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Prunus mume extract exhibits antimicrobial activity against pathogenic oral bacteria

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Objectives. *Prunus mume* is a common fruit in Asia, which has been used in traditional Chinese medicine. In this study, we focused on the antimicrobial properties of *Prunus mume* extract against oral pathogens related to dental caries and periodontal diseases.

Study design. A total of 15 oral pathogens including *Streptococcus mutans*, *S. sobrinus*, *S. mitis*, *S. sanguinis*, *Lactobacillus acidophilus*, *P. gingivalis*, *Aggregatibacter actinomycetemcomitans*, and *Candida* species were included in the study. Initially, agar diffusion assay was performed to screen the antimicrobial activities of *Prunus mume* extract. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were then determined for sensitive species. Effect of *Prunus mume* extract on human oral keratinocytes (HOK) viability was also tested.

Result. In the agar diffusion assay, drug suspension of 2 g/mL was able to inhibit all the bacterial species tested, but not the fungal species. MIC and MBC range of *Prunus mume* extract against the oral bacteria was 0.15625–0.0003 g/mL and *P. gingivalis* being the most susceptible species. Prune extract did not cause any detrimental effect on HOK.

Conclusion. *Prunus mume* extract may be a potential candidate for developing an oral antimicrobial agent to control or prevent dental diseases associated with oral pathogenic bacteria.

Introduction

Two major dental diseases in the world are dental caries and periodontal disease[1]. Microorganisms in the form of plaque biofilms are a prerequisite for the development of aforementioned dental diseases. Gram-positive bacteria such as, *Streptococcus mutans*, *Streptococcus sobrnius*, Lactobacillus spp. are major causative pathogens related to dental caries, whereas Gram-negative anaerobic bacteria, such as *Aggregatibacter actinomycetemcomitans*, *Porphyromonas gingivalis*, are closely related to the periodontal diseases[2].

Mouth rinses that contain various forms of antimicrobial agents have been developed to control the level of these pathogenic oral bacteria; however, emerging antimicrobial drug resistance has posed a major global challenge with increasing number of resistant strains including that of pathogenic oral bacteria against commonly used antimicrobials. Therefore, there is an urgent need of novel antimicrobial agents, which prompted us to search for new antimicrobial compounds from various other sources. Traditional Chinese medicines (TCMs) are a rich natural source of 'traditional' antimicrobial agents, which have been used in China to treat various infectious diseases for more than 4000 years. TCM possess a spectrum of different properties that make them ideal candidates for novel drugs. A number of research workers in the past have shown potential antimicrobial properties of TCM[3,4]. In a pilot study of large-scale TCM screening, we found that *Prunus mume* possess considerable antimicrobial properties. Hence, this study was devised to study the antimicrobial properties of *Prunus mume* against pathogenic oral bacteria.

Prunus mume (also known as Japanese Apricot, a species of *Prunus*) is a common edible fruit in Asia. Aged *Prunus mume* is used as an

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herbal medicine in TCM. Hence, in TCM, *Prunus mume* served internally to relieve cough and externally for warts and corns removal. Interestingly, a recent study showed that *Prunus mume* extract could be an effective agent to inhibit *Helicobacter pylori*, a bacterium associated with gastritis and peptic ulcers[5]. Although *Prunus mume* has been documented to be antibacterial, no studies have been specifically undertaken on its action against oral bacteria. Therefore, the aim of this study was to evaluate the antimicrobial activity of *Prunus mume* against a range of oral bacterial and fungal pathogens. Moreover, we also evaluated the high-performance liquid chromatography (HPLC) spectrum of the *Prunus mune* to assess the presence of active components. Further, we tested whether the *Prunus* extract has any detrimental effect on primary human oral keratinocytes (HOK) to assess its safety.

