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Full Length Research Paper

Polistes olivaceous decreases biotic surface colonization

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The objective of this investigation was to evaluate the anti-bacterial efficacy of the honeycomb of *Polistes olivaceous* on oral biotic surface (biofilm) model by means of pH response, population of oral bacteria and enamel mineralization. Three copies of a three-organism-bacterial consortium was grown on hydroxyapatite (HA) surfaces in a continuous culture system and exposed to repeated solution pulses of sucrose solution every 12 h to construct a cariogenic biofilm on the HA discs in the flow cells. One flow cell was only pulsed with 500 µmol/ml of sucrose (S group). The second flow cell was pulsed with 500 µmol/ml sucrose and 2.5 mg/ml *P. olivaceous* extract (P group). The third flow cell was pulsed with 500 µmol/ml sucrose, 230 mg/L sodium fluoride and 0.2% chlorohexidine digluconate (C group). During the course of carbohydrate supplement, the pH of the S group dropped sharply compared with the others. The P group demonstrated pH recovery to baseline more easily than the S group ($p < 0.05$). The C group demonstrated very little pH drop. The P group displayed a lower level of colonization than the S group, which was reflected by a lower cariogenic bacterial count and a less compact biofilm especially after the third pulse. *P. olivaceous* suppresses bacteria growth and accelerates pH recovery. *P. olivaceous* may have stabilizing effect against cariogenic shift on the oral biofilm, preventing tooth decay.

Key words: *Polistes olivaceous,* oral biotic surface (biofilm), hydroxyapatite, cariogenic bacteria, sucrose.

INTRODUCTION

A biotic surface (biofilm) is defined as a community of bacteria intimately associated with each other within an exo-polymer matrix. This biological unit exhibits its own properties that are quite different from those shown by each individual species in planktonic form (Bortolaia and Sbordone, 2002). The micro-organisms which have been correlated with the most common oral pathologies reside in the biofilm (Bortolaia and Sbordone, 2002). The importance of the biofilm mode of growth for the expression of specific physiological characteristics has been established in a number of studies (Brown and Gilbert, 1993; Hardie, 1993; Wennstrom and Lindhe, 1985). It is reported that micro-organisms within biofilms tend to be more resistant to antimicrobial agents (up to 1000-fold), even though these same bacteria are sensitive to the agents if grown under planktonic conditions (Brown and Gilbert, 1993; Gander, 1996). The reason for this is not clear, however, some speculations include the inactivation of antimicrobial agents or the failure of the antimicrobial agent to penetrate into the biofilm. In addition, it is also reported that biofilm increases the opportunity for gene transfer between bacteria (Xie et al., 2000).

Cariogenic bacteria, such as Streptococcus mutans etc, can colonize tooth surface and initiate plaque formation by synthesizing extracellular polysaccharides from sucrose. The polysaccharide matrix of biofilm forms a barrier that prevents the diffusion of acids produced by the bacteria. The acids accumulate *in situ* and decalcify minerals in the enamel and result in dental caries (Bradshaw et al., 1989).

Many researchers have emphasized the importance of controlling dental plaque to prevent dental caries and to maintain oral health (Kanchanakamol et al., 1993;

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Sauvetre et al., 1995; Sharma et al., 1994; Yankell et al., 1996). A wide variety of sources have been explored in the search for some effective anti-plaque agents (Marsh and Bradshaw, 1997; Southard et al., 1984; Wennstrom and Lindhe, 1985). In recent years, reports on the isolation and identification of natural plaque inhibiting substances have generated much interest in the research of caries prevention (Gander, 1996; Taniguchi and Kubo, 1993). A recent study has demonstrated antimicrobial activity against selected oral pathogens from natural sources and herbs (Yankell et al., 1993).

From the native American plant *Ceanothus americanus*, ceanothic acid and ceanothetric acid demonstrated growth-inhibitory effects against *S. mutans*, *A. viscosus* and *P. gingivalis* (Li et al., 1997). Bakuchiol, isolated from *P. corylifolia,* have antimicrobial activities against oral microorganisms *in vitro* (Katsura et al., 2001). Ethanolic extracts of propolis showed antimicrobial activity against three oral microorganisms (Koo et al., 2000; Xiao et al., 2006). Most of these studies focused on antimicrobial activities against batch cultivated cariogenic bacteria *in vitro* (Katsura et al., 2001; Koo et al., 2000; Kostiuchenkov and Farashchuk, 1991). Some natural herbs have been given as a dentifrice and were proven to show antimicrobial effects (Lee et al., 2004).

