

Antimicrobial Activity of Traditional Chinese Medicines on Common Oral Bacteria

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Received January 24, 2011; revised March 8, 2011; accepted March 15, 2011

Abstract

Objective: To evaluate twenty Traditional Chinese Medicines (TCM) against four oral bacteria. **Methods:** Twenty TCM were tested for sensitivity against *Streptococcus mitis*, *Streptococcus sanguis*, *Streptococcus mutans* and *Porphyromonas gingivalis*. Aliquots of suspension of each bacterial species were inoculated on a horse blood agar (HBA) plate, 6 mm diameter paper disks soaked in different drug suspensions were placed concentrically on a HBA plate. Disks soaked in 0.2% w/v chlorhexidine were used as positive controls. These HBA plates were incubated for 48 hours anaerobically and the diameters of growth inhibition of three different areas were measured using a calibrated computer software and the mean diameter obtained for each bacteria. Broth microdilution assay was used to determine minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). The experiment was repeated on three separate occasions. **Results:** The TCMs that consistently against *Porphyromonas gingivalis*, included *Folium artemisiae argyi*, *Fructus crataegi*, *Rhizoma dryopteris crassirhizomae*, *Flos magnoliae*, *Rhizoma polygoni cuspidati*, *Radix scrophulariae ningpoensis*, *Galla chinensis*, *Radix scutellariae baicalensis* and *Rhizoma coptidis*; against *Streptococcus mutans* included *Fructus crataegi*, *Galla chinensis* and *Rhizoma coptidis*; against *Streptococcus mitis* and *Streptococcus sanguis* included *Galla chinensis* and *Rhizoma coptidis*. **Conclusion:** *Rhizoma coptidis* and *Galla chinensis* had inhibitory effects on *Streptococcus mitis*, *Streptococcus sanguis*, *Streptococcus mutans* and *Porphyromonas gingivalis* *in vitro*.

Keywords: Chinese Medicine, Antimicrobial Activity, *Streptococcus mutans*, *Streptococcus sanguis*, *Streptococcus mitis*, *Porphyromonas gingivalis*, Oral Biofilm

1. Introduction

Dental caries is a common human disease that affects a vast majority of people. It is a chronic endogenous infection caused by the normal oral commensal flora [1]. Oral biofilm develop on all natural or artificial shedding and non-shedding surfaces of the oral cavity. Microorganisms in oral biofilm are the major aetiological agents of dental caries. Other than caries, oral biofilm can cause many oral infections including periodontal disease and candidiasis. Plaque formation involves initial colonization and multiplication by pioneer species, followed by secondary colonization by other species and finally becoming a climax community [2]. The “pioneer species” of oral biofilm are *Streptococcus oralis*, *Streptococcus mitis* and *Streptococcus sanguis*. There are also specific bacteria that are closely

related to specific dental diseases, for example, *Streptococcus mutans* and *Porphyromonas gingivalis* are associated with dental caries and periodontal disease, respectively [1].

Traditional Chinese Medicines (TCM) has been used in China to treat various infectious diseases for more than four thousand years. Different from the western-medicine, TCM works as a formula of herbs that is tailored to individual patient under their specific condition.

They are designed in the form of remedy that uses one or two main ingredients that target the illness, with many other ingredients also added to adjust the formula to suit patient’s condition [3]. Recently the mechanism of one of these formulae has been investigated at molecular, cellular and organism levels [4]. TCM possess a variety of biological properties that has the potential to be de-

veloped as effective drugs. Certain TCM have been shown to have antibacterial properties and so far none has shown to have any known resistance. Currently, a number of TCMs has already been used in oral health-care products such as toothpaste according to their effects. Yet few studies have been performed to screen these TCM and evaluate their effectiveness against oral bacteria forming oral biofilm.

The twenty TCMs are selected according to their antibacterial properties that enable them to treat infection and disease at different part of body. The aim of this study was to evaluate in vitro twenty TCMs that are currently used to treat infectious diseases; for their antimicrobial activity against oral biofilm bacteria and caries and periodontal disease. The hypothesis is to investigate the TCMs investigated in the study have effect against the four common oral bacteria.

2. Methods

2.1. Organism and Culture Condition

Frozen isolates of type cultures of *Streptococcus mitis* (ATCC 15914), *Streptococcus sanguis* (ATCC 10556), *Streptococcus mutans* (ATCC 35668) and *Porphyromonas gingivalis* (ATCC 33277) were thawed and their identity reconfirmed using standard methodology. They were then inoculated onto horse blood agar (HBA) and incubated anaerobically at 37°C for 3 days. For sensitivity studies the bacterial cultures were suspended in phosphate buffered saline at a concentration of 1×10^6 cells/mL (0.5 MacFarland Standard Units).

