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Activity of Quercetin and Kaemferol against *Streptococcus mutans* Biofilm

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Abstract

Objective: *Nidus Vespa* (NV) is the honeycomb of *Polistes Olivaceous*, *P. Japonicus Saussure*, and *Parapolybiavaria Fabricius*. Previously, we have shown the extract and chemical fractions from NV demonstrated remarkable capacities of inhibiting the acid production of oral bacteria at sub-minimum inhibitory concentration (MIC) concentrations. In searching the most potent anti-caries compounds in NV, we further separated the NV CHL/MeOH fraction and obtained two purified compounds: quercetin and kaemferol. The objective of this study was to assess the effectiveness of quercetin and kaemferol against *S. mutans* biofilm formation.

Methods: The MIC, minimum biofilm inhibition concentration (MBIC₅₀) and minimum biofilm reduction concentration (MBRC₅₀) against *Streptococcus mutans* were examined for NV-derived of quercetin and kaemferol. The effectiveness of inhibiting *S. mutans* biofilm formation was further examined using *in vitro* biofilm model.

Results: Both quercetin and kaemferol compounds demonstrated anti-biofilm activities when compared to the negative control. They are capable of reducing biofilm dry-weight, total protein, viable cells measured by colony forming unit (CFU), insoluble and soluble glucans formation. The *in situ* culture pH was less acidic when the biofilms were treated by quercetin and kaemferol. The quercetin and kaemferol demonstrated comparable capability of *S. mutans* killing in biofilms, compared to chlorhexidine.

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JX and JL contributed to the study design, JX, YZ and JL performed the data acquisition and analysis. JX, YZ, AN, HA, JL contributed to the data interpretation, manuscript writing and critical revision of the manuscript.

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Declaration of interests: None

Conclusions: The results of this study showed inhibitory activity of quercetin and kaemferol against *S. mutans* biofilms, suggesting that quercetin and kaemferol might be considered as alternative anti-caries agents in searching novel anti-caries therapeutics.

Keywords

Streptococcus mutans; Caries; Kaemferol; Quercetin; Biofilm inhibition; Natural product

Introduction

Dental caries is a diet-modified bacterial infectious disease initiated from cariogenic dental plaques/biofilms formed on tooth surfaces. It is one of the most prevalent chronic diseases worldwide (Paes Leme, Koo, Bellato, Bedi, & Cury, 2006; Selwitz, Ismail, & Pitts, 2007; Takahashi & Nyvad, 2008). *Streptococcus mutans* is the most well known culprit for dental caries (Caufield, Cutter, & Dasanayake, 1993; Kanasi et al., 2010; Klein, Florio, Pereira, Hofling, & Goncalves, 2004; Klinke et al., 2014; Li, Caufield, Dasanayake, Wiener, & Vermund, 2005; Slayton, 2011; Zhan, Tan, Den Besten, Featherstone, & Hoover, 2012). During the development of dental caries, *S. mutans* can adhere to enamel surfaces, synthesize biofilm extracellular matrix, and metabolize dietary carbohydrates to organic acids. Persistent acidification in dental biofilms favors the selection of acid uric species, e.g. *S. mutans* and *Lactobacillus* species, and further lowers the plaque pH. When the plaque pH drops under the critical pH 5.5, demineralization of enamel and degradation of dentin organic matrix takes place, and caries occurs (Takahashi & Nyvad, 2011, 2016).

To prevent and treat this bacterial infectious disease, antimicrobial therapies have been used for centuries. Although antibiotics are effective in preventing caries *in vitro* and in animal models, excessive use of antibiotics can lead to alterations of oral and intestinal flora (Goldin & Gorbach, 1984) and development of bacteria resistance (Dickinson & Surawicz, 2014; Gopinath, Lichtman, Bouley, Elias, & Monack, 2014; Vennila et al., 2014). As an alternative, plant-based compounds derived from natural products have shown potential therapeutic uses in caries control (Groppa et al., 2008; Newman, 2008). Several plant derived compounds emerged with potential anti-biofilm effects against cariogenic bacteria, such as epigallocatechin-3-gallate and farnesol (da Silva et al., 2012; L. Guo et al., 2015).

