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**The antimicrobial efficacy of Fructus mume extract on orthodontic bracket: a monospecies-biofilm model study *in vitro***

**Key word: Antimicrobial, Fructus mume, Orthodontic**

## **Abstract**

**Objective:** The aim of this study was to evaluate the antimicrobial efficacy of Traditional Chinese Medicine Fructus mume on a monospecies-biofilm model established on orthodontic brackets *in vitro*.

**Materials and methods:** The antimicrobial effect of Fructus mume aqueous extract on the planktonic *Streptococcus mutans* (*S.mutans*) was tested by microdilution method (MIC). The cell viability of *S.mutans* biofilm on Damon3 MX bracket (Ormco, USA) after exposed to Fructus mume extract was quantified by XTT reduction assay. Visualization of the samples was performed by fluorescence microscope and confocal laser scanning microscopy (CLSM).

**Results:** HPLC analysis revealed the main compounds of Fructus mume are organic acids. The MIC of Fructus mume extract on the planktonic *S.mutans* was 50mg/mL. XTT assay indicated that 250mg/mL Fructus mume extract reduced about 24% cell viability of *S.mutans* biofilm on bracket within 1 min exposure. Microscope image showed Fructus mume extract obviously increased the amount of dead bacteria on the surface of bracket.

**Conclusion:** Fructus mume extract showed antimicrobial effect on *S.mutans* biofilm on orthodontic bracket *in vitro* which may indicate its potential use as an oral antimicrobial agent for orthodontic patients.

## Introduction

Previous research showed the placement of orthodontic brackets does create new locations for plaque retention, thereby increasing plaque adhesion and the inflammatory response (1, 2). Moreover, the morphology and architecture of the orthodontic bracket, as well as the ligation mode, may play a role by providing new areas of retention and protection from plaque-removal forces thus facilitating dental plaque accumulation and maturation (3, 4).

Bacterial plaque plays an essential role in the etiology of periodontal disease and dental caries (5). Aside from daily mechanical hygiene practices, chemical antimicrobial agents have been widely used to improve efficiency of mechanical plaque control in recent decades (6, 7). The main advantage of mouthwashes is its ability to deliver antimicrobial ingredients and benefits to all accessible surfaces in the mouth, including interproximal hard and soft tissues, and remains active for extended periods (8, 9). Since it is hard to fully clean the bracket by mechanical measurement, orthodontic patients are likely to benefit more from daily using mouthrinse (10, 11).

Chlorhexidine (CHX) is regarded as the most effective antibacterial agent for chemical plaque control and has been routinely recommended to orthodontic patients (12). However, side effects to CHX treatment such as alteration taste sensation, tooth discoloration, desquamation and soreness of the oral mucosa have limited its long term usage (13). Therefore, the interest to explore alternative agent, especially agents from natural plants which people are viewed as being safer than synthetic chemicals, and have become increasing in recent decades (14, 15).

Fructus mume (also known as Japanese Apricot, the smoked fruit of *Prunus mume*) was used to relieve cough, treat ulceration and improve digestive function in Traditional Chinese Medicine (TCM) for thousands of years (16). In recent years, Fructus mume extract was reported to have strong inhibition effect on human influenza A virus and suppressive effects on *Helicobacter pylori*-induced glandular stomach lesions in Mongolian gerbils (17, 18). However, its antimicrobial activity for oral pathogens has not been fully investigated yet. Therefore, the aim of this study was to evaluate the antimicrobial efficacy of Fructus mume extract on a monospecies-biofilm model established on orthodontic brackets in vitro.

## **Materials and methods**

### ***Fructus mume aqueous extract preparation***

Fructus mume was purchased from a Chinese medicine store in Hong Kong and voucher specimens were identified under standard procedure (16). The flesh of Fructus mume (20 g) was dried at room temperature and extraction was carried out by boiling with distilled water (100 mL) for a period of 2 hours by heating procedure. Extracts were filtered and the solvent was evaporated on the rotary evaporator under reduced pressure at 4 °C and reaching a concentration 1g/mL solution, and then diluted to 250mg/mL (this concentration Fructus mume extract is used as an internal medicine in TCM.)