Materials and methods

Organisms and culture conditions

In this study, we used a total of 15 Gram-positive, Gram-negative bacteria, and fungal species were used for the study. *Streptococcus mutans* (ATCC 35668), *Streptococcus mutans* (ATCC 700610), *Streptococcus mitis* (ATCC 15914), *Streptococcus sanguinis* (ATCC 10556), *Streptococcus sobrinus* (ATCC 33478), *Lactobacillus acidophilus* (ATCC 9224), and *L. acidophilus* (WT-1) were included as Gram-positive bacteria. *Porphyromonas gingivalis* (ATCC 33277) and *A. actinomycetemcomitans* (ATCC 43718) were included as Gram-negative bacteria. Fungal species were *Candida albicans* (ATCC 90028), *Candida glabrata* (ATCC 90030), *Candida krusei* (ATCC 6258), *Candida tropicalis* (ATCC 13803), *Candida parapsilosis* (ATCC 22014), and *Saccharomyces cerevisiae* CAN-1. All bacterial and fungal isolates were obtained from the archival collection of Oral Biosciences, Faculty of Dentistry, The University of Hong Kong. Frozen isolates were thawed, and the identity reconfirmed using standard methodology. Bacterial species were inoculated on Horse blood agar (HBA) plates and incubated in an anaerobic chamber (5% CO_2, 10% H_2 and 85% of N2) at 37 °C for 2–5 days as appropriate. The bacterial cultures were harvested and suspended in phosphate-buffered saline at a concentration of 1×10^6 cells/mL (0.5 MacFarland Standard Units) for the sensitivity studies.

Antimicrobial susceptibility testing

For the antimicrobial susceptibility testing, desired drug concentration was prepared in distilled water and the mixture was autoclaved before each experiment. The standard agar diffusion assay for sensitivity testing on *Prunus mume* was performed according to a standard protocol[6,7]. Twenty microlitre aliquots of suspensions of each bacterial species were inoculated on a HBA plate using a glass rod and afterwards, a 6-mm-diameter paper discs soaked in 10 μL of (2 g/mL) of each of the drug suspensions were placed concentrically on the HBA plate. Discs soaked in 10 μL of 0.2% w/v chlorhexidine were used as positive controls. These HBA plates were incubated for 48 h (for *P. gingivalis* more than 5 days) anaerobically at 37 °C. Afterwards, naked eye measurement of growth inhibition zone, if any, was evaluated using a finely calibrated ruler (Quanzhou Light Industry, Quanzhou City, Fujian, China). The diameter of growth inhibition of three different areas was measured, and the mean diameter obtained for each organism. The experiment was repeated on three separate occasions.

MIC and MBC determination

The organisms sensitive to the drug in disc diffusion assay were selected for minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) determination. Broth macrodilution and broth microdilution assays were performed according to the standard NCCLS criteria and with slight modifications[8]. In brief, bacteria were cultured anaerobically in HBA plates for 3–7 days at 37 °C. The bacteria cultures were harvested and suspended BHI at a concentration of 1×10^6 cells/mL (0.5 McFarland Standard Units) for the broth macrodilution assay. Drugs were serially double diluted in 1.5-ml Eppendorf tubes and then bacterial suspensions were added as 1 : 1 ratio. These were incubated for 48 h at 37 °C.

The lowest concentration without visual growth was recorded as the MIC. After MIC determination, 20 μL of was inoculated in HBA and kept for 48 h for observation. The lowest drug concentration that yielded no growth was documented as MBC. Aforementioned protocol was slightly different for of *P. gingivalis* taking its slow growth into consideration. Thus, *P. gingivalis* was prepared in '*P. gingivalis* broth' (TSB 30 g/litre, Yeast extract 5 g/litre, hemin 0.005 g/L, Vit K 0.001 g/L, dH2O 1 L) for the optimal growth of the organism. Initial inoculum of *P. gingivalis* was 1×10^8 cells/mL (2 McFarland Standard Units), which incubated with drugs for 5 days for MIC determination. Thereafter, a 20 μl bacterial suspension was inoculated in HBA and kept for 5 days for MBC determination as described earlier.

High-performance liquid chromatography (HPLC)

Dried power of *Prunus mume* was obtained commercially by Nongs Company Ltd (http://www.nongs.com). High-performance liquid chromatography profile of the Prunus power was obtained using reverse phase HPLC. Isolation was performed using a C18 coated silica gel column. The mobile phase was 20% of ammonium phosphate and 80% of methanol, and the flow rate was 1 mL/min. Dissolved *Prunus mume* powder in water (100 mg/mL) was tested along with some of the organic acids viz. citric acid, oxalic acid, tartaric acid, fumaric acid, and succinic acid. Antimicrobial activity of some active components was tested against *S. mutans* using disc diffusion assay as described earlier.