Nidus Vespae (NV) is the honeycomb of *Polistes Olivaceous*, *P. Japonicus* Saussure, and *Parapolybiavaria Fabricius*. It is known to have a number of pharmacological effects, including antimicrobial, anti-inflammatory, anti-virus, anti-tumor and anesthetic properties (Xiao et al., 2006). Previously, we have shown the extract and chemical fractions from NV demonstrated remarkable capacities of inhibiting the acid production of oral bacteria at sub-MIC concentrations (Xiao et al., 2006). Among several NV chemical fractions, the chloroform/methanol (Chl/MeOH) fraction demonstrated the highest capability of inhibiting the cariogenic bacterial acidogenicity, biofilm extracellular matrix synthesis and dental biofilm formation (Xiao, Zhou, Feng, Hao, & Li, 2007; Xiao, Zuo, et al., 2007). In searching the most potent anti-caries compounds in NV, we further separated the (Chl/MeOH) fraction and obtained two purified compounds: quercetin and kaemferol. The objective of this study

was to assess the effectiveness of quercetin and kaemferol against *S. mutans* biofilm formation.

Materials and Methods

Compounds isolation and purification from *Nidus Vespae*-Chl/MeOH fraction

The NV Chl/MeOH fraction was prepared as previously described by Xiao (Xiao et al., 2006). In this study, the NV Chl/MeOH fraction was dissolved in methanol, followed by depigmentation using MCI (Mitsubishi Chemical Industries, Ltd., Tokyo, Japan) column and compounds separation using gel column chromatography. Eight sub-fractions were obtained after purification and separation, and two purified compounds (Figure 1) were obtained from the eighth sub-fraction (Figure S1).

Compounds identification using high performance liquid chromatography-photodiode array-electrospray source ionization-multistage mass spectrometry (HPLC-PDA-ESI-MSⁿ) analysis

Eight NV Chl/MeOH sub-fractions and two purified compounds were identified using a Thermo Finnigan LCQ DECA mass spectrometer with an electrospray ionization source coupled by a Thermo Finnigan TSP HPLC system. HPLC was equipped with an AS3000 autosampler, a P4000 quaternary gradient pump and an UV6000LP UV detector (with PDA). Chromatographic separation was carried out in a Hypersil BDS C₁₈ column (150×2.1 mm, 5 μm) by gradient elution at a flow rate of 1 mL/min. Gradient elution was performed with the following parameters: 0-90 min 5% to 70% methanol, 90-100 min 100% methanol and 100 - 110 min 100% to 5% methanol. Injection volume was set to 20 μL. The column oven temperature was set to 35 °C. The detection wavelength was set to 190-400 nm. The LCQ-ESI-MSⁿ analysis was performed in both positive and negative electrospray ionization modes. The MSⁿ conditions were as follows: spray voltage 3.50 kV; sheath gas (nitrogen) flow rate 80 arbitrary units (AU); aux gas (nitrogen) flow rate 15 AU; collision gas was helium; capillary temperature 350 °C; capillary temperature 320 °C; capillary voltage ±15V. Full scans were performed at m/z 100-1000. The MSⁿ collision energy was 40% MS² and 45% MS³-MS⁴.

The UV-PDA results of the two purified compounds were showed in Figure 1B and 1C, the retention time of the 2 purified compounds were 65.13 min and 73.42 min respectively. The combination data from the HPLC-PDA-ESI-MSⁿ analysis, retention time, UV wavelength and the MS/MS² data indicated that the 2 purified compounds were Quercetin and Kaemferol, see Figure 1.

Bacterial strain

S. mutans UA159 (ATCC 700610) was stored at -80°C in the laboratory of the Eastman Institute for Oral Health, University of Rochester. To prepare the inoculation, *S. mutans* was first grown on brain-heart infusion (BHI; Oxoid, Basingstoke, UK) agar plates for 48 hours. Subsequently, several colonies were inoculated into 10 ml of buffered tryptone yeast extract broth (pH 7.0) with 1% of glucose, and incubated at 37°C for 18 hours.