2.2. Identification and Preparation of TCM Extracts

The twenty TCM were purchased from a local Chinese Medicine store and were identified morphologically, histologically and chemically using standard Chinese herbal identification procedures [5].

Aqueous extracts of TCM were prepared using standard protocol [5]. Briefly, 4mL distilled water was added to 10 g of TCM powder. The mixture was boiled with constant stirring for 4 hours with occasional adding of distilled water to prevent drying. Distilled water was added at the end 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 twenty TCM chosen for the study were *Rhizoma coptidis*, *Radix arnebia*, *Herba artemisiae*, *Flos magnoliae*, *Radix bupleuri*, *Galla chinensis*, *Folium artemisiae argyi*, *Radix scrophulariae ningpoensis*, *Radix scutellariae baicalensis*, *Rhizoma polygoni cuspidati*, *Folium*

isatidis, *Fructus crataegi*, *Herba patriniae cum radice*, *Rhizoma dryopteris crassirhizomae*, *Spica prunellae vulgaris*, *Radix sophorae*, *Fructus gardeniae jasminoidis*, *Anemarrhena aspodeloidea Bunge*, *Cortex fraxini* and *Tarxacum mongolicum*. Chlorhexidine gluconate, a common oral antiseptic, at a concentration of 0.2% w/v was used as a positive control for all experiments.

2.3. Agar Diffusion Assay

The standard agar diffusion assay for sensitivity testing was performed according to a standard protocol [1]. 20 μ L aliquots of suspension of each bacterial species were inoculated on horse blood agar (HBA) plates using glass rods, then 6 mm diameter paper disks soaked in 10 μ L of each of the 2.5 g/mL TCM extract were placed concentrically on the HBA plate. Positive controls were disks soaked in 10 μ L of 0.2% w/v chlorhexidine placed in the HBA plates. These HBA plates were incubated anaerobically for 48 hours at 37°C. After that, measurements of any growth inhibition zone were evaluated using a calibrated computer software (Image J 1.40 for Windows, NIH Image, Maryland, USA). The diameters of growth inhibition of three different directions were measured and the mean diameter of growth inhibition was calculated for each organism. The experiment was repeated on three separate occasions. In cases where no clear bacteria growth inhibition could be seen but the bacteria surface appearance changed, partial inhibitory effect was recorded.

2.4. 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 [6]. Inocula of 24 hours bacteria cultures were standardized to a turbidity equivalent of 0.5 McFarland standard at 520 nm with a spectrophotometer. The suspensions were further diluted in Rosewell Park Memorial Institute (RPMI) 1640 medium (Life technologies, New York, USA) to yield an inoculum concentration of approximately 1×10^4 CFU mL⁻¹. MIC assay was performed in 96-well round-bottomed microtiter plates (Iwaki, Tokyo, Japan) and each of the bacteria was exposed to a double dilution of each of TCM agents. The plates were covered with a lid and incubated for 72 hours at 37°C in an anaerobic chamber to evaluate the growth kinetics. The MIC of each TCM drug was defined as the lowest concentration that prevents visible turbidity of the broth. Same procedure had also been carried out on a

horseblood agar plate to evaluate the minimal bactericidal concentration (MBC) which classified as the lowest concentration of drug that kills at least 99.9% of the CFUs contained in the original inoculums. Experiments were repeated on three different occasions with duplicate determinations on each occasion.

2.5. Statistic Analysis

Data were analysed with a statistical analysis computer software (SPSS 15.0 for Windows©, Chicago, USA). Data were performed with one-way ANOVA to compare the effects of different TCMs. Differences of pair-wise comparison of each TCM with the positive control were considered significant when the p value was less than 0.05. All TCMs that were showed effective against individual bacteria in this experiment proved to be significant except when *Folium artemisiae argyi* was tested against *P. gingivalis* (Table 1).

3. Results

3.1. Initial Biofilm forming Bacteria (*Streptococcus mitis*, *Streptococcus sanguis*)

The mean of inhibition zones of chlorhexidine measured were 7.4 mm against *Streptococcus mitis* compared to *Galla chinensis* and *Rhizoma copitidis*, which were of 5.8 mm and 6.2 mm respectively (Table 1, Figures 1 and 2). The average mean of inhibition zone measured in diameter of chlorhexidine was 9.1 mm against *Streptococcus sanguis*, where as *Galla chinensis* and *Rhizoma copitidis* were measured as 10.6 mm and 7.6 mm. *Rhizoma copitidis* demonstrated a comparable effect to chlorhexidine against *Streptococcus mitis*, while *Galla chinensis* showed sign of stronger effect than the chlorhexidine (9.1 mm) against *Streptococcus sanguis*.