### ***High performance liquid chromatography of Fructus mume extract***

High performance liquid chromatography (HPLC) profile of the Fructus mume extract 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 1mL/min. Fructus mume extract was tested with a serial of the organic acids including citric acid, oxalic acid, tartaric acid, fumaric acid and succinic acid, the total running time last for 25 min.

### ***Microorganisms and culture conditions***

*Streptococcus mutans* strain (ATCC 35668) was obtained from the archival collection of Oral Bioscience, 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<sub>2</sub>, 10% H<sub>2</sub> and 85% of N<sub>2</sub>) at 37 °C for 2 days, and then, microorganism cultures were harvested and suspended in Brain Heart Infusion (BHI) at a concentration of 10<sup>7</sup>–10<sup>8</sup> CFU/mL (4.0 McFarland standard at 660nm) for the experiment.

### ***Determination of minimum inhibitory concentration (MIC)***

The minimum inhibitory concentration (MIC) value of the crude extract was determined using a broth microdilution assay in line with NCCLS guidelines with few modifications.(19) Briefly, Fructus mume extract were serial twofold diluted in BHI to achieve concentrations ranging from 250 to 3.9mg/mL. The microorganism suspension was inoculated with serial dilution antimicrobial agent and was adjusted to yield final a cell concentration of 10<sup>5</sup>–10<sup>6</sup>

CFU/mL (0.5 McFarland standard at 660nm), each well containing 200  $\mu$ L inocula finally, 2% CHX and PBS served as positive and negative control. The microplates were prepared in triplicate and incubated at 37 °C 24 h. The MIC end point was identified as the lowest concentration at which there was no visible growth by naked eyes.

### ***Biofilm development on orthodontic bracket***

The lower incisor brackets (uniform size) of Damon3 MX (Ormco, USA) were chosen for the test. *Streptococcus mutans* biofilm developed on bracket follow the previous protocol of biofilm formation on microtiter plate with slight modification (20). Briefly, The sterilized brackets which base were placed on red utility wax (this step avoiding the formation of *S. mutans* biofilm on bracket base) were transferred into 24-well plate and incubated with 1000 $\mu$ L microorganism suspension BHI with 1% sucrose at a concentration of  $10^5$ – $10^6$  CFU/mL (0.5 McFarland standard at 660nm), and the plates were incubated anaerobically at 37 °C as a static culture for 24 h.

### ***XTT reduction assay***

After 24 hours incubation, twelve brackets were divided into three equal groups (test, positive and negative control groups) and placed in a 96-well plate, and then washed with 200 $\mu$ L BHI twice to flush the non-adhered bacteria. By washing the biofilm like this, the devastating effect of an air/liquid interface was avoided, as this could give rise to severe errors in enumeration. Thereafter, 200 $\mu$ L Fructus mume extract was added to test group and then discarded after 1 minute static exposure, 2% CHX and BHI were served as positive and negative control followed the same procedure above. Thereafter, each bracket was washed with 200 $\mu$ L BHI twice and 200 $\mu$ L XTT solution (containing 158 $\mu$ l PBS, 40 $\mu$ L XTT ({2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2H-tetrazolium hydroxide}), 2 $\mu$ L Diluted menadione) was added (21). The 96-well plate was incubated statically for 3h at 37 °C, in the dark. Then, each solution was transferred into a new 96-well plate and the absorbance read at 490 nm in spectrometer. The test above was performed in triplicate.

### ***Fluorescence microscopy and confocal laser scanning microscopy***

For fluorescence microscopy and confocal laser scanning microscopy (CLSM) imaging, *S. mutans* biofilms were developed on brackets and exposed to different agents using a protocol similar to above description. Thereafter the biofilms on brackets were gently washed twice with BHI and stained using the Molecular Probes' Live/Dead BacLight viability kit comprising SYTO-9 and propidium iodide (PI) (Molecular Probes, Eugene, OR). The SYTO-9 is a green-fluorescent nucleic acid stain, generally labeling both live and dead microorganisms. PI, in contrast, is a red-fluorescent nucleic acid stain and penetrates only the cells with damaged membranes, thus visualizing only the dead microbes. The biofilms were incubated with SYTO-9 and PI for 20 min in the dark before the microscope examinations. Subsequently, the biofilms on brackets were visualized by microscope analyze of their structure and the bacterial vitality by using Fluorescence microscope and CSLM system (FLUOVIEW FV 1000, Olympus, Tokyo, Japan)

### ***Statistical analysis***

Statistical analysis was performed by SPSS 17.0 version (Chicago, IL, USA). The mean values of the viability of *S.mutans* biofilm after exposure to different agents were analyzed by **repeated measures ANOVA assay**. Statistically significant tests were set at a  $P < 0.05$ .