MIC, MBIC₅₀ and MBRC₅₀

The minimum inhibitory concentration (MIC) of quercetin and kaemferol against *S. mutans* ATCC 25175 was determined by microdilution assay according to procedures developed by the National Committee of Clinical Laboratory Standards (NCCLS 2000), detailed previously (Xiao et al., 2006). The effect of quercetin and kaemferol on *S. mutans* biofilm formation and reduction was examined by MBIC₅₀ and MBRC₅₀, detailed previously (Xiao, Zuo, et al., 2007). The minimum biofilm inhibition concentration (MBIC₅₀) was defined as the lowest agent concentration that showed 50% or more inhibition on the formation of biofilm. The minimum biofilm reduction concentration (MBRC₅₀) was defined as the lowest agent concentration that showed reduction of the biofilm by 50% or more.

Biofilm preparation

Hydroxyapatite (HA) discs (surface area of $3.14 \pm 0.2 \text{ cm}^2$, BDH Chemical Ltd, Poole, England) were upright placed in a 12-well polystyrene cell culture plate (Costar, Corning inc. USA) and incubated for 2 h at 37°C with 300 µl of sterile saliva under agitation (120 rpm). After the formation of a pellicle, saliva was removed and replaced by buffered tryptone yeast extract broth (pH 7.0) with addition of 1% (w/v) sucrose. Each well was inoculated with a suspension of *S. mutans* [final concentration, 10^8 colony forming unit (CFU)/ ml] and the plates were incubated at 37°C for 24 h.

Biofilms treatments

During the first 24 h, *S. mutans* were grown undisturbed to allow for initial biofilm formation. The biofilms (24 h old) were then treated twice daily (3 min exposure, at 9 a.m. and 5 p.m.) until the 5th day of the experimental period (120 h) with one of the following: 1) 16 mg/ml quercetin; 2) 8 mg/ml kaemferol; 3) 12% ethanol containing 3% DMSO, as the negative control and 4) 0.12% chlorhexidine as the positive control. Each biofilm was exposed to the respective treatment with a total of 8 times. The culture medium was replaced daily.

Biofilm analyses

At the end of the experimental period, the pH of the culture medium was measured. The biofilms were dip-washed three times, and then gently swirled in 0.89% NaCl (w/v) to remove loosely adherent material. The HA surfaces were gently scraped with a sterile spatula to remove the formed biofilms; the biofilms were suspended in 2.5 ml of sterile saline solution. The removed biofilms were subjected to sonication using a Branson Sonifier 450 (Emerson, Missouri, US), three 10 s pulses with 5 s intervals at 50 W. The homogenized suspension was used for the measurement of dry weight, total protein, bacterial viability and polysaccharide composition, detailed previously by Xiao et al. (Xiao, Zhou, et al., 2007).

Confocal laser scanning microscopy (CLSM)

At 120 h, each HA disc was placed in a Petri dish (5 cm in diameter) with 5 ml of saline containing live/dead fluorescence dye (Kits L13152, Molecular Probes, USA). After 30 minutes incubation in the dark, the biofilms were examined with CLSM (Leica TCS SP2, Germany) equipped with a 20× air objective. Each disc was scanned at five randomly

selected positions away from the disk edge. Three independent biofilm experiments were performed and 10 image stacks (512 × 512 pixel tagged image file format) per experiment were collected. The biofilm biovolume and layer coverage distribution were quantified from the confocal stacks by the image-processing software COMSTAT (Heydom *et al.*, 2000).

Scanning electron microscopy (SEM)

120h agent treated biofilms were rinsed once with distilled water and fixed using 2.5% buffered glutaraldehyde at room temperature for 2 h. Fixed samples were then dehydrated through a graded series of ethanol concentrations, air dried, mounted and sputter coated with gold. Samples were imaged at several magnifications (×50 and ×1000).

Statistical analyses

The data of biofilm analysis were analyzed using ANOVA, and the F-test was used to test for significant differences between the treatment groups. When significant differences were detected, pairwise comparisons were made between all the groups using Tukey's method to adjust for multiple comparisons. The chosen level of significance for all statistical tests was $P < 0.05$. Statistical computation was performed using the IBM SPSS software (SPSS Incorporated, Chicago).

