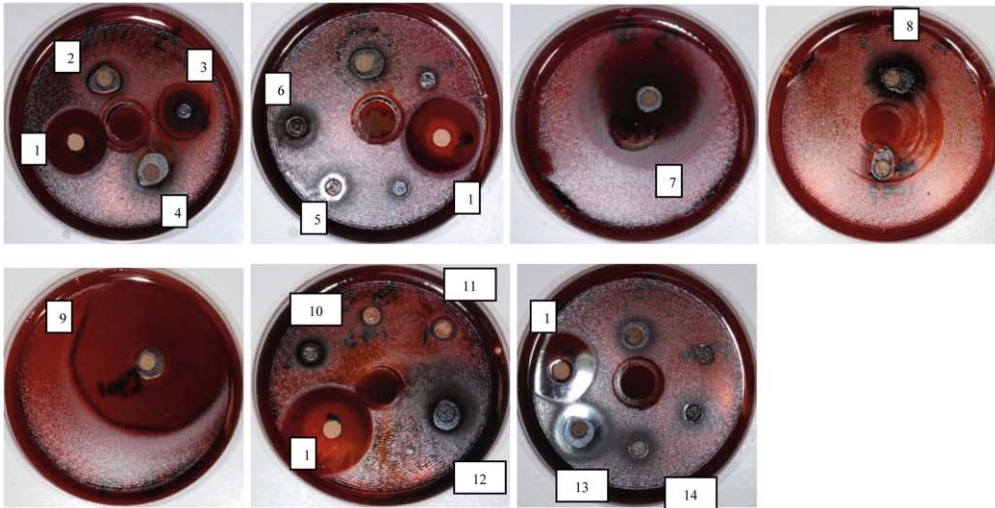
Streptococcus mutans*Streptococcus mitis**Streptococcus sanguis**Porphyromonas gingivalis*

Fig. 5. Results for all bacteria. *S. mutans*: (1) Chlorhexidine, (2) *Cortex phellodendri*, (3) *Fructus armeniaca mume*. *S. mitis*: (1) Chlorhexidine, (2) *Radix et rhizoma rhei*, (3) *Fructus armeniaca mume*. *S. sanguis*: (1) Chlorhexidine, (2) *Fructus armeniaca mume*, (3) *Radix et rhizoma rhei*. *P. gingivalis*: (1) Chlorhexidine, (2) *Cortex magnoliae officinalis*, (3) *Fructus armeniaca mume*, (4) *Radix gentianae longdancao*, (5) *Ramulus cinnamomi cassiae*, (6) *Herba cum radice violae yedoensis*, (7) *Radix et rhizoma rhei*, (8) *Rhizoma cimicifugae*, (9) *Cortex phellodendri*, (10) *Flos lonicerae japonicae*, (11) *Cortex magnoliae officinalis*, (12) *Fructus forsythiae suspensae*, (13) *Pericarpium granati*, (14) *Herba menthae haplocalycis*.

Herba ephedrae (ma huang) demonstrated weak antimicrobial effects against *P. gingivalis* (Table 1).

Statistical analysis

Data were analyzed with statistical analysis computer software (SPSS 15.0 for

Windows[®], Chicago, USA). A one-way ANOVA was used to compare the effects of different TCMs. Differences were considered significant when the *p*-value was less than 0.05. All the TCM results that were shown to be effective against individual bacteria in this experiment proved to be significant (Table 1).

MIC

The MIC values for *Fructus armeniaca mume* (wu mei) against *S. mutans*, *S. mitis* and *S. sanguis* were 0.0781 g/ml. The MIC value against *P. gingivalis* was 0.0003 g/ml (Table 2). The MBC values for *Fructus armeniaca mume* (wu mei) against *S.*

Table 2. MIC and MBC of *Fructus armeniaca mume* (wu mei) against oral microorganisms.

