Sains Malaysiana 52(1)(2023): 199-210
http://doi.org/10.17576/jsm-2023-5201-16

In Vitro Antimicrobial, Antiglycolytic, and Antibiofilm Activities of Synthetic 1,4-Naphthoquinone Derivatives against Cariogenic Bacteria

(Aktiviti Antimikrob, Antiglikolitik dan Antibiofilem *In Vitro* bagi Terbitan 1,4-Naftokuinon Sintetik terhadap Bakteria Kariogenik)

PICHAYAPORN RATTI[1], JUTHARAT MANUSCHAI[1], JIRAPORN KARA[2,3], LUELAK LOMLIM[2,3] & SUPAWADEE NAORUNGROJ[1,*]

[1]*Department of Conservative Dentistry, Faculty of Dentistry, Prince of Songkla University, Hat Yai, Songkhla, Thailand*
[2]*Department of Pharmaceutical Chemistry, Faculty of Pharmaceutical Sciences, Prince of Songkla University, Hat Yai, Songkhla, Thailand*
[3]*Phytomedicine and Pharmaceutical Biotechnology Excellent Center (PPBEC), Faculty of Pharmaceutical Sciences, Prince of Songkla University, Hat Yai, Songkhla, Thailand*

Received: 11 June 2022/Accepted: 29 September 2022

ABSTRACT

This study investigated the potential anticaries properties of synthetic 1,4-naphthoquinone derivatives. Synthetic 1,4-naphthoquinone derivatives (**2**-**4**) were designed and synthesized by employing lawsone methyl ether (LME, **1**), a plant-derived 1,4-naphthoquinone, as a lead compound. The synthetic compounds were characterized by infrared spectroscopy, ^{1}H-nuclear magnetic spectroscopy, ^{13}C- nuclear magnetic spectroscopy, and high-resolution mass spectrometry. Minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC), and growth curves were determined to assess their antibacterial effects against *Streptococcus mutans*, *Lacticaseibacillus casei*, and *Actinomyces naeslundii*. The pH drop assay was also performed on these three bacterial species. The effect on *S. mutans* biofilm formation was evaluated by crystal violet assay. From the microdilution assay, 2-(prop-2-ynyloxy)naphthalene-1,4-dione (compound **2**) showed potent antimicrobial activity against *S. mutans* and *A. naeslundii* (MIC of 1.56 and 3.125 µg/mL, respectively) in the same range as chlorhexidine (MIC of 1.95 and 1.95 µg/mL, respectively). The 1,4-naphthoquinone derivatives showed low antibacterial activity against *L. casei*. LME (compound **1**) and 2-(prop-2-ynyloxy)naphthalene-1,4-dione (compound **2**) inhibited pH reduction from *S. mutans*. The compounds at sub-MIC concentrations showed a potent inhibitory effect against *S. mutans* biofilm formation in a dose- and time-dependent manner. These results suggested that the synthetic 1,4-naphthoquinone derivatives are promising compounds that could be developed as a novel alternative or adjunctive anticaries therapies.

Keywords: Acid production; antibacterial activity; dental biofilm; dental caries; 1,4-naphthoquinone

ABSTRAK

Penyelidikan ini mengkaji potensi sifat antikaries bagi terbitan 1,4-naftokuinon sintetik. Terbitan 1,4-naftokuinon sintetik (**2-4**) telah direka dan disintesis dengan menggunakan lawsone metil eter (LME, **1**), 1,4-naftokuinon yang berasal daripada tumbuhan, sebagai sebatian plumbum. Sebatian sintetik telah dicirikan oleh spektroskopi inframerah, spektroskopi magnet nuklear ^{1}H, spektroskopi magnet nuklear ^{13}C dan spektrometri jisim resolusi tinggi. Kepekatan perencatan minimum (MIC), kepekatan bakteria minimum (MBC) dan lengkung pertumbuhan ditentukan untuk menilai kesan antibakteria mereka terhadap *Streptococcus mutans, Lacticaseibacillus casei* dan *Actinomyces naeslundii*. Ujian penurunan pH juga dilakukan ke atas ketiga-tiga spesies bakteria ini. Kesan ke atas pembentukan biofilem *S. mutans* dinilai dengan ujian kristal violet. Daripada ujian pencairan mikro, 2-(prop-2-yniloksi)naftalena-1,4-dion (sebatian **2**) menunjukkan aktiviti antimikrob yang kuat terhadap *S. mutans* dan *A. naeslundii* (masing-masing MIC 1.56 dan 3.125 µg/mL) dalam julat yang sama seperti klorheksidin (masing-masing MIC 1.95 dan 1.95 µg/mL). Terbitan 1,4-naftokuinon menunjukkan aktiviti antibakteria yang rendah terhadap *L. casei.* LME (sebatian **1**) dan

2-(prop-2-yniloksi)naftalena-1,4-dione (sebatian **2**) menghalang pengurangan pH daripada *S. mutans*. Sebatian pada kepekatan sub-MIC menunjukkan kesan perencatan yang kuat terhadap pembentukan biofilem *S. mutans* dalam cara yang bergantung kepada dos dan masa. Keputusan ini mencadangkan bahawa terbitan 1,4-naftokuinon sintetik adalah sebatian yang menyakinkan dan boleh dibangunkan sebagai alternatif baru atau terapi antikaries tambahan.