Results

MIC, MBIC₅₀ and MBRC₅₀

The MIC, MBIC₅₀ and MBRC₅₀ of quercetin and kaemferol compound are shown in Table 1. 16mg/ml and 32mg/ml of quercetin are the MBIC₅₀ and MBIC₅₀ against *S. mutans* biofilms. Compared to quercetin, kaemferol was more potent in the inhibition and reduction of *S. mutans* biofilms, with an 8 mg/ml MBIC₅₀ and a 16mg/ml MBRC₅₀.

Biofilm biochemical composition

Both quercetin and kaemferol compound demonstrated anti-biofilm activities when compared to the negative control. They are capable of reducing biofilm dry-weight, total protein, viable cells measured by CFU, insoluble and soluble glucans formation. The *in situ* culture pH was less acidic when the biofilms were treated by quercetin and kaemferol. Interestingly, when compared to the positive control chlorhexidine, quercetin demonstrated stronger inhibition of biofilm formation (measured by dry-weight), total protein, viable cells, insoluble glucans formation; quercetin demonstrated comparable efficacy in the inhibition of soluble glucans formation and pH drop. In comparison to chlorhexidine, kaemferol demonstrated stronger inhibition of biofilm formation (dry-weight), protein production, insoluble glucans formation and comparable capability of inhibiting viable cells, soluble glucans formation and culture pH drop.

Biofilm structure

The inhibition of biofilm formation by quercetin and kaemferol compound was further confirmed in the SEM images. Figure 2 revealed detail structure of *S. mutans* biofilm. Quercetin and kaemferol treated biofilms had more porous and less interlinked bacteria-

extracellular matrix when compared to the negative control group and even the chlorhexidine treated biofilms.

The quercetin and kaemferol demonstrated comparable capability of *S. mutans* killing in biofilms, compared to chlorhexidine. The 120h biofilms treated by quercetin and kaemferol had more dead cells vs. live cells when compared to the negative control group, shown in CLSM images (Figure 3), which was measured and confirmed by quantifying the live and dead cells using COMSTAT (Figure 4A). The vertical biofilm coverage distribution was shown in Figure 4B–E. *S. mutans* biofilm treated by quercetin was the thinnest; the bulk of biofilms was accumulated at around 15um above the biofilm-HA disc interface (Figure 4D). The biofilm treated by kaemferol had the least horizontal converge, with only approximate 17% coverage at the most abundant layer (20um above the biofilm-HA disc interface) (Figure 4E).

Discussions

Quercetin and kaemferol were often found together in natural products and both of them have shown anti-oxidation, anti-inflammatory and anti-nociceptive activities (C. J. Guo et al., 2013; Nazaruk, Chledzik, Strawa, Bazydlo, & Wajs-Bonikowska, 2017; Nwido, Airhihen, & Ahmadu, 2016). Both flavonoids quercetin and kaemferol were found to be capable of modulating inducible nitric oxide synthase, cyclooxygenase-2 and reactive C-protein expression, and inducing changes in the nuclear factor kappa B pathway in the human hepatocyte-derived cell line Chang Liver (Garcia-Mediavilla et al., 2007). To our knowledge, this is the first time that NV-derived quercetin and kaemferol were shown to be effective against *S. mutans* biofilms.

In this study, in searching the active antibacterial compounds of NV, quercetin and kaemferol were isolated and purified, and were found as the main active compounds against *S. mutans* biofilm formation, and shown comparable antibacterial effect when compared to an oral antimicrobial gold standard, 0.12% chlorhexidine. It is speculated that quercetin and kaemferol are active against *S. mutans* virulence properties and biofilm formation through the following mechanisms: 1) inhibit bacterial growth and metabolism; 2) inhibit acid production; and 3) inhibit glucan synthesis, which contributes to less biofilm extracellular matrix formation.