Effect on human oral keratinocytes

Next, we tested whether the Prune extract has any detrimental effect on HOK using viability assay. Human oral keratinocytes (ScienCell Research Laboratories, Carlsbad, USA) were seeded to presterilized polystyrene 96-well plates at a cell concentration of about 2×10^5 cells/well in Dulbecco's Modified Eagle-Ham's F-12 (DMEM) (ScienCell) and incubated in 8% CO_2 atmosphere at 37 °C until they were about 90% confluent. Thereafter, cells were exposed to 0.2 g/mL of Prune extract and 0.02% chlorhexidine for 5 min. Then, extract suspension was aspirated, cells were washed with PBS, and incubated with 0.5 mg/mL MTT [3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide] for 4 h. Then, equal volume of DMSO was added to each well and further incubated for 30 min. Optical density of the wells were measured at 570 and 690 nm[9].

Statistical analysis

The data are presented as the mean ± SD. Statistical significance of disc diffusion and MIC between the controls and Prunus mume was evaluated by Mann–Whitney *U* test using the Statistical package for Social Sciences version 17 (SPSS Inc., Chicago, IL, USA) for windows. Statistical significance of cell viability assay for keratinocytes between groups was evaluated by ANOVA, and post hoc Bonferroni test. $P < 0.05$ was considered significant.

Results

Antibacterial susceptibility testing

Standard agar diffusion assay preformed to screen the sensitivity of microorganisms to the drug suspension of 2 g/mL was able to significantly inhibit ($P < 0.05$) all the bacterial species tested including *S. mutans*, S. *mitis*, *S. sanguinis*, *P. gingivalis*, *L. acidophilus*, *A. actinomycetemcomitans* compared to the negative control (Table 1). None of the tested fungal species, i.e., *Candida* species and *S. cerevisiae*, were sensitive to Prunus mume extract at this concentration (i.e., there were no inhibition zones in agar diffusion assay). Broth microdilution assay revealed that Prunus extract was effective against all putative oral pathogenic bacteria tested compared to the control ($P < 0.05$), *P. gingivalis* being more sensitive than the other species (Table 2).

HPLC profile of Prunus mume

HPLC profile showed that serial organic acids are the main ingredients in the Prune mume extract, and citric acid (14863043), tartaric acid (1151546), and oxalic acid (517743) were present in the HPLC profile (Fig. 1). Therefore, as the next step we tested the antimicrobial

Microorganism	Inhibition zone (mean ± SD in mm)	
	0.2% Chlorhexidine	Prunus mume
Streptococcus mutans ATCC 35668	8.23 (± 0.1)	18.0 (± 0.1)
S. mutans ATCC 700610	8.22 (± 0.1)	12.38 (± 0.1)
S. mitis ATCC 15914	8.28 (± 0.1)	8.59 (± 0.2)
S. sobrinus ATCC 33478	11.0 (± 0.1)	12.0 (± 0.1)
S. sanguinis ATCC 10556	11.05 (± 0.2)	5.95 (± 0.2)
P. gingivalis ATCC 33277	11.94 (±0.3)	20.44 (± 0.1)
Lactobacillus acidophilus ATCC 9224	9.1 (±0.1)	8.77 (±0.2)
L. acidophilus WT-1	9.1 (±0.1)	8.76 (±0.2)
Aggregatibacter actinomycetemcomitans ATCC 43718	13.5 (±0.2)	15.5 (±0.2)

Table 1. Agar diffusion assay of Prunus mume against oral pathogens. Inhibitory zones were statistically significant ($P < 0.05$) compared to that of controls.

Microorganism	MIC (g/mL)	MBC (g/mL)
S. mutants ATCC 35668	0.078125	0.15625
S. mutants ATCC 700610	0.078125	0.15625
S. mitis ATCC 15914	0.078125	0.15625
S. sobrinus ATCC 33478	0.05	0.05
S. sanguinis ATCC 10556	0.078125	0.078125
P. gingivalis ATCC 33277	0.0003	0.0003
Lactobacillus acidophilus ATCC 9224	0.15625	0.15625
L. acidophilus WT-1	0.15625	0.15625
Aggregatibacter actinomycetemcomitans ATCC 43718	0.078125	0.078125

Table 2. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of Prunus mume extract against oral pathogenic bacteria. MIC values were significant ($P < 0.05$) compared to the controls.