Kata kunci: Aktiviti antibakteria; biofilem gigi; karies gigi; penghasilan asid; 1,4-naftokuinon

Introduction

Dental caries, a biofilm-mediated tooth disease, is widely regarded as the most common oral disease. *Streptococcus mutans* is a major etiological pathogen of dental caries, which plays a vital role in developing cariogenic biofilms. If these biofilms are not removed from the tooth surface and are frequently exposed to dietary carbohydrates, *S. mutans* and other acid-producing bacteria within the biofilm community will metabolize sucrose to organic acids (Jeon et al. 2011). The low-pH environment subsequently created at the tooth-biofilm interface results in demineralization of the enamel resulting in the initiation/progression of caries (Takahashi & Nyvad 2016).

Actinomyces spp. are also early colonizers that play an essential role in developing the biofilm (Li et al. 2004; Takahashi & Nyvad 2016). *Actinomyces naeslundii* has been implicated in the formation of root caries lesions on the human dentition (Howell et al. 1965). Lactobacilli are also well-known as etiological pathogens of dental caries (Caufield et al. 2015; Tanner et al. 2018). Due to the relatively low affinity for teeth, caries lesion is necessary for Lactobacilli colonization. It provides a retentive, stagnant site with an acidic environment rich in carbohydrates, where Lactobacilli can thrive. In such a habitat, Lactobacilli metabolize carbohydrates, produce lactic acid, and generate a lower pH. For this reason, Lactobacilli represent a significant contributor to caries progression (Caufield et al. 2015).

One strategy for preventing and treating dental caries is to use antimicrobial agents to reduce or eliminate bacteria associated with caries (Simon-Soro & Mira 2015; ten Cate & Zaura 2012). Systemic antibiotics showed potential efficacy in preventing dental caries (Alaki, Burt & Garetz 2009; Vohra et al. 2016), but their application has gradually reduced because many of them were not specifically developed for oral diseases. Although several antibiotics/antimicrobial agents are effective for dental caries prevention, they may cause undesirable effects such as hypersensitivity, renal toxicity, as well as drug resistance. Therefore, other antimicrobial agents developed specifically for treating oral diseases, such as fluoride, chlorhexidine, quaternary ammonium salts and antimicrobial peptides (AMPs) have been used more widely (Qiu et al. 2020).

Lawsone and lawsone methyl ether (LME) are plant-derived bioactive 1,4-naphthoquinones with antimicrobial activity (Panichayupakaranant, Septama & Sinviratpong 2019). The compounds possess antifungal activity and activity against a wide range of bacteria, including *S. mutans* (Panichayupakaranant, Septama & Sinviratpong 2019; Sakunphueak & Panichayupakaranant 2012; Yang et al. 2001). Besides the natural-occurring 1,4-naphthoquinone derivatives, the synthesis and biological activities of synthetic 1,4-naphthoquinones has been reported (Lopez et al. 2014). Antibacterial activity of lawsone and its derivatives arises from the quinone moiety. It can undergo a cascade redox reaction in the mitochondria of the microorganism, react with oxygen molecules, and generate harmful reactive oxygen species (ROS). Furthermore, the carbon atom at position 3 of the quinone ring represents an electrophilic site in Michael addition reaction with bacterial biomolecules and leads to their malfunction (Anaissi-Afonso et al. 2018; Lopez et al. 2014). A previous study suggested that incorporation of a lipophilic group on the hydroxyl group at position 2 of lawsone structure i.e., LME enhanced the absorption through the microbial cell membrane and thus increased its antimicrobial potency (Sakunphueak & Panichayupakaranant 2012). Therefore, due to their antimicrobial activity, 1,4-naphthoquinone derivatives could be a candidate for an anticaries agent.

This study aims to investigate the effect of lipophilic substituents at the 2-position of the 1,4-naphthoquinone system on antibacterial activity against cariogenic bacteria. Moreover, we also assess the effect of the synthetic 1,4-naphthoquinone derivatives on bacterial acid production and the effect on biofilm formation of *S. mutans*.

Materials and Methods

Synthesis of 1,4-naphthoquinone derivatives

Chemicals used in the preparation of the compounds

were purchased from Merck AG (Darmstadt, Germany). Monitoring and primary characterization of products were achieved by TLC on aluminium sheets coated with silica gel 60 F_{254} purchased from E. Merck using dichloromethane:hexane (20:80, 40:60, 20:80) as an eluent. Eluted TLC's were shown under UV (254 nm). Melting points (mp, °C) were recorded using the Stuart SMP11 (Cole-Parmer, Staffordshire, UK). To confirm the functional groups of the synthesized compounds. The samples were prepared as thin films or KBr pellets and investigated on a Perkin Elmer Spectrum One (Connecticut, USA) Infrared (IR) spectrophotometer. The Nuclear Magnetic Resonance (NMR, ^{1}H-NMR 500 MHz, ^{13}C-NMR 125 MHz) NMR spectroscopy was performed on the Bruker Ascend 500/Avance Neo (Massachusetts, USA) using $CDCl_3$ as a solvent. Chemical shifts (δ) are expressed in ppm relative to $CDCl_3$. Multiplicity is indicated as *s* (singlet), *d* (doublet), *t* (triplet) and *m* (multiplet). Electrospray Ionization Mass Spectrometry (ESI-MS) spectra were recorded on a Thermo Finnigan MAT 95XL (Bremen, Germany) using a dual electrospray ionization (ESI) source operating in positive modes. IR analysis was carried out at the Departmet of Pharmaceutical Chemistry, Faculty of Pharmaceutical Sciences, Prince of Songkla University, Songkhla, Thailand. NMR analysis was carried out at the Office of Scientific Instrument and Testing, Prince of Songkla University, Songkhla, Thailand. ESI-MS was carried out at the Science Lab Center, Faculty of Science, Naresuan University, Phitsanulok, Thailand.