Polistes olivaceous, extracted from honeycomb of *De Geer*, is generally used in clinics of conventional Chinese Medicine as an anti-oxygenation agent for treat-ing renal deficiency (Kostiuchenkov and Farashchuk, 1991). It has been reported to show antineoplastic effects in combination with other herbs used to treat parotid gland cancer, mastocarcinoma, gastric and liver cancer *in vivo* and *in vitro* in traumatology to treat microorganism infection (Zhang and Zhou, 2004). The application of the powdered form of *P. olivaceous* directly to carious teeth for relieving toothache has been well known among Chinese medical and dental practitioners.

Although *P. olivaceousis* is widely used, only few studies have been carried out to investigate the effect of *P. olivaceous* has on dental biofilm bacterial colonization (Xiao et al., 2006; Xiao et al., 2007). The cariostatic efficacy of *P. olivaceous* was evaluated by studying the influence of *P. olivaceous* on pH response, the population of cariogenic organisms and enamel mineralization within a biofilm model invented by Hodgson (Hodgson et al., 2001).

MATERIALS AND METHODS

The preparation of *P. olivaceous*

P. olivaceous was provided by the School of Chinese Medicine, the University of Hong Kong and was tested by Société Générale de Surveillance (SGC) in Hong Kong to be free of micro-organisms. The solid *P. olivaceous* was dried and grounded into a powder which was then extracted with methanol (Lin et al., 2001). Prior to use, it was dissolved in a sucrose solution at a final concentration of 2.5 mg/ml (Huang et al., 2003) and filtered with 0.22 µM Cellulose Acetate (Corning, NY, USA) for sterilization.

Streptococcus sanguis (*S. sanguis*) ATCC 10557, *Streptococcus salivarius* (*S. salivarius*) ATCC 25975 and *Streptococcus mutans* (*S. mutans*) ATCC 25175 were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA).

They were cultured in our laboratory. The mixed bacterial culture was grown in a 250 ml vessel, operated at 37°C under a gas phase of 5% (v/v) CO_2 in nitrogen, at a dilution rate of 0.1 h^{-1} for 20 h. The pH was maintained at 7.0 by the automated addition of 0.5 N NaOH.

Total aerobic (15% CO_2 in air atmosphere) and anaerobic counts were performed on supplemented Brain Heart Infusion (BHI) Agar (BioMerieux, Inc. Wilsonville, Oregon, USA) containing 5% (v/v) defibrinated horse blood (BHIS). *S. sanguis* and *S. salivarius* were counted on tryptone-yeast extract cysteine (TYC) agar (Scharlab S.L. Chemical Plant; Barcelona, Spain) and *S. mutans* on tryptone-yeast-cysteine-sucrose-bacitracin (TYCSB) agars (Microdiagnostic, Brisbane, Australia) (Wan et al., 2002; Van Palenstein Heldermanet al., 1983). Identification was based on colony morphology and Gram staining reaction (Holt, 1984).

Biofilm preparation

The growth biofilm medium contained 2.5 mg/ml hog gastric mucin Type III (Sigma-Aldrich, MO, USA), 2 mg/ml proteose peptone (Sigma-Aldrich, MO, USA), 1 mg/ml trypticase peptone (Sigma-Aldrich, MO, USA), 1 mg/ml yeast extraction (Sigma-Aldrich, MO, USA), 0.5 mg/ml glucose, 2.5 mg/ml KCl, 0.1 mg/ml cysteine-HCl (Sigma-Aldrich, MO, USA). The medium was filtered with 0.22 Micro Cellulose Acetate (Corning, NY, USA) for sterilization and adjusted to pH 7.5 (Harvey, 2000).