Quercetin, a naturally derived plant extract, was previously isolated from propolis, and was shown capable of inhibiting *S. mutans* growth in planktonic condition with a MIC >1250 micromoles (Bhatavadekar Nilkanth B, 2004). Quercetin was also incorporated into a commercially developed dental adhesive (Yang et al., 2017). This quercetin-doped adhesive at the concentration of 500 µg/mL preserved its bonding properties against collagenase ageing, suggesting that quercetin may work as a cross-linker of dentin collagen. In the meantime, this quercetin-doped adhesive inhibited the growth of *S. mutans* biofilm, on immediate and thermo cycled specimens. Efficient bonding interface sealing ability, matrix metalloproteinase inhibition and acceptable biocompatibility were also achieved. However, it is not clear the type of quercetin from Sigma (which sells several formulations), whether ethanol was added to the control adhesive.

Kaempferol is a natural flavonoid found in various natural products, including fruits, edible plants, and several traditional medical herbs; some epidemiological studies have found a positive association between the consumption of foods containing kaempferol and a reduced risk of developing several disorders such as cancer and cardiovascular diseases (Calderon-Montano, Burgos-Moron, Perez-Guerrero, & Lopez-Lazaro, 2011a). Kaempferol and kaempferol glycosides have a wide range of physiological activities, including antioxidant, anti-inflammatory, anti-microbial, anticancer, anti-diabetic, and anti-obesity activities (Calderon-Montano, Burgos-Moron, Perez-Guerrero, & Lopez-Lazaro, 2011b; Devi et al., 2015). It is speculated that kaempferol may help even prevent cancer by augmenting the human's antioxidant defense against free radicals (Chen & Chen, 2013; Gao, Yin, Rankin, & Chen, 2018; Jeong, Phan, & Choi, 2017). Kaempferol was found capable of reducing the number of *Helicobacter pylori* colonies inoculated in the stomach of the gerbils after 10 days oral administration (Habhu, Mahadevan, Shastry, & Manjunatha, 2009). Kaempferol and its glycosides were also found demonstrated antimicrobial activity to other oral disease related bacteria reviewed by (Calderon-Montano et al., 2011a); These bacteria include: *Enterococcus faecalis*, *Porphyromonas gingivalis* and *Prevotella intermedia*. In addition, kaempferol and its glycosides can also act synergistically with antibiotics (e.g. rifampicin, vancomycin, methicillin, erythromycin and clindamycin) against antibiotic-resistant bacteria (Lim, Kim, & Seo, 2007; Otsuka et al., 2008; Xu & Lee, 2001).

One limitation of this study lies in that we have not tested the antimicrobial and antibiofilm effect of the quercetin and kaempferol combination, which could have shown more potency against *S. mutans* biofilm since both quercetin and kaempferol co-present in NV. In the future, we will consider testing the antimicrobial and antibiofilm formation activities using commercially available quercetin and kaempferol, with the combination of both compounds, and the combination of these compounds and other current commonly used anticaries agents, e.g. fluoride products.

Conclusions

The results of this study showed effective activity of quercetin and kaempferol against *S. mutans* biofilms, suggesting that quercetin and kaempferol might be considered as alternative anti-caries agents in searching novel anti-caries therapeutics.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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Abbreviations

NV

Nidus Vespae

MIC	Minimum inhibitory concentration
MBIC₅₀	Minimum biofilm inhibition concentration
MBRC₅₀	Minimum biofilm reduction concentration
CFU	Colony forming unit
Chl/meoh	Chloroform/methanol
HPLC-PDA-ESI-msⁿ	High performance liquid chromatography-photodiode array-electrospray source ionization-multistage mass spectrometry
CLSM	Confocal laser scanning microscopy
SEM	Scanning electron microscopy

Reference

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Highlights

- Plant-derived quercetin and kaemferol
- Potent in inhibiting *Streptococcus mutans* biofilm

Fig. 1. HPLC-PAD-ESI-MSⁿ analysis of *Nidus Vespae* Chl/MeOH fraction and UV-PDA analysis of the purified compounds quercetin and kaemferol.

(A) HPLC-PAD-ESI-MSⁿ total ion chromatogram of *Nidus Vespae*-Chl/MeOH fraction with identified peaks. Two purified compounds were obtained and identified as quercetin and kaemferol using the combination of retention time, UV wavelength and the MS/MS² data.