Lawsone methyl ether (LME) (compound **1**) and three 1,4-naphthoquinone derivatives (compounds **2**-**4**) were synthesized via *O*-alkylation reaction at the 2-hydroxyl group of lawsone. Lawsone methyl ether was prepared via *O*-methylation of lawsone under acidic conditions using the method previously described (Panichayupakaranant & Reanmongkol 2008). Briefly, a mixture of lawsone (1 g) and conc. hydrochloric acid (0.8 mL) in absolute methanol (50 mL) was heated under reflux for 4 hours. The reaction mixture was cooled down to room temperature and the precipitate was collected by vacuum filtration. Recrystalization of the precipitate in a mixture of ethyl acetate and methanol yielded yellow needles of lawsone methyl ether. 2-(Prop-2-ynyloxy) naphthalene-1,4-dione (compound **2**), 2-(but-2-ynyloxy)naphthalene-1,4-dione (compound **3**), and 2-(4-phenylbut-2-ynyloxy)naphthalene-1,4-dione (compound **4**) were prepared by *O*-alkylation of lawsone with the corresponding halide derivatives according to Anaissi-Afonso et al. (2018) with minor modification. In their method, a solution of lawsone (1.0 mmol) in dimethylformamide (10 mL) and potassium carbonate (2.0 mmol) was stirred for 15 min at room temperature. Propargyl bromide (1.2 mmol) was added dropwise, and the reaction mixture was stirred overnight. After completion of the reaction, water was added and the mixture was extracted with ethyl acetate. The crude product was purified by column chromatography using ethyl acetate/hexane (3:7) to yield the desired product. In our method, the mole ratio of the starting materials and reaction duration were slightly changed. Different eluents for column chromatography and recrystallization solvent were used in our method. The synthesis pathway of the 1,4-naphthoquinone derivatives is illustrated in Figure 1.

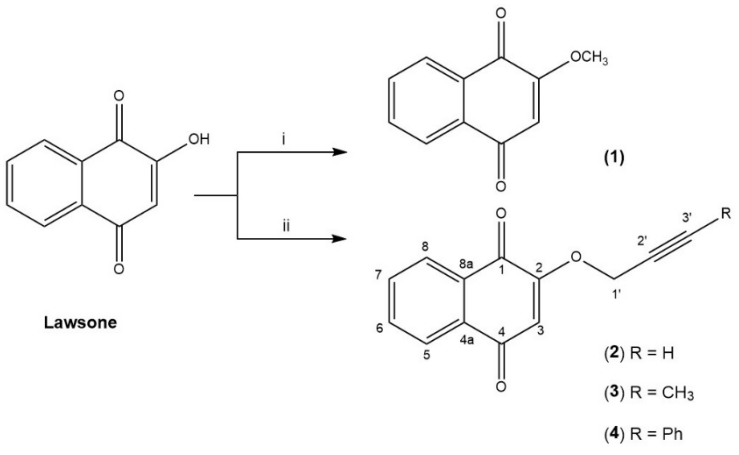

FIGURE 1. Synthesis pathway of the 1,4-naphthoquinone derivatives
(i) CH_3OH/conc. HCl/reflux 4 h; (ii) K_2CO_3/corresponding halide/DMF/r.t. 72 h

THE GENERAL METHOD FOR THE SYNTHESIS OF COMPOUNDS **2-4**

Based on method of Anaissi-Afonso et al. (2018) with minor modifications, lawsone (100 mg, 0.58 mmol) was dissolved in *N,N*-dimethyl formamide (7.5 mL). Potassium carbonate (K_2CO_3; 79.36 mg, 0.58 mmol) was added into the solution. The reaction mixture was stirred at room temperature for 15 min and then the corresponding alkyl halide (0.58 mmol) was added. The reaction mixture was further stirred for 72 h. The progress of the reaction was monitored with silica gel 60 F254 thin-layer chromatography (TLC). When the reaction was completed, distilled water (30 mL) was added to the reaction mixture, and the product was extracted by ethyl acetate (3×30 mL). The combined organic phases were dried over anhydrous sodium sulphate (Na_2SO_4) and concentrated under reduced pressure to give the crude product. The product was purified by silica gel column chromatography (using dichloromethane:hexane (2:8) for compound **2** and **4**, (4:6) for compound **3** as eluent) and recrystallized from hexane/methanol. The purified compounds were analyzed and the results obtained from respective analyses as described below.

2-(prop-2-ynyloxy)naphthalene-1,4-dione (*Compound* **2**) Yellow solid. 89 mg (73%); m.p. 149-151 °C; IR (cm^{-1}, thin film): 3252.1, 3054.1, 2923.8, 1681.9, 1605.7, 1458.4, 1257.4, 1016.2, 722.2, 696.3; ^{1}H-NMR (ppm, $CDCl_3$): 2.65 (1H, *s*), 4.81 (2H, *s*), 6.36 (1H, *s*), 7.74 (2H, *m*), 8.07 (1H, *d*), 8.14 (1H, *d*); ^{13}C-NMR (ppm, $CDCl_3$): 56.74 (1'), 75.44 (3'), 78.20 (2'), 111.61 (3), 126.24 (5), 126.75 (8), 131.05 (8a), 131.89 (4a), 133.48 (6), 134.38 (7), 158.05 (2), 179.83 (1), 184.71 (4); HR-MS (m/z, $[M+1]^+$): 213.0531 (calcd for $C_{13}H_8O_3$, 212.0473)