## **Results**

### ***HPLC profile of Fructus mume extract***

HPLC profile showed that serial organic acids are the main ingredients in the Fructus mume mume extract, citric acid (14863043), tartaric acid (1151546) and oxalic acid (517743) were present in the HPLC profile. (Figure.1)

### ***Determination of minimum inhibitory concentration (MIC)***

Broth microdilution assay demonstrated that Fructus mume extract was effective against *S.mutans* in planktonic state, MIC is 50mg/mL.

### ***XTT reduction assay***

The OD values, measured by XTT reduction assay from *S.mutans* biofilms after 1min exposed to 250mg/mL Fructus mume extract < BHI (-) ( $p < 0.01$ ) (Figure.2). Subsequently, 250mg/mL Fructus mume extract reduced about 24% cell viability of *S. mutans* biofilms on the orthodontic brackets within 1 min exposure.

### ***Fluorescence microscope and Confocal laser scanning microscopy***

Microscopic observations of the *S.mutans* biofilms after treatment with Fructus mume extract for 1 min, revealed an obvious increasing of the amount of dead bacteria on the surface of orthodontic bracket compared to the BHI solution in both of microscopes. The images were presented in (Figure. 3 and Figure. 4)

### **Discussion**

There is a growing demand from the public for evidence-based natural products that achieve the desired antimicrobial and anti-inflammatory effects for the oral health care to complement and enhance the mechanical removal of plaque; the consumers who gravitate toward using herbal products often view these products as being safer than synthetic products (22). Fructus mume has not only been safely used in Chinese medicine for thousand years, but also has a long history as a favorite fruit in China and Japan. In this study, HPLC result showed the main ingredients of Fructus mume extract are organic acids which make its pH value equal to 2.4, this acidic property may be one of its mechanisms of antimicrobial effect and its main compound citric acid had been reported anti-bacterial activity against microbial plaque deposits present on periodontal disease root surfaces in previous research (23, 24). However, we found that Fructus mume extract showed stronger antimicrobial effect when compared to citric acid solution (0.6%, pH=2.2) in our laboratory, therefore, further studies need to be conducted to explore the other antimicrobial mechanisms of Fructus mume extract.

It has long been recognized that rate of enamel dissolution increases with decreasing pH for all acids (25). However, factors such as the exposure time, saliva buffer and salivary pellicle on tooth surface, and enamel remineralization need to be considered when evaluating the possible enamel damage caused by Fructus mume extract (26, 27). One study suggested that a complete rehardening of the softened enamel after exposure to citric acid (pH 3.2) was reached after a remineralization time of 6 hour (28). Another in vitro study reported that compared with the original enamel before erosion, no remarkable difference in surface morphology was observed after 1 min exposure to pH 3.2 citric acid solution, and the erosion depth was near zero, even after 3 min, only a few indistinct keyhole-like rods appeared on the enamel surface, while the erosion depth was still very small (29). Although it has been reported that frequent and excessive consumption of acidic citrus fruits, pure fruit juices (lemon juice, pH ~2.0) or carbonated sports drinks (cola, pH~2.1) may cause irreversible and pathological tooth wear, dietary acid attacks do not cause immediate dissolution of dental



enamel because the tooth surface is covered with the acquired salivary pellicle (30). Based on the result above, Fructus mume extract may be a safety mouthwash for twice a day.

In this study, XTT reduction assay showed that the antimicrobial effect of 250mg/mL Fructus mume extract was less than that of CHX-containing mouthrinse. However, the plant extract of Fructus mume inhibit the *S.mutans* biofilm in a concentration dependent manner, and it was found that 1g/mL Fructus mume extract could reduce about 80% cell viability of biofilm in our lab (date not showed). Since such high concentration of Fructus mume extract is not feasible for clinical use, 250mg/mL Fructus mume extract which is common used as internal medicine in TCM was used in this study. *S.mutans* is the primary causative agent involved in dental caries in humans. In previous report, the amount of *S.mutans* was dramatically increasing in patient's saliva and dental plaque after the bonding of orthodontic appliances (3, 31). The inhibitory capability of Fructus mume extract on *S.mutans* which indicate its potential use as an oral antimicrobial agent for orthodontic patients.