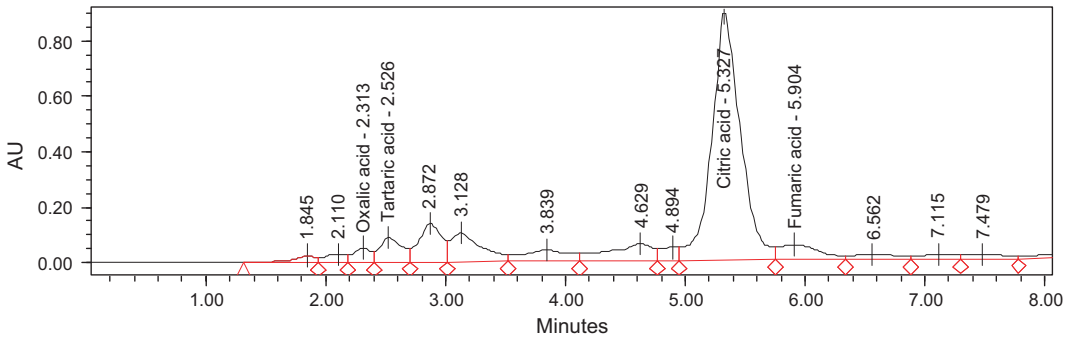

Fig. 1. High-performance liquid chromatography profile showed that citric acid is the main ingredients in the Prune mume extract, 25 min run time.

activity of critic acid, tartaric acid, and oxalic acid against *S. mutans*. Disc diffusion assay of these acids produced inhibition zones of 12, 14.5, and 16 mm, respectively, compared to 18-mm inhibition zone of Prune (Fig. 2).

Effect on HOK

Effect of *Prunus mume* on HOK was evaluated to determine the safety of the extract for mammalian cells. *Prunus mume* extract did not cause any deleterious effect on HOK as indicated by the activity of MTT assay[9] (Fig. 3). Hence, metabolic activity of the HOK was as same as the control and chlorhexidine exposed cells, which indicated that the viability of cells is similar to that of un-exposed control cells.

Discussion

Dental caries is one of the most prevalent diseases worldwide, problem affecting all age groups throughout the life time[1]. Mutans group streptococci (e.g., *S. mutans*, *S. mitis*) and *Lactobacilli* are the main cariogenic pathogens that cause tooth decay. Evidence from traditional medicine as well as emerging research evidence suggests that consumption of natural fruits in natural form may render a protective

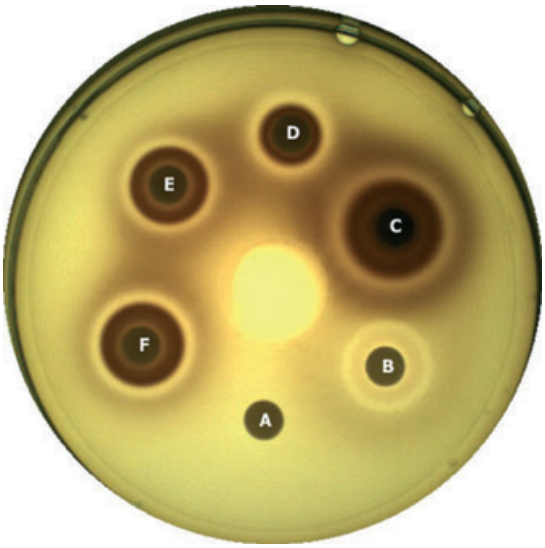

Fig. 2. Disc diffusion assay of critic acid (D), tartaric acid (E) and oxalic acid (F) against *Streptococcus mutans* along with Prunus mume extracts (C). Inhibition zones of the acids were 12, 14.5 and 16 mm compared to that of 18 mm of Prunus mume. PBS was used as the negative control (A) and Chlorhexidine as positive control (B).

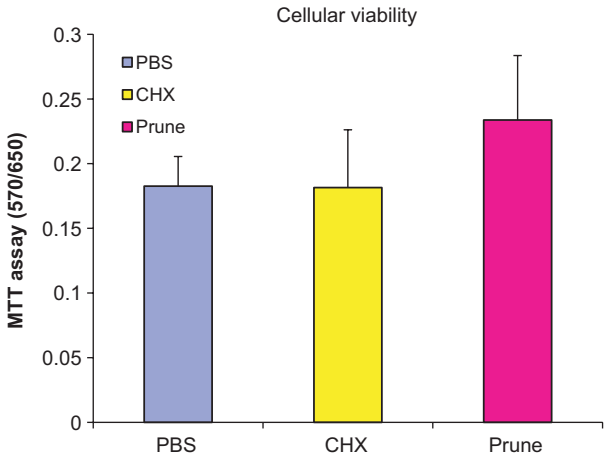

Fig. 3. Viability of human oral keratinocytes following 5 min exposure to the Prunus mume extract measured by MTT reduction assay (mean ± SD)[9]. 0.2% chlorhexidine (CHX) and phosphate-buffered saline (PBS) were used as positive and negative controls, respectively.