Inoculation of organism

When a stable population containing all three organisms had been established, three parallel connecting flow cells were inoculated simultaneously with a mixture of the planktonic phase from the continuous culture and fresh medium (in a 1:9 ratio). After inoculation, the consortium was allowed to establish for at least 7 days prior to the start of biofilm experiments. The constant liquid level was maintained by means of a weir system (20 ml working volume). The growth medium was supplemented with 5 µmol/ml phosphate buffer, pH 8.0. A total flow rate of 15 ml/h of combined inoculums or fresh medium was used to obtain a dilution rate of 0.80 h^{-1}. The contents of the flow cells were re-circulated (60 ml/min) to achieve constant mixing. Liquid feeds to the flow cell, recirculation of contents and removal of waste were controlled by peristaltic pumps. The pH of the planktonic phase was monitored over the course of the experiments using electrodes; these were cleaned in sodium hypochlorite and washed with normal saline prior to use.

Polistes olivaceous minimal and optimal concentration determination

The pilot studies to find out the minimal and optimal concentration of *P. olivaceous* had been conducted in the Hospital of Ningxia Medical University, Yinchuan, China, a total of 550 university students were randomly recruited in the test between 2007 and 2009. The ethics committees at Ningxia Medical University approved the research protocols, also informed consents were obtained from these subjects before the joint study. The minimal and optimal concentration of *P. olivaceous* to be used in the pulse experiment was determined by doses response tests from 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4 and 5 mg/ml in total 450 patients. Another 50 subjects used oral rinses of 0.9% normal saline to serve as a negative control and

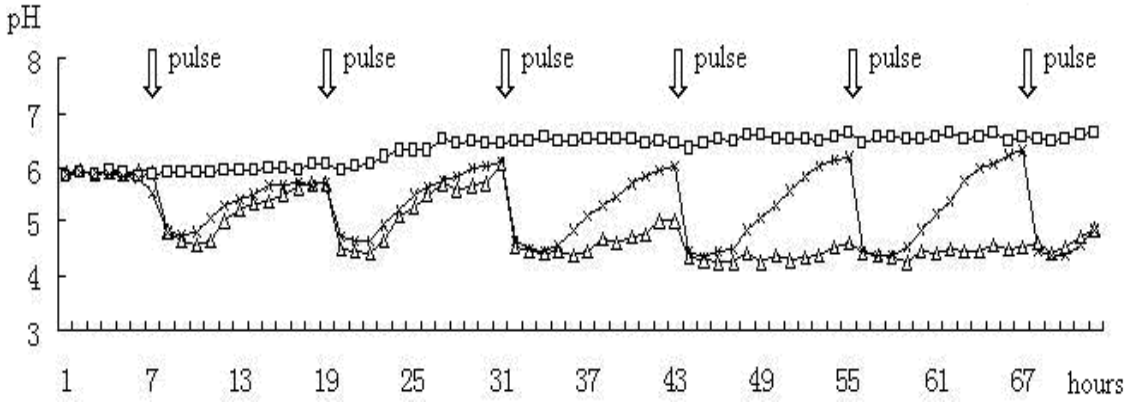

Figure 1. pH response to pulses of sucrose alone, sucrose with *Polistes olivaceous* and sucrose with sodium fluoride and cholorhexidine digluconate. ⇩: indicates one pulse to the flow cell; △: sucrose pulsed group (S); □: sucrose, sodium fluroide and chlorohexidine digluconate pulsed group (C); I<: Sucrose and *Polistes olivaceous* pulsed group (P).

50 used oral rinses of 0.2% chlorohexidine to serve as a positive control. Each dose group consisted of 25 males and 25 females; they were aged between 20 and 35 with the mean age 26.8.

Each tested concentration of *P. olivaceous* was rinsed by 10 individuals for three days at three times per day. After three days, an oral swab from the enemal surface was used for a bacterial culture to determine the number of colony forming units (CFU). Observations from the culture results (data did not show in this study) determined that 2.5 mg/ml of *P. olivaceous* was the optimal and minimal concentration which was statistically different from the CFU obtained by the saline rinse ($p < 0.05\%$). As the concentration increased from 2.5 mg/ml, the CFU counts approached those obtained by patients who used the chlorohexidine rinse, but concentrations above 2.5 mg/ml caused discomfort to the subjects. The oral bacteria were unresponsive to concentrations of *P. olivaceous* below 2.5 mg/ml and CFU counts were similar to patients who used the 0.9% normal saline. Thus 2.5 mg/ml serving as the optimal and minimal concentration of *P. olivaceous* was used for the pulse experiments.

Pulse conditions

Three cariogenic biofilms on hydroxyappatite (HA) discs in the flow cells were created, each of them was pulsed every 12 h for 72 h with a sucrose solution (500 µmol/ml in water) to produce an initial concentrations of 50 µmol/ml in the bulk liquid phase.