In this study, two microscope systems were used to observe the biofilm, fluorescence microscope system is able to create high-end images for biofilm on the surface of whole bracket. Compared with fluorescence microscopy, confocal laser scanning microscopy has the ability to control depth of field, elimination or reduction of background information away from the focal plane (that leads to image degradation), and the capability to collect serial optical sections from thick specimens (32). However, in this study the biofilm on the surface of orthodontic bracket could be observed under the both microscopes, the bacteria state in the bracket slot could not be detected. This shortcoming was compensated by the XTT reduction assay which could detect the whole cell viability of the biofilm on orthodontic bracket. Microscope image showed Fructus mume extract obviously increased the amount of dead bacteria on the surface of bracket. This phenomenon corresponding with the XTT result which indicated the cell viability of *S. mutans* on biofilm has about 24% decreasing.

A monospecies biofilm-based model was introduced to assess the antimicrobial effect of plant extract on orthodontic brackets in this study. The described model showed that Fructus mume extract has inhibitory effect on *S.mutans* biofilm and this model may be effectively used to assess other mouthrinses in the future. However, although the efficacy and safety of the Fructus mume extract for oral using has been explained above, a pilot randomized clinical

trial will be next step. Moreover, we should be very cautious to extrapolate results from in vitro studies to the *in vivo* situation.

### **Conclusion**

Fructus mume extract showed antimicrobial effect on *Streptococcus mutans* biofilm on orthodontic bracket *in vitro* which may indicate its potential use as an oral antimicrobial agent for orthodontic patients.

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**Figure 1.** HPLC profile showed that citric acid is the main ingredients in the Fructus mume extract, 25min run time.

**Figure 2.** The repeated measure values of the cell viability (OD<sub>1</sub>, 2, 3) of *S.mutans* biofilms on orthodontic bracket Damon3 MX (Ormco, America) upon exposure to test solutions: Fructus.M (Fructus mume extract, 250mg/mL), 2% CHX (+), and BHI (-). Bars and error bars indicate mean and SD, respectively.

**Figure 3.** Fluorescence microscope images of *S.mutans* biofilms on orthodontic bracket Damon3 MX (Ormco, America) upon exposure to test solutions: BHI (A), 2% CHX (B), and Fructus mume extract (C). Biofilms were rinsed 1min statically. Visualization was performed immediately after the last rinse step. Samples were stained with two fluorescent dyes which selectively stain vital bacteria green and dead bacteria red. Scale bars represent 1mm

**Figure 4.** Confocal images of *S.mutans* biofilms on orthodontic bracket Damon3 MX (Ormco, America) upon exposure to test solutions: BHI (a), 2% CHX (b), and Fructus mume extract (c). Biofilms were rinsed 1min statically. Visualization was performed immediately after the last rinse step. Scale bars represent 100  $\mu$ m