2-(but-2-ynyloxy)naphthalene-1,4-dione (*Compound* **3**) Yellow solid. 71 mg (55%); m.p. 168-169 °C; IR (cm^{-1}, thin film): 3103.2, 2920.0, 1681.3, 1608.4, 1440.4, 1259.9, 1015.5, 777.7, 724.0; ^{1}H-NMR (ppm, $CDCl_3$): 1.84 (3H, *s*), 4.73 (2H, *s*), 6.32 (1H, *s*), 7.67-7.74 (2H, *m*), 8.05-8.11 (2H, *m*); ^{13}C-NMR (ppm, $CDCl_3$): 3.73 (CH_3), 57.62 (1'), 71.15 (3'), 86.61 (2'), 111.51 (3), 126.15 (5), 126.69 (8), 131.08 (8a), 131.92 (4a), 133.35 (6), 134.28 (7), 158.35 (2), 180.04 (1), 184.92 (4); HR-MS (m/z, $[M+1]^+$): 227.0703 (calcd for $C_{14}H_{10}O_3$, 226.0630)

2-(4-phenylbut-2-ynyloxy)naphthalene-1,4-dione (Compound **4**) Yellow solid. 78 mg (47%); m.p. 169-170 °C; IR (cm^{-1}, thin film): 3074.6, 2963.7, 1681.1, 1608.0, 1457.3, 1244.9, 1023.5, 779.5, 721.7; ^{1}H-NMR (ppm, $CDCl_3$): 5.05 (2H, *s*), 6.47 (1H, *s*), 7.32-7.47 (5H, *m*), 7.73-7.80 (2H, *m*), 8.11-8.18 (2H, *m*) ; ^{13}C-NMR (ppm, $CDCl_3$): 29.68 (1′), 57.67 (3'), 80.57 (2'), 89.56 (1′′), 111.60 (3), 126.19 (3′′, 5′′), 126.72 (2′′, 6′′, 4′′), 128.35 (5), 129.18 (8), 131.07 (8a), 131.92 (4a), 133.40 (6), 134.33 (7), 158.28 (2), 179.99 (1), 184.80 (4); HR-MS (m/z, $[M+1]^+$): 289.0858 (calcd for $C_{19}H_{12}O_3$, 288.0786)

BACTERIA AND GROWTH CONDITIONS

S. mutans (DMST 41283) was purchased from the National Institute of Health, Department of Medical Sciences, Ministry of Public Health, Thailand. *L. casei* (TISTR 1463) and *A. naeslundii* (TISTR 2426) were purchased from the Thailand Institute of Scientific and Technological Research, Ministry of Higher Education, Science, Research and Innovation, Thailand. *S. mutans* and *A. naeslundii* were grown on Brain Heart Infusion (BHI) agar plates (HiMedia Laboratories, Mumbai, India) at 37 °C in the presence of 5% CO_2, and *L. casei* were grown on deMan, Rogosa, Sharpe (MRS) agar plates (HiMedia Laboratories, Mumbai, India) at 37 °C in the presence of 5% CO_2. All strains were grown for two days on agar media before being transferred to liquid media and grown in the same growth conditions used throughout the study.

To measure an exponential bacterial growth curve of bacterial strains used in this study, 15 mL of overnight culture was inoculated in a 135 mL sterile broth and incubated. The absorbance values of bacterial suspensions were measured hourly using a spectrophotometer (Genesys 10s UV-VIS, Thermo Fisher Scientific, Waltham, MA, US) at a wavelength of 620 (Wilkins, Homer & Beighton 2002), 600 (Dieterle et al. 2016), and 660 nm (Kawashima et al. 2013) for *S. mutans*, *L. casei,* and *A. naeslundii*, respectively. To obtain bacteria growth curves, graphs were plotted as a function of time in hours on the X-axis versus optical density on the Y-axis. The drop plate method was used to estimate the number of viable bacteria cells as a colony-forming unit (CFU) at the mid-exponential phase of each strain. The estimated numbers of viable cells at the mid-exponential phase are 10^8 CFU/mL for *S. mutans* (6 h) and *L. casei* (12 h), and 10^7 CFU/mL for *A. naeslundii* (19 h). Cell culture absorbance values of each strain are as follows: OD_{620nm} = 0.5±0.1 for *S. mutans*, OD_{600nm} = 2.0±0.1 for *L. casei*, and OD_{660nm} = 0.5±0.1 for *A. naeslundii*.

To prepare bacterial cultures for all experiments, overnight cultures of each strain were subcultured and grown until they entered the mid-exponential phase. Absorbance values were measured before being used in any assays. CFU counts of bacterial suspension for each experiment were also done by the drop plate method to verify the numbers of viable cells.

EFFECTS OF SYNTHETIC 1,4-NAPHTHOQUINONE DERIVATIVES ON THE BACTERIAL GROWTH

Antibacterial activities of 1,4-naphthoquinone derivatives against *S. mutans*, *L. casei*, and *A. naeslundii* were assessed by obtaining minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values using the microdilution technique in 96-well microtiter plates. Bacterial cultures were adjusted to approximately 10^5 CFU/mL using sterile broth as diluent. Lawsone derivatives were 2-fold serially diluted with broth solution, which was dispensed in 96-well microtiter plates, to obtain the final concentration range of 0.195 – 100 µg/mL. The positive control for antibacterial activity was chlorhexidine (CHX), while the negative control was broth solution. Bacterial strains were inoculated in each well. After well-mixing, 96-well microtiter plates were incubated for 24 h. The MICs were determined by the lowest concentrations of agents that completely inhibited the bacteria growth in wells which appears as no increased turbidity of suspensions compared to sterile broth solution. Bacterial culture media from wells without increased turbidity were collected, dropped onto agar plates, and incubated for 24 h. The MBCs were determined by the lowest concentration yielding a negative bacterial growth. The experiment was independently repeated three times.