The first flow cell was pulsed with sucrose alone (S group). The second flow cell was pulsed with sucrose, 230 mg/L sodium fluoride and 0.2% chlorohexidine digluconate (C group). The final flow cell was pulsed with 2.5 mg/ml *P. olivaceous* extract (initial concentration of 0.5 mg/ml) together with 500 µmol/ml sucrose solution (P group).

Scanning electron microscopy

The HA discs were fixed by modified Karnovsky's fixative (2.5% glutaraldehyde, 2% paraformaldehyde in 0.1 M sodium cacodylate buffer, pH 7.4). Samples were fixed at 4°C for 12 h. All equipments were then rinsed briefly in buffer, post-fixed in 1% buffered osmium tetroxide and dehydrated in graded ethanol. Specimens were then rinsed in propylene oxide then infiltrated and embedded in Araldite resin. Representative sections were prepared using an LKB/Wallac 8801A Ultrotome III Microtome (Diversified Equiment Company, Inc. Lorton, VA, USA) with a diamond knife. Thick sections (1 µ) were stained with toluidine blue stain (Newcomer Supply, Middleton, WI, USA). Thin sections (800 Å) were stained with aqueous urangyl acetate, counterstained with Reynolds' lead citrate and examined by a Leica Stereoscan S440 Scanning Electron Microscope (Leica Microsystems GmbH, Wetzlar, Germany) in the Electron Microscope Unit, Queen Mary Hospital, the Univeristy of Hong Kong.

Statistical analysis

Statistical analysis of the mean colony formation units per ml ($\log_{10}$ cfu/mL) on the agar plates was calculated by SPSS 15(USA) using one-way ANOVA test.

RESULTS

pH response to pulses

The flow cells resided at a steady-state of pH 6.0 during the initial phase of biofilm growth. After the first pulse, the S group presented with a pH drop from 6.0 to 4.3, which recovered to 6.0 before the second pulse. The pH recovered to 6.0 after the second pulse, but remained at 4.3 from the third pulse onwards. The P group demonstrated similar pH drops and recoveries as the S group. However, unlike the S group, the pH continued to recover after every pulse and did not remain at 4.3. Also, after around 30 h, there was a significant difference ($p < 0.05$) between S and P group. The pH of S group fluctuated less, stayed around 4 and 5 and became less dependent to the pulse after 31 h while the pH for P group continued to demonstrate a pH drops and recoveries after 31 h. Meanwhile the pH value of the C group did not show any sharp pH drops and remained stable at about pH 6.2 (Figure 1).

Table 1. Bacterial count of S. mutans, S. sanguis and S. salivarus in each flow.

Bacteria	Log_{10} CFU/disc		
	S group	C group	P group
S. mutans	8.20 ± 0.15*	6.10 ± 0.10	6.50 ± 0.11 *
S. sanguis	10.00 ± 0.18*	7.40 ± 0.12	9.10 ± 0.12
S. salivarius	9.10 ± 0.17*	8.40 ± 0.15	8.20 ± 0.09

Values are mean ± standard deviation.
*Statistically significant ($p < 0.05$).
S group: pulses of sucrose alone, P; group: pulses of sucrose and *Polistes olivaceous;* C; group: pulses of sucrose, sodium fluoride and chlorohexidine digluconate.

Response of biofilm populations to pulses

The bacterial population was cultured after the biofilm experiment was performed for four days. The mean colony formation units (CFU) was calculated and expressed in terms of log_{10} cfu/mL of organism in planktonic phase (Table 1).

A significant increase ($p < 0.05$) in total biofilm population for all three species was observed between the biofilms of the S and the C group.

While between the S and the P group, the population of *S. mutans* was significantly inhibited ($p < 0.05$).

Biofilm microstructure of (Scanning Electron Microscope) SEM imaging

The HA discs were taken out from the flow cells after 72 h for observation under a SEM. In the C group, the circumscription was rather clear without sucrose pulsed in (Figure 2a). While in the micro-radiography of the S group, the biofilm had much more extracellular polysaccharide, which created a fuzzy appearance indicating a sign of demineralization (Figure 2b). As compared to the S group, the P group showed less demineralization (Figure 2c).