3.2. Caries Causing Bacteria (*Streptococcus mutans*)

The mean of inhibition zones of chlorhexidine measured were 12.1 mm against *Streptococcus mutans*. Three out of twenty TCM extracts tested demonstrated consistent antimicrobial activities with zones of growth inhibition ranging from 5 mm to 12 mm against *Streptococcus mutans*. The average mean of inhibition zones measured in diameter of *Fructus crataegi*, *Galla chinensis* and *Rhizoma copitidis* to *Streptococcus mutans*, are 5.4 mm, 9.6 mm and 11.4 mm respectively (Table 1, Figure 3). When compared to the positive control, chlorhexidine, which is a highly potent antibacterial agent, *Rhizoma copitidis* demonstrated a similar effect to chlorhexidine (12.1 mm).

Table 1. Table showing antimicrobial property of TCMs.

	<i>S. mutans</i>	<i>S. mitis</i>	<i>S. sanguis</i>	<i>P. gingivalis</i>
<i>Cortex fraxini</i>	-	-	-	P
<i>Flos magnoliae</i>	-	-	-	9.6 (± 0.1)*
<i>Folium artemisiae argyi</i>	-	-	-	7.2 (± 0.6)
<i>Folium isatidis</i>	-	-	-	P
<i>Fructus crataegi</i>	5.4 (± 0.3)*	-	P	7.3 (± 0.1)*
<i>Fructus gardeniae jasminoidis</i>	-	-	-	-
<i>Galla chinensis</i>	9.6 (± 0.1)*	5.8 (± 0.2)*	0.6 (± 0)*	20.2 (± 0.2)*
<i>Herba artemisiae</i>	-	-	-	P
<i>Herba patriniae cum radice</i>	-	-	-	-
<i>Herba taraxaci mongolici cum radice</i>	-	-	P	P
<i>Radix arnebia</i>	-	-	-	P
<i>Radix bupleuri</i>	-	-	-	-
<i>Radix scrophulariae ningpoensis</i>	-	-	P	19.4 (± 0.1)*
<i>Radix scutellariae baicalensis</i>	-	-	P	25.3 (± 0.1)*
<i>Radix sophorae</i>	-	-	P	-
<i>Rhizoma anemarrhenae</i>	-	-	-	-
<i>Rhizoma copitidis</i>	11.4 (± 0)*	6.2 (± 0.2)*	7.6 (± 0)*	56.3 (± 0.2)*
<i>Rhizoma dryopteris crassirhizomae</i>	-	-	-	9.2 (± 0.3)*
<i>Rhizoma polygoni cuspidati</i>	-	-	-	12.1 (± 0.1)*
<i>Spica prunellae vulgaris</i>	-	-	-	P
<i>Control</i>	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)

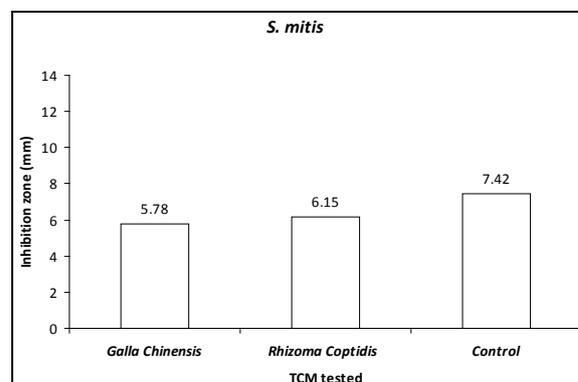


Figure 1. Sensitivity of *S. mitis* to 2 out of 20 TCM tested shown in mean value.

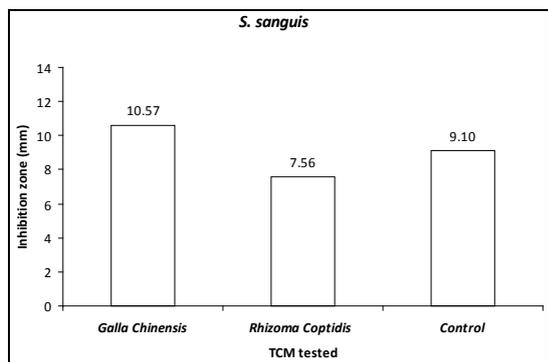


Figure 2. Sensitivity of *S. sanguis* to 2 out of TCM tested shown in mean value.