The effect of 1,4-naphthoquinone derivatives on bacterial growth kinetics was assessed by growth curve assay. Bacterial strains (10^5 CFU/mL) were placed in 96-well microtiter plates and incubated in different concentrations of lawsone derivatives (as described earlier). The absorbance value of each well, at a wavelength of 620, 600, and 660 nm for *S. mutans*, *L. casei,* and *A. naeslundii*, respectively, was recorded by a microplate reader (Multiskan GO, Thermo Fisher Scientific, Waltham, MA, US) every 2 h until the bacterial growth enters a stationary phase of each strain.

EFFECTS OF SYNTHETIC 1,4-NAPHTHOQUINONE DERIVATIVES ON ACID PRODUCTION

Bacterial cells were harvested from 24 mL mid-exponential phase bacterial culture by centrifugation (3,000 g, 4°C) (Labofuge 400 R, Heraeus, Hanau, Germany) for 10, 5 and 20 min for *S. mutans*, *L. casei,* and *A. naeslundii*, respectively. Cell pellets were washed with a salt solution containing 50 mM KCl and 1 mM $MgCl_2$ before being resuspended in the same salt solution. Lawsone derivatives were added to obtain the final concentrations of 100 µg/mL. Salt solution without lawsone derivative was added to the control mixture. Glucose was added to obtain the final concentration of 1% (w/v). The pH of the mixture was adjusted by 0.2 M KOH to 7.3 ± 0.1. The pH was monitored by pH meter (FiveEasy™ F20 pH/mV Meters, Mettler Toledo, Greifensee, Switzerland) at 10 min intervals over 120 min. The experiment was independently repeated three times.

EFFECTS OF SYNTHETIC 1,4-NAPHTHOQUINONE DERIVATIVES ON *S. mutans* BIOFILM FORMATION

S. mutans (10^5 CFU/mL) were placed in 96-well microtiter plates and incubated in different concentrations of 1,4-naphthoquinone derivatives (1/2 – 1/32 MIC) with the presence of 5% (w/v) sucrose. Wells without 1,4-naphthoquinone derivatives were used as controls. After 6, 12, and 24 h incubation periods, media and unbound cells were decanted. The remaining planktonic cells were removed by gently rinsing with distilled water. The attached cells (biofilms) were stained with 0.1% crystal violet for 15 min at room temperature. After two rinses with distilled water, 95% ethyl alcohol was added, and the plates were shaken for 10 min to allow full release of the dye. The absorbance of extracted crystal violet in ethyl alcohol was read at $absorbance_{595nm}$ (Taff, Nett & Andes 2012). The percentages of inhibition were calculated using the following formula:

$$\%\text{inhibition} = 100 - \left(\frac{\text{sample OD}}{\text{control OD}} \times 100\right)$$

STATISTICAL ANALYSIS

Descriptive statistics (mean and standard deviation) were used to summarize the data of the initial and terminal pH of three strains in the pH drop assay. Bacterial growth curves and pH drop profiles were displayed by line graphs. The percentages of biofilm inhibition were shown by bar charts. The data were also analyzed with a repeated measure ANOVA test to determine the effect of lawsone derivatives on pH values over time. The Kruskal-Wallis H test was used to determine the difference in the percentage of biofilm inhibition among lawsone derivatives at each time point. All analyses were performed using STATA version 13.1 (StataCorp, College Station, Texas). Differences at $P < 0.05$ were considered to be significant.

RESULTS AND DISCUSSION

To the best of our knowledge, this is the first study to investigate anticaries properties of 1,4-naphthoquinone

derivatives. We investigated the effects of 1,4-naphthoquinone derivatives on virulence properties associated with cariogenicity of three bacterial strains. Our study suggests that 1,4-naphthoquinone derivatives are potential anticaries candidates due to their ability to inhibit the growth of tested bacterial strains, and reducing acid production as well as biofilm formation of *S. mutans*.

SYNTHESIS OF 1,4-NAPHTHOQUINONE DERIVATIVES

Compounds **2**-**4** were prepared via a one-step reaction and purified by column chromatography and recrystallization to afford the synthesized compounds as a pure substance in good yields. Spectroscopic data (IR, ^{1}H-NMR, ^{13}C-NMR, and HR-MS) confirmed the chemical structure of the desired products.

Natural products have been extensively studied as they are a large source of bioactive molecules that may be used in alternative or adjunctive therapy for dental caries. Lawsone (2-hydroxy-1,4-naphthoquinone) is an organic compound found in nature that belongs to the 1,4-naphthoquinones class. Various synthetic 1,4-naphthoquinone derivatives have demonstrated a wide range of biological activities, including antibacterial activity (Lopez et al. 2014). In the present study, four 1,4-naphthoquinone derivatives were synthesized. Compound **1** is an artificial compound with the same structure as naturally occurring LME, found in the plant *Impatiens balsamina* L. In compound **2**, the methyl group of compound **1** was replaced with a more hydrophobic propargyl moiety. In compounds **3** and **4**, the terminal alkyne proton of compound **2** was substituted with methyl and phenyl groups, respectively. The synthetic lawsone derivatives were conveniently prepared via a one-step reaction as the pure substance in good yield. This can benefit the further development of these compounds as anticaries agent in terms of purity, quantification, and quality control.