(B) UV-PDA results of the 2 purified compounds obtained from the eighth *Nidus Vespae*-Chl/MeOH sub-fraction.

Fig. 2. SEM of agents treated 120h *S. mutans* biofilms

The inhibition of biofilm formation by quercetin and kaemferol compound was shown. Quercetin (C-1 and C-2) and kaemferol (B-1 and B-2) treated biofilms had more porous and less interlinked bacteria-extracellular matrix when compared to the negative control group (A-1 and A-2) and even the chlorhexidine treated biofilms (D-1 and D-2).

Fig. 3. Confocal laser scanning microscopy of agents treated 120h *S. mutans* biofilms
 The 120h *S. mutans* biofilms treated by quercetin and kaempferol had more dead cells vs. live cells when compared to the negative control group.

Fig.4. Biovolume and layer distribution of live/dead cells in 120h agents treated *S. mutans* biofilms

The biovolume of live and dead cells (A) was measured using the image-processing software COMSTAT (Heydorn *et al.*, 2000). The vertical biofilm distribution was shown B-E. *S. mutans* biofilm treated by quercetin was the thinnest; the bulk of biofilms was accumulated at around 15 μm above the biofilm-HA disc interface (D). The biofilm treated by kaemferol had the least horizontal converge, with only approximate 17% coverage at the most abundant layer (20 μm above the biofilm-HA disc interface) (E).

Table. 1.

Effects of quercetin and kaemferol on the formation (MBIC₅₀^a) and reduction (MBRC₅₀^b) of *S. mutans* biofilm

Test agent	MBIC ₅₀ ^a (mg/ml)	MBRC ₅₀ ^b (mg/ml)	MIC (mg/ml)
Quercetin	16	32	16
Kaemferol	8	16	8

^aMBIC₅₀, minimum biofilm inhibition concentration, the lowest agent concentration that showed >50% inhibition on biofilm formation.

^bMBRC₅₀, minimum biofilm reduction concentration, the lowest agent concentration that showed >50% reduction of biofilm.

Table 2.*S. mutans* biofilm composition and acidogenicity after Quercetin and Kaemferol treatment

Biofilm analyses	Treatment groups * (Mean $\pm$ Standard deviation)			
	Control	Quercetin 16mg/ml	Kaemferol 8mg/ml	Chlorhexidine
Dry-weight (mg)	8.61 $\pm$ 0.56 ^a	4.55 $\pm$ 0.05 ^b	3.08 $\pm$ 0.06 ^c	6.57 $\pm$ 0.87 ^d
Total protein (mg)	1.78 $\pm$ 0.06 ^a	1.24 $\pm$ 0.14 ^b	0.62 $\pm$ 0.29 ^c	1.54 $\pm$ 0.21 ^a
CFU (10 ⁷)	1.83 $\pm$ 0.07 ^a	1.07 $\pm$ 0.04 ^b	1.31 $\pm$ 0.08 ^c	1.34 $\pm$ 0.11 ^c
Insoluble glucans (mg)	2.34 $\pm$ 0.14 ^a	1.24 $\pm$ 0.13 ^b	1.09 $\pm$ 0.12 ^b	1.56 $\pm$ 0.21 ^c
Soluble glucans (mg)	1.08 $\pm$ 0.05 ^a	0.78 $\pm$ 0.03 ^b	0.74 $\pm$ 0.03 ^b	0.79 $\pm$ 0.10 ^b
In situ pH	4.62 $\pm$ 0.34 ^a	5.4 $\pm$ 0.22 ^b	5.68 $\pm$ 0.08 ^b	5.3 $\pm$ 0.32 ^b

* Twice daily with three-minute exposure for each treatment at 9 AM and 5 PM.

* Two samples were used for each treatment group in each experiment. Experiments were repeated three times with a total of six samples for each treatment group.

^{abcd} Values followed by the same superscripts are not significantly different from each other (P>0.05; ANOVA, comparison for all pairs using Tukey-Kramer HSD).