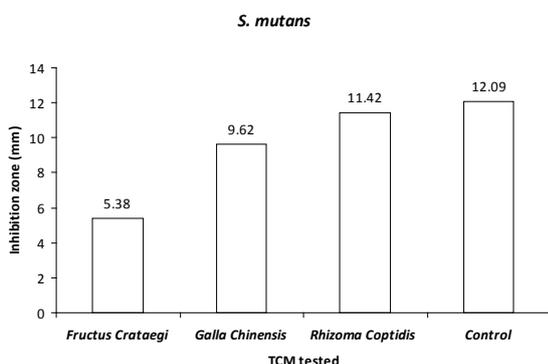


Figure 3. Sensitivity of *S. mutans* to 3 out of 20 TCM tested shown in mean value.

3.3. Periodontal Disease Causing Bacteria (*Porphyromonas Gingivalis*)

Chlorhexidine was measured to have 23.7 mm in average mean against *Porphyromonas gingivalis*. Nine of the twenty TCM extracts also demonstrated consistent antimicrobial activity with zones of growth inhibition ranging from 7 mm to 57 mm against *Porphyromonas gingivalis*. The sequences of drug in ascending orders of effectiveness towards *Porphyromonas gingivalis* were *Folium artemisiae argyi* (7.2 mm), *Fructus crataegi* (7.3 mm), *Rhizoma dryopteris crassirhizomae* (9.2 mm), *Flos magnoliae* (9.6 mm), *Rhizoma polygoni cuspidati* (12.1 mm), *Radix scrophulariae ningpoensis* (19.4 mm), *Galla chinensis* (20.2 mm), *Radix scutellariae baicalensis* (25.3 mm) and *Rhizoma coptidis* (56.3 mm). *Galla chinensis* (20.2 mm) and *Radix scutellariae baicalensis* (25.3 mm) demonstrated comparable effective to chlorhexidine (28.7 mm), while *Rhizoma Coptidis* shows a significant antimicrobial effect compares with chlorhexidine against *Porphyromonas gingivalis* (Table 1, Figure 4).

3.4. Partial Effects

For the remaining TCMs tested, *Fructus crataegi*, *Radix*

scutellariae baicalensis, *Radix scrophulariae ningpoensis*, *Radix sophorae* and *Herba taraxaci mongolici cum radice* showed weak antimicrobial effects against *Streptococcus sanguis*. While *Cortex fraxini*, *Herba artemisiae*, *Folium isatidis*, *Spica prunellae vulgaris*, *Radix arnebia* and *Herba taraxaci mongolici cum radice* demonstrated weak antimicrobial effects against *Porphyromonas gingivalis*.

3.5. Minimum Inhibitory Concentrations

The MIC and MBC values for *Rhizoma Coptidis* against *S. mutans* were 0.039 g/mL and 0.156 g/mL respectively. It stated as 0.020 g/mL and 0.156 g/mL while against *S. mitis*. And 0.039 g/mL for both MIC and MBC values when against *S. sanguis* (Tables 2 and 3).

Galla chinensis when hold against *S. mutans* had the MIC value as 0.0391 g/mL and 0.078 g/mL as MBC value. For *S. mitis*, the MIC value was 0.010 g/mL and MBC value was 0.078 g/mL. Both MIC and MBC values were the same when *Galla chinensis* against *S. sanguis*, which is 0.039 g/mL (Tables 2 and 3).

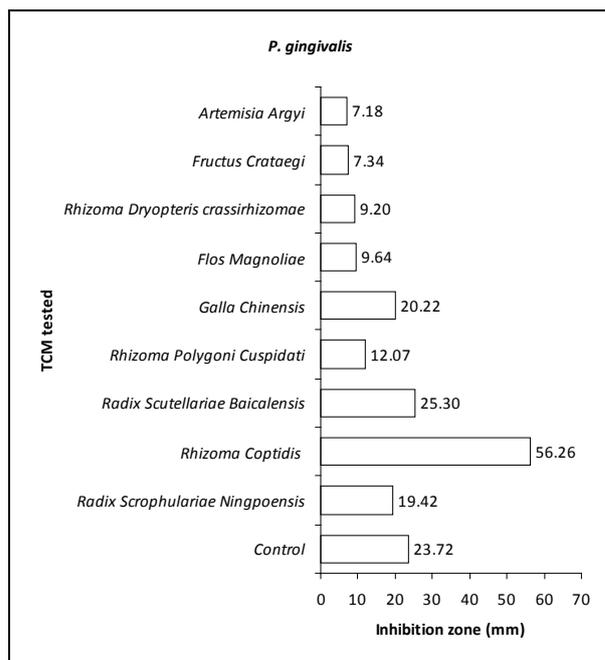


Figure 4. Sensitivity of *P. gingivalis* to 9 out of 20 TCM tested shown in mean value.