EFFECTS OF SYNTHETIC 1,4-NAPHTHOQUINONE DERIVATIVES ON THE BACTERIAL GROWTH

Bacterial susceptibility of the 1,4-naphthoquinone derivatives against *S. mutans, L. casei*, and *A. naeslundii* are shown in Table 1. The 1,4-naphthoquinone derivatives exhibited the most potent antibacterial activity against *S. mutans*, followed by *A. naeslundii*, and were the least active against *L. casei*. Compounds **1**, **2** and **3** (MIC of 1.56 µg/mL) exhibited comparable antibacterial activity to CHX (MIC of 1.95 µg/mL) against *S. mutans*. Compound **1** inhibited *L. casei* with moderate potency (MIC of 50 µg/mL) while *L. casei* was resistant to other lawsone derivatives. Compound **2** inhibited *A. naeslundii* (MIC of 3.125 µg/mL) with high potency in the same range as CHX (MIC of 1.95 µg/mL). The growth curves of *S. mutans*, *L. casei* and *A. naeslundii* showed that 1,4-naphthoquinone derivatives at the concentrations of 1/2 MICs prolonged the lag phase, reduced the slope of the exponential phase, and decreased the peak absorbance (Figure 2).

Previous studies evaluated antibacterial activity of compound **1** against *S. mutans*. MIC and MBC values of LME against *S. mutans* were reported as 31.25 µg/mL and 62.5 µg/mL, respectively (Sakunphueak & Panichayupakaranant 2012). A previous study evaluated the antibacterial activity of oral spray containing LME and reported the same MIC and MBC values of LME against *S. mutans* (Nittayananta et al. 2018). The MIC value of compound **1** against *S. mutans* in the present study is much lower, 1.56 µg/mL. However, the MBC value of compound **1** against *S. mutans* was higher than 100 µg/mL. Differences in *S. mutans* strains and the form of a compound may be the root of the inconsistent findings.

Antibacterial activity of 1,4-naphthoquinones arises from the quinone moiety. Primary mechanisms which have been discussed in previous studies are: quinone moiety generates harmful reactive oxygen species (ROS) by the redox cycle under aerobic conditions; and quinones act as electrophiles in Michael addition reaction with bacterial macro biomolecules (Anaissi-Afonso et al. 2018; Lopez et al. 2014). However, ROS generation is a more important mechanism for lawsone derivatives than the others. The mitochondrial respiratory chain is the target of 1,4-naphthoquinone derivatives, where ROS are generated by redox cycling in the presence of oxygen (Anaissi Afonso et al. 2018). This is consistent with the previous study, which implied that the oxygen consumption of microorganisms involves the antibacterial activity of LME (Sakunphueak & Panichayupakaranant 2012). This could explain the different degrees of sensitivity to 1,4-naphthoquinone derivatives that was found among the three bacterial strains tested in the present study. *S. mutans* and *A. neaslundii* are facultative anaerobes, whereas *L. casei* is an oxygen-tolerant anaerobe with an ability to shift from anaerobic growth to aerobic/respiratory growth (Ahn, Wen & Burne 2007; van der Hoeven & van den Kieboom 1990; Zotta, Parente & Ricciardi 2017). Thus, these three strains use oxygen differently, and for this reason, lawsone derivatives exhibit antibacterial activity with different potency among them under the microaerobic growth condition in this study.

The 1,4-naphthoquinone derivatives synthesized in this study resulted from the addition of different functional groups into the 2-hydroxy-1,4-naphthoquinone structure. The chemical modification altered the electronic, lipophilic and steric properties of the 1,4-naphthoquinone system, resulting in varying antibacterial properties of 1,4-naphthoquinone derivatives. It has been suggested that the polar hydroxyl group in the structure of lawsone hinders its penetrability through the plasma membrane (Anaissi-Afonso et al. 2018). The incorporation of a less polar group at position 2 of the lawsone structure enhanced the absorption through the microbial cell membrane and thus increased its antimicrobial potency. Therefore, based on the aforementioned remarks, increased lipophilicity of the functional group at position 2 of the lawsone structure is assumed to enhance the antimicrobial potency. Among four 1,4-naphthoquinone derivatives in this study, the lipophilicity of the functional groups incorporated in the 1,4-naphthoquinone structure increases progressively from compounds **1** to **4**. However, compound **4** showed the least inhibitory effect on the growth of three bacterial strains. This implies that, besides lipophilicity, steric properties of the side chain on 2-position are also a determinant of antibacterial potency of 1,4-naphthoquinone derivatives. The steric side chain might hinder binding interactions between 1,4-naphthoquinone derivatives with their biological target in bacteria and lead to a lower inhibitory effect.

Generally, antibacterial drugs are divided into two groups based on their effect on bacteria, which are bacteriostatic and bactericidal. In the present study, only compounds **2** and **3** were able to kill *S. mutans* at the highest tested concentration (100 µg/mL), and only compounds **1** and **2** were bactericidal against *A. naeslundii* at the concentration of 100 µg/mL. However, their MBC values were much higher than their corresponding MIC values (16- to 64-fold higher). The MBC of bactericidal drugs is usually not more than 4-fold higher than the MIC (Levison 2004). Therefore, all lawsone derivatives in our study are considered bacteriostatic agents, which means they require the aid of host defences to eliminate the residual pathogens otherwise those bacteria will continue to grow as soon as lawsone derivatives are no longer available at the target, for instance, in the oral cavity or dental biofilm. However, dental biofilm and the resident oral pathogens are regularly removed mechanically by tooth brushing and the usage of oral hygiene aids, which are recommended to be performed twice daily. Thus, despite their bacteriostatic effect, when combined with an adequate oral hygiene routine, lawsone derivatives in our study are still an interesting candidate for an alternative anticaries agent.