DISCUSSION

It is now clear that dental plaque plays an essential role in the pathogenesis of dental caries. When supplied with carbohydrate, there will be a shift from a symbiotic biofilm to a cariogenic biofilm, which contains a larger population of cariogenic bacteria (De Stoppelaar et al., 1970; Dennis et al., 1975; Minah et al., 1985; Staat et al., 1975). These effects have also been demonstrated in animal models (Beighton and Hayday, 1986).

To control dental plaque, a wide variety of sources have been screened. The use of natural products has been one of the most successful strategies for the discovery of new medicines (Harvey, 2000). Seventy eight percent of new antibiotics and 61% of new antitumor drugs approved by the Food and Drug Administration, USA or comparable entities in other countries from 1983 to 1994 were natural products or derived from natural products (Cragg et al., 1997).

Extensive studies showed *P. olivaceous* have antimicrobial activity against oral microorganisms and can inhibit the activity of glucosyltransferase *in vitro* (Koo et al., 2000; Koo et al., 2002). In this context, we studied *P. olivaceous* influences on the cariogenic shift of oral biofilm.

The pH measurements made in the planktonic phase of flow cells showed pulse-like responses to sucrose challenge. With sucrose pulsed in, the pH dropped quickly. Acid was cleared over a 12 h period, with about four hours at minimum pH; this was compared with the C group which had no significant pH drop. Since the third pulse, the pH of the S group had been inhibited at a low level of pH 4-5, which was the critical pH of enamel decalcification (Ingram and Silverstone, 1981). While in the P group, though there was the same concentration of sucrose pulsed in, the pH could be recovered 6 - 7 h after every pulse, which might contributed to the effect of *P. olivaceous* extract.

Biofilm structure was also clearly affected by the sucrose pulsing, which produced a more compact biofilm structure compared with the C group. These findings confirmed previous work using a similar biofilm system (Bradshaw et al., 1989; Singleton et al., 1997). Increased density of biofilm structure had been implicated as a barrier to host immune responses (Anwar et al., 1992). Furthermore, extracellular polysaccharides produced, as a result of sucrose metabolism, might play a role in enhancing the cariogenic challenge. These polysaccharides influenced sugar and acid diffusions (Dibdin and Shellis, 1988; McNee et al., 1982), or facilitate fermentable substrate penetration to deeper regions of the biofilm, where the reduced fixed buffer effect from lower numbers of bacteria may allow a more pronounced pH drop at the plaque enamel interface (Dibdin and Shellis, 1988; Zero et al., 1986). While with the *P. olivaceous* pulsed together with sucrose, the biofilm of the P group was less compact than that of the S group even though both groups contained the same amount of sucrose.

The ecological plaque hypothesis for dental caries (Marsh and Bradshaw, 1997) states that generation of a low pH environment from sugar fermentation results in an ecological shift to a more cariogenic microflora. The results presented here, using the continuous culture system, confirm that the ecological responses were consistent with this hypothesis. With carbohydrate supplement in the flow cell, the population of cariogenic bacteria decreased significantly, together with more extracellular polysaccharides and more compact biofilm structure. While with the *P. olivaceous* pulsed in (P group), the growth of *S. mutans*, one of the primary cariogenic organisms, was inhibited significantly when compared with