effect against dental caries[10]; however, only a few studies have investigated the antimicrobial activity of the fruits in connection with dental caries. Some workers have shown that propolis, a resinous Apis mellifera bees' product, possess antimicrobial activity against mutans streptococci[11]. Moreover, nidus vespae extract have been shown to possess antistreptococcus activity[12]. Some studies have analysed the effect of medicinal plant extract against oral bacteria[13,14]. These studies found that essential oil derived from these plants is effective against *C. albicans* and *S. mutans*; however, aforementioned studies have not investigated the effect of these natural products on other oral bacterial species. Therefore, it appears that this study is the first to demonstrate antimicrobial activity of an edible fruit on multiple species of oral microorganisms.

Periodontal disease, together with caries, affect up to 90% of the human population[15]. *P. gingivalis* and *A. actinomycetemcomitans* bacteria are associated with periodontal diseases. In particular, *P. gingivalis* is well known as 'red-complex' bacteria, which is strongly involved in the pathogenesis of periodontal disease; however, there is no evidence in the English literature to our knowledge on the antiperiodontal effect of natural products. Some studies have shown TCM mixtures have antibacterial effects on periodontal bacteria[2]; however, these studies have not extensively characterized the effect of these products on oral or periodontal bacterial pathogens. Here, we provide primary data that *Prunus mume* extract could be a potentially utilized as an ingredient to control both dental caries and periodontal diseases.

TCM essentially depends on the plant extract, and it has been used from ancient time for infectious disease. Some of these herbal products have been a subject of extensive research in the recent past, which has resulted in isolating active components. Therefore, it is tempting to speculate that, in future, *Prunus mume* could be use as a key ingredient of an antibacterial mouth rinse or other antimicrobial agent incorporated into tooth paste to prevent and control caries and periodontal diseases. As it is a natural compound, it is less likely that products incorporating *Prunus mume* will produce undesirable side effects compared to artificial, synthetic products such as the widely used chlorhexidine-containing derivatives. *Prunus mume* is known to contain active components with antioxidative properties and organic acids[16]. HPLC profile of the Prune mume showed that it contained only a few organic acids and other components are remained to be determined. Although the acidic components of the *Prunus mume*

showed some antibacterial activity, it is lesser compared to the original extract. Therefore, there are other active components in the *Prunus* extract that possess considerable antibacterial activity. Hence, acidic property may be one of its mechanisms of antimicrobial effect; however, for clinical application more molecular studies need to elucidate the mechanism and other active components that are attributed to the antimicrobial activity of *Prunus mume*. There are other studies showing that active components derived from medicinal plants such as Carvacrol and dimer chalcons combination may be used to develop antimicrobial gels for dental diseases[14]. In the present study, it was found that there is no harmful effect on HOK with exposure to *Prunus mume* extract. Therefore, it could be assumed that *Prunus* extract in the form of mouthwash at the given concentration will not cause any detrimental effect on oral tissues; however, it should be noted that we have only used the crude drug preparation of the *Prunus mume*. Next, logical extension of the study is to elucidate the active components and their activity against pathogenic bacteria and oral cells/tissues.

In conclusion, we report here for the first time the antibacterial effect of edible fruit *Prunus mume* against a wide range of oral pathogens. More research into the isolation of active compounds of the *Prunus mume* is warranted to elucidate the mechanisms of its activity against oral bacteria.

What this paper adds

- This study is the first report for the *Prunus mume* as an effective antimicrobial agent to control the oral pathogen.
- This study has provided an insight into a traditional herb medicine as an alternative antimicrobial agent to control the oral pathogen.
- This study provides a new way to prevent dental caries and periodontal diseases.

Why this paper is important to paediatric dentists

- Paediatric dentists should be cautious about the shortcomings of the chemical plaque control.
- The antimicrobial agent extracted from nature plant may be an alternative clinical choice, especially for young patients.
- Oral pathogens are the main aetiology factor for dental caries and periodontal diseases.

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