TABLE 1. The MIC and MBC values of synthetic 1,4-naphthoquinone derivatives against *S. mutans*, *L. casei,* and *A. naeslundii*

Compounds	*S. mutans*		*L. casei*		*A. naeslundii*	
	MIC (µg/mL)	MBC (µg/mL)	MIC (µg/mL)	MBC (µg/mL)	MIC (µg/mL)	MBC (µg/mL)
1	1.56	>100	50	>100	6.25	100
2	1.56	100	>100	>100	3.125	100
3	1.56	100	>100	>100	6.25	>100
4	6.25	>100	>100	>100	50	>100
CHX	1.95	15.6	15.6	31.25	1.95	3.9

MIC, Minimum inhibitory concentration; MBC, Minimum bactericidal concentration; CHX, Chlorhexidine gluconate

EFFECTS OF SYNTHETIC 1,4-NAPHTHOQUINONE DERIVATIVES ON ACID PRODUCTION

Due to their antibacterial activity against all tested bacterial strains, compounds **1** and **2** were selected for glycolytic pH drop assay. The initial and final pH of the bacterial suspension of each strain is shown in Table 2. Compounds **1** and **2** tended to retard the acid production rate and increased the terminal pH of *S. mutans* suspensions compared to control (Figure 3(A)). However, pH profiles of *L. casei* and *A. naeslundii* were not affected (Figure 3(B), 3(C)). In the analysis, the effect of the compounds on the pH drop profiles across time is not statistically significant for all tested bacterial strains (P >0.05).

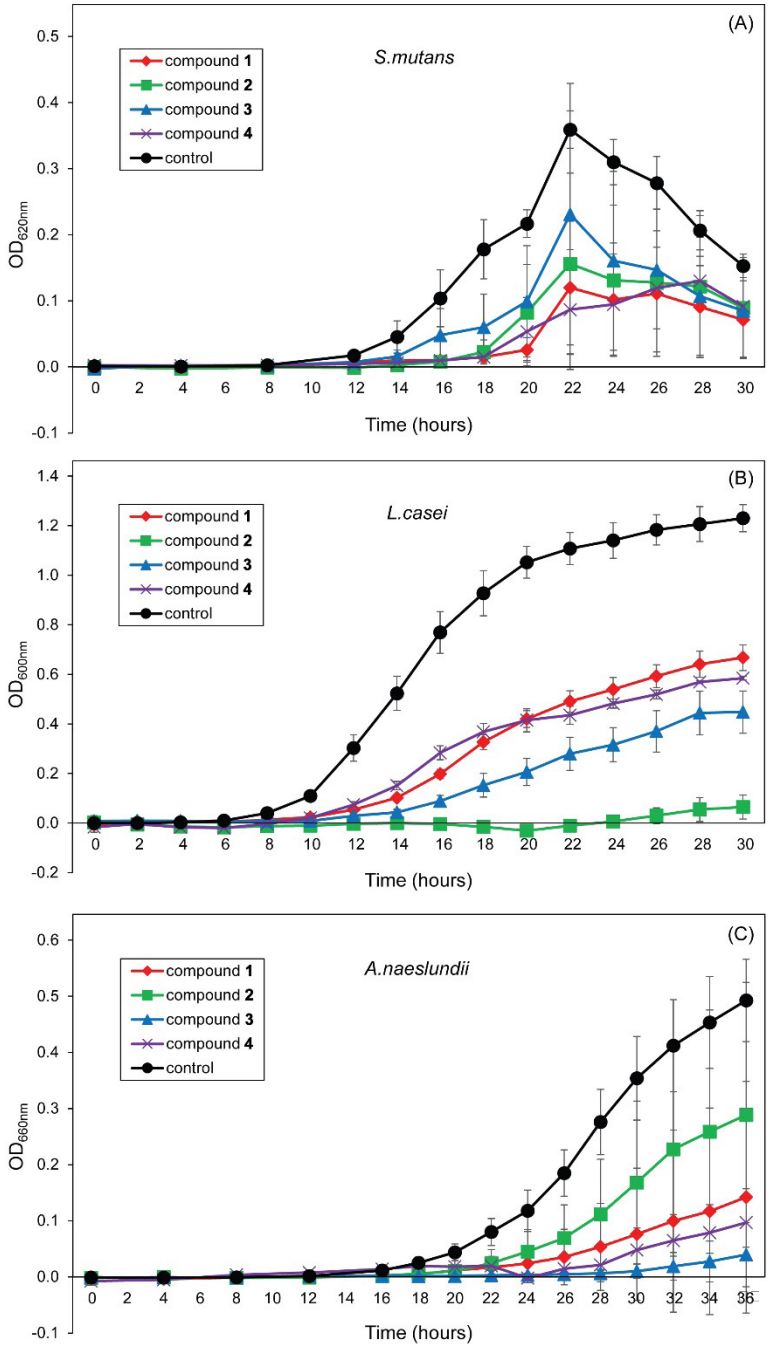

FIGURE 2. Growth curves of bacterial strains with the presence of synthetic 1,4-naphthoquinone derivatives at the concentrations of 1/2 MICs; (A) *S. mutans,* (B) *L. casei*, and (C) *A. naeslundii*