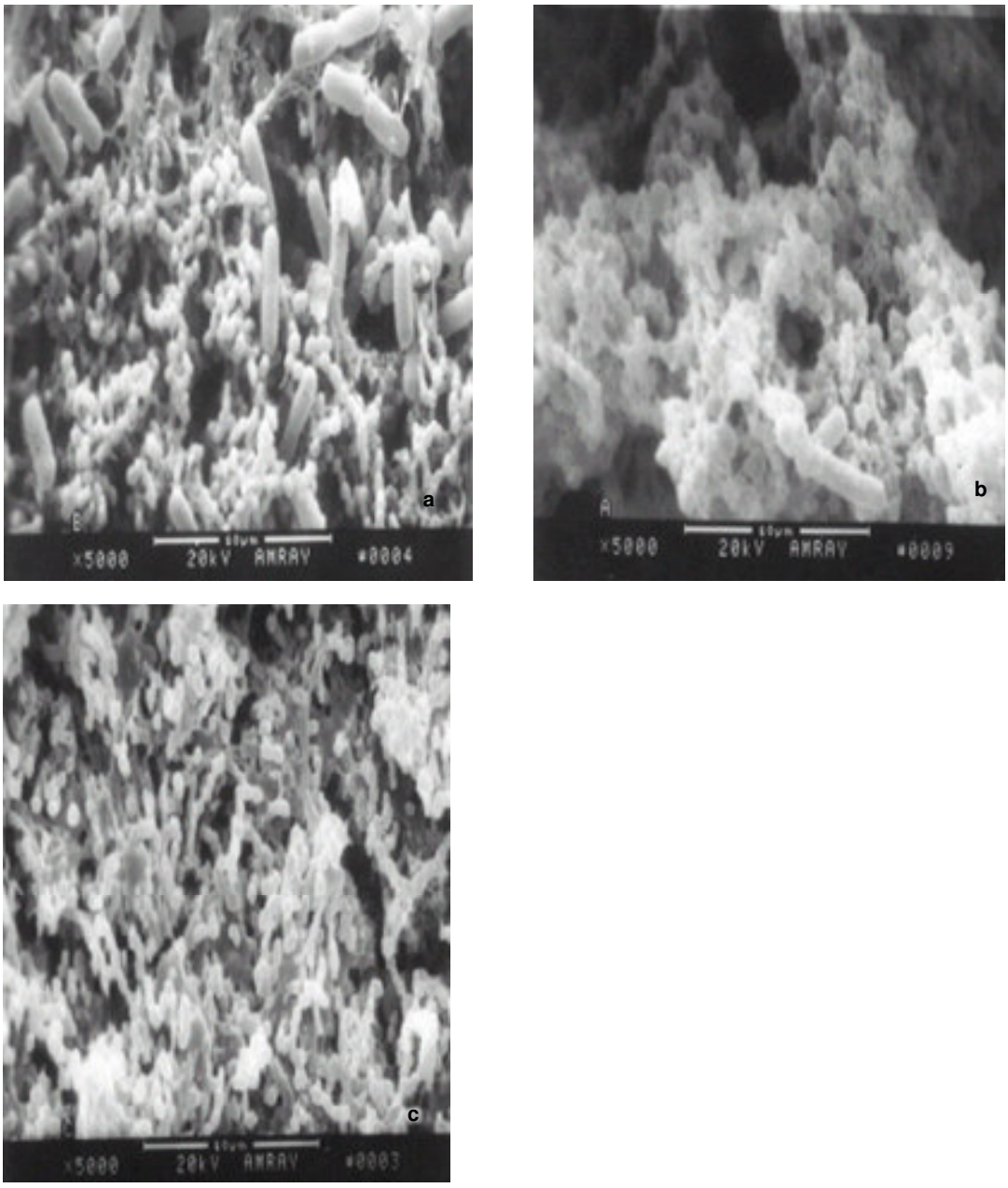

Figure 2. The micro-radiograph taken from scanning electron microscope of the enamel sections of HA discs under a) pulses of sucrose, sodium fluoride and chlorohexidine digluconate, b) pulses of sucrose alone and c) pulses of sucrose and *Polistes olivaceous* (Magnification 5000x).

that of the S group. The microstructure of the biofilm was also less compact and pH recovery was accelerated. All these results indicated that cariogenic shift of plaque was inhibited to some extent by the pulsed medicine extract, *P. olivaceous*.

Current oral rinses contain chlorohexidine which may induce discomfort after long term use. Further studies may involve monitoring of the long term use of *P. olivaceous* to determine its suitability and long term safety profile.

In summary, *P. olivaceous* may help in the clearance of acid accumulation from carbohydrate metabolism. A lower level of colonization of the HA surface was observed in the *P. olivaceous* pulsed group and this was reflected in both the total viable count and biofilm imaging, which has less cariogenic bacteria and a less compact biofilm (Xiao et al., 2006, 2007). These revealed that *P. olivaceous* could suppress bacteria growth, as well as accelerating pH recovery. These events could reduce the pain due to acids and cytotoxins produced by oral bacteria and could

help patients recover from tooth decay (Xiao et al., 2006, 2007). From the present study, *P. olivaceous* may have inhibitory effects on the carigenic shift of oral biofilm and it appears to be a promising source of new agents that may prevent dental caries. A phase I clinical trail would soon be conducted to investigate the efficacy of direct application of *P. olivaceous* in mouth rinse.

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