**Figure 1.**

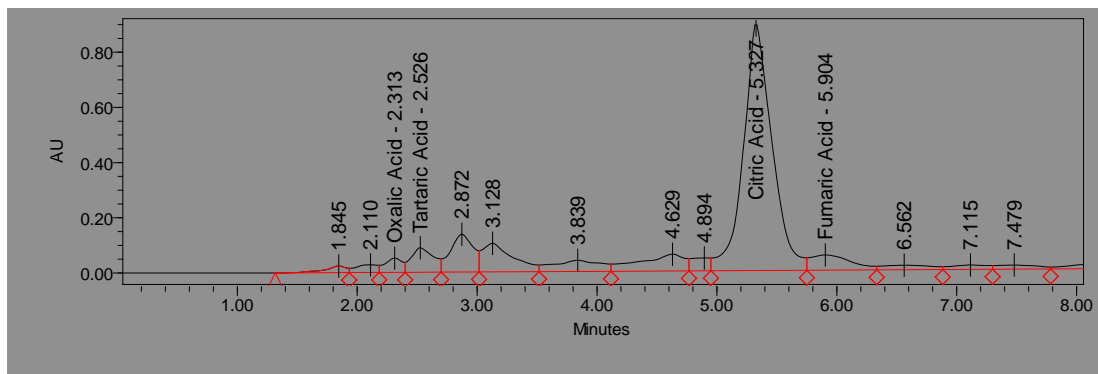
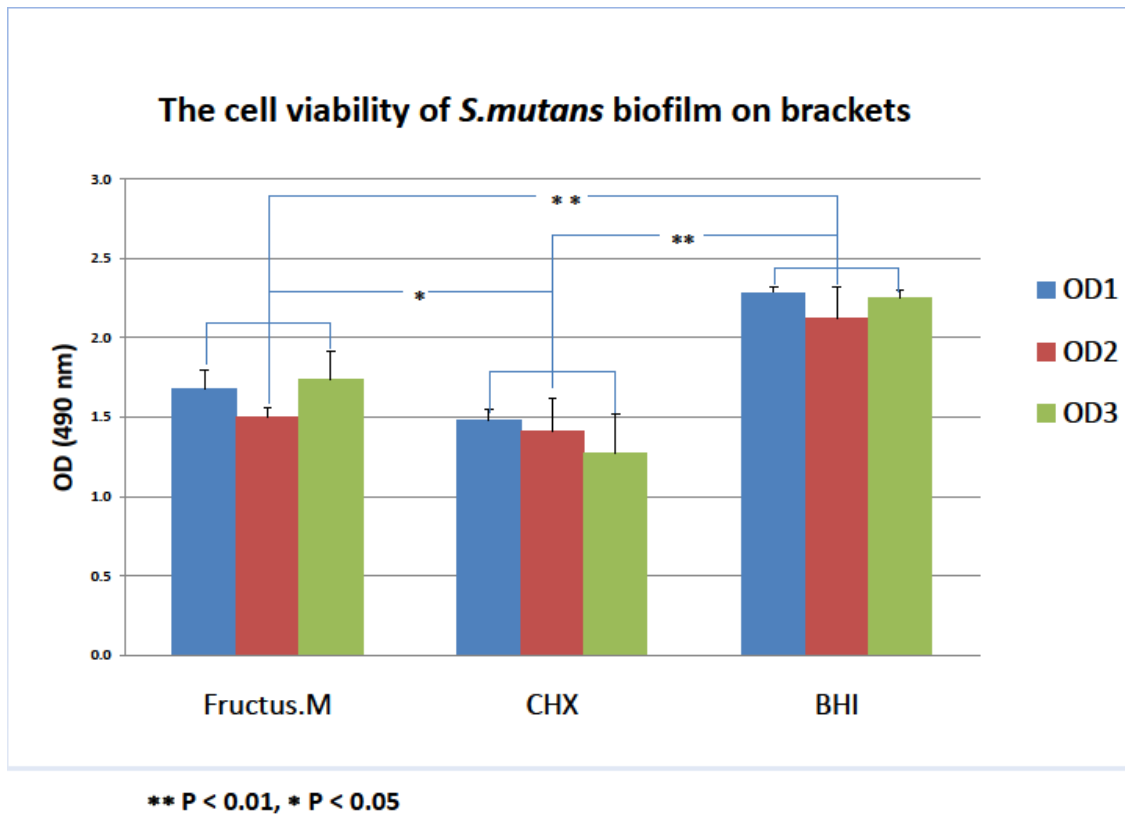
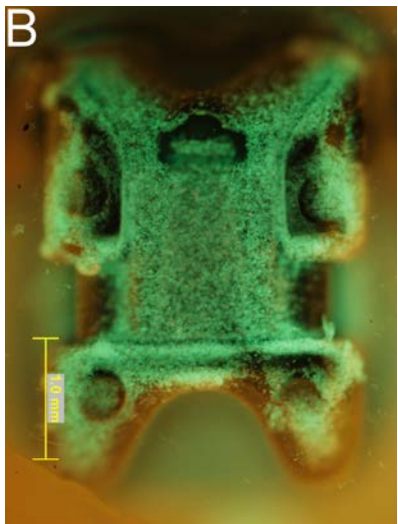


Figure 2.





**Figure 3.**



**Figure 4.**