TABLE 2. Initial and terminal pH of bacterial suspension with the presence of 1% glucose and synthetic 1,4-naphthoquinone derivatives (at concentration of 100 µg/mL)

	S. mutans		*L. casei*		*A. naeslundii*	
	Initial pH Mean (±SD)	Terminal pH Mean (±SD)	Initial pH Mean (±SD)	Terminal pH Mean (±SD)	Initial pH Mean (±SD)	Terminal pH Mean (±SD)
Control	7.26 (0.040)	3.98 (0.055)	7.26 (0.051)	4.00 (0.198)	7.29 (0.070)	5.60 (0.257)
Compound **1**	7.36 (0.051)	4.20 (0.110)	7.26 (0.047)	4.05 (0.212)	7.32 (0.020)	5.55 (0.250)
Compound **2**	7.26 (0.047)	4.19 (0.151)	7.30 (0.067)	4.18 (0.169)	7.27 (0.067)	5.75 (0.316)

SD, Standard deviation

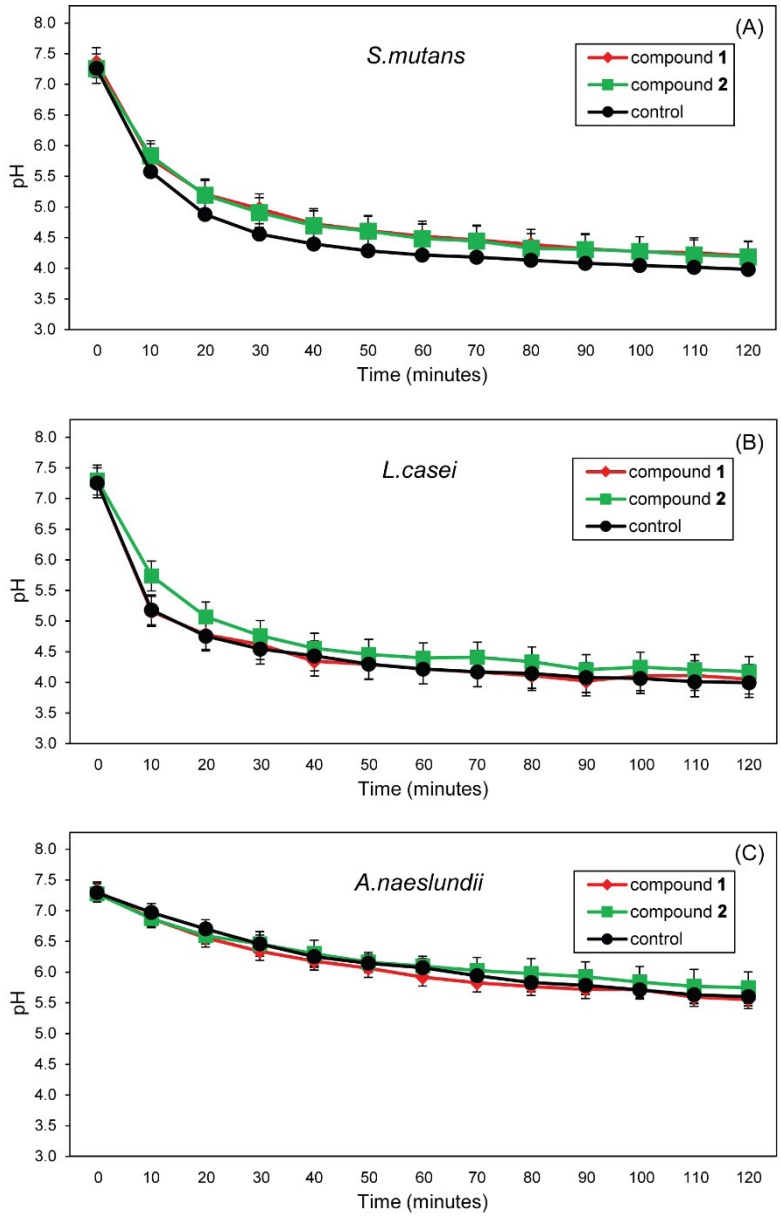

FIGURE 3. pH drop profile of bacterial strains with the presence of compounds **1** and **2** at the concentration of 100 µg/mL; (A), *S. mutans* (B) *L. casei*, and (C) *A. naeslundii*

In the glycolytic pH drop assay, the pH drop profile of *S. mutans* showed that compounds **1** and **2** at the concentration of 100 µg/mL reduced the rate of pH drop and increased the terminal pH of *S. mutans* (Figure 3(A)), indicating that 100 µg/mL compounds **1** and **2** may have an ability to inhibit acid production which is one of the cariogenic virulence factors of *S. mutans*. However, the pH drop assay is influenced by various factors, such as enzyme activity under changing pH conditions and the buffer capacities of the reaction mixture components (Han et al. 2021). The possible causes of our finding include: lawsone derivatives inhibiting glycolytic metabolism of *S. mutans*, maybe via inhibition of lactate dehydrogenase enzyme and cell membrane-associated sugar uptake enzyme system, the PEP-PTS; lawsone derivatives possess buffer capacity. Thus, other assays are needed to justify either the effect of lawsone derivatives on the acidogenicity of *S. mutans*

or their buffer capacity. However, the finding of this study was