Species	MIC (g/ml)	MBC (g/ml)
<i>S. mutans</i>	0.0781	0.1563
<i>S. mitis</i>	0.0781	0.1563
<i>S. sanguis</i>	0.0781	0.0781
<i>P. gingivalis</i>	0.0003	0.0003

mutans and *S. mitis* were 0.1563 g/ml. Its MBC value against *S. sanguis* was 0.0781 g/ml and 0.0003 g/ml against *P. gingivalis* (Table 2).

Discussion

In this study, 20 TCM extracts were evaluated for their antimicrobial activities against four common bacterial species present in the oral cavity, which are important in biofilm formation (*S. mitis*, *S. sanguis*), as a cause of dental caries (*S. mutans*) or as a cause of periodontal disease (*P. gingivalis*).

This research showed that some TCM are active against oral bacteria. This is one of the first studies to show this possible association. Further research is needed to substantiate this and to evaluate the principles of TCM when applied to antibacterial treatment.

Fructus armeniaca mume (wu mei) was shown to be very effective against all four bacteria; this study is one of the first to show its effects specifically on oral bacteria. *Fructus armeniaca mume* (wu mei), also known as *Prunus mume* or Japanese Apricot, is a common fruit in Asia, which is eaten in its raw form and used as a herbal medicine after it has been cooked and aged. As a TCM, *Fructus armeniaca mume* (wu mei) is taken internally to relieve cough and used externally for the removal of warts and corns.¹⁵ Its antibacterial effect on some bacteria is known, but no research has been carried out on its action against oral bacteria. It has low toxicity compared with inedible oral antiseptic mouth rinses (e.g. chlorhexidine), costs little and is widely available. If it is effective in inhibiting the bacteria commonly found in the oral biofilm, it may be a safe agent for the effective reduction of the oral biofilm and the prevention of dental caries and periodontal disease.

The authors compared the antimicrobial activity of TCMs with one effective oral antiseptic, chlorhexidine. The authors showed that they have comparable effects. This research is the first study that investigates TCMs specifically on oral bacteria. TCMs are promising agents in the development of new antibacterials for oral microorganisms. This study is a key step

in the discovery of new drugs/treatments using TCM. Further research is needed to identify the active components related to the antibacterial action, to determine the range of action, and to investigate the mechanisms involved. The antibacterial actions include the MIC and MBC. It is also necessary to test the antibacterial action on other oral bacteria and fungi. TCM extracts that are less effective against oral bacteria, have potential for further investigation because the active components inside the extracts may only present in very low concentration, so although they may be very effective, the overall antibacterial effect of the extracts is small.

Further research will focus on four strategies. The active components in the antimicrobial action of *Fructus armeniaca mume* should be identified and the mechanisms investigated. It contains a number of acids (e.g. citric acid, malic acid, oxalic acid, succinic acid, fumaric acid, tartaric acid, picric acid); these might create a low pH environment that may account for its antibacterial effect. Several herbs that have an antibacterial action can be combined. This may allow more effective control of microorganisms, and it may be possible to combat the newly emerging resistant microorganisms. TCM with similar properties to *Fructus armeniaca mume* should be screened for their antimicrobial actions. *In vivo* and clinical studies should be carried out to optimize the clinical use of *Fructus armeniaca mume* in mouth rinses, toothpaste, chewing gum and other oral products.

In this study, *Fructus armeniaca mume* was found active against all four oral bacteria. This result is unexpected as *Fructus armeniaca mume* is used for cough suppression in TCM. This shows that only investigating herbs with strong suggestions of antimicrobial action is not enough as some important sources of antimicrobial action may be missed. In conclusion, *Fructus armeniaca mume* had inhibitory effects on *S. mitis*, *S. sanguis*, *S. mutans* and *P. gingivalis* *in vitro*. It is possible that its acidic content accounts for its wide antibacterial spectrum.

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Competing interests

The author(s) declare that they have no competing interests.

Ethical approval

Not required.

Authors' contributions

MY performed TCM collection, method development, validation, result analysis and manuscript drafting. CS performed MIC and MBC data collection. RW and UH participated in the design of the study, selection of TCM for testing and manuscript drafting. RW, UH and LS conceived the idea of the study and participated in its design and coordination. All authors approved the final manuscript.

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