Table 2. MIC of *Rhizoma copiditis* and *Galla chinensis* against oral micro-organisms.

Species	<i>Rhizoma copiditis</i> (g/mL)	<i>Galla chinensis</i> (g/mL)
<i>S. mutans</i>	0.039	0.039
<i>S. mitis</i>	0.020	0.010
<i>S. sanguis</i>	0.039	0.039
<i>P. gingivalis</i>	<0.001	<0.001

Table 3. MBC of *Rhizoma copiditis* and *Galla chinensis* against oral micro-organisms.

Species	<i>Rhizoma copiditis</i> (g/mL)	<i>Galla chinensis</i> (g/mL)
<i>S. mutans</i>	0.156	0.078
<i>S. mitis</i>	0.156	0.078
<i>S. sanguis</i>	0.039	0.039
<i>P. gingivalis</i>	<0.001	<0.001

Both MIC and MBC for *Rhizoma Coptidis* and *Galla chinensis* against *P. gingivalis* were below <0.001 g/mL, which reached the lowest boundary for both MIC and MBC (Tables 2 and 3).

4. Discussion

In this study, twenty TCM extracts were evaluated for their antimicrobial activities against four common bacterial species presented in oral cavity which had been considered as important in biofilm formation (*Streptococcus mitis*, *Streptococcus sanguis*), or causing dental caries (*Streptococcus mutans*) or causing periodontal disease (*Porphyromonas gingivalis*).

Chlorhexidine was showed to be effective against all four tested bacteria. Of these, two TCM extracts, *Rhizoma copiditis* and *Galla chinensis* were shown to have similar effects. Although the first one has gained much attention due to its wide range of antimicrobial activities [5,7-9], ours is the first to show their effects specifically on oral bacteria. It has shown antimicrobial activity against periodontopathogenic bacteria including *Porphyromonas gingivalis*, *Prevotella intermedia*, *Prevotella nigrescens* and *Actinobacillus actinomycetemcomitans* *Actinomyces naeslundii*, but had less inhibitory effect on the growth of *Streptococcus* and *Lactobacillus* [5]. One active component, berberine alkaloid has been shown to have potent activity against all tested strains of methicillin-resistant *Staphylococcus aureus* (MRSA) [7]. Other studies demonstrated the antibacterial effect of *Rhizoma copiditis* and its alkaloids against *Propionibacterium* and *Helicobacter pylori* [8,9]. Results of the Control Group were published in a contemporary study evaluating another group of TCMs [10].

Results showed that both *Rhizoma copiditis* and *Galla chinensis* have comparable effects with chlorhexidine. When comparing the two TCMs, both *Galla chinensis* and *Rhizoma Coptidis* demonstrated the same MBC values against *S. sanguis* (Tables 2 and 3). *Galla chinensis* showed a lower MBC value against *S. mitis* and *S. sanguis* compared to *Rhizoma Coptidis*. This demonstrated that *Galla chinensis* has a higher bacteriocidal activity than *Rhizoma Coptidis* against the two bacteria. For *P. gingivalis*, both TCMs reached the lowest concentration boundary for the MIC and MBC values, further investigations are needed to determine the exact MIC and MBC

values for both TCMs. This study is the first study that investigates TCMs specifically on oral bacteria. Therefore these TCMs are promising agents that can develop to new antibacterials for oral micro-organisms. Further research is needed to identify the specific active components that are related to the antibacterial action, to determine the range of action, and to investigate the mechanisms involved.

5. Conclusions

Both *Rhizoma copiditis* and *Galla chinensis* had inhibitory effects on *Streptococcus mitis*, *Streptococcus sanguis*, *Streptococcus mutans* and *Porphyromonas gingivalis* *in vitro*.

6. Acknowledgements

We thank Ms Joyce Yau on her technical assistance. This study was supported by the University Research Grant No.: 10207346.15633.08003.323.01, The University of Hong Kong.

7. Conflict of Interest Statement

The authors declare that there are no potential conflicts exist between the authors and the products mentioned in the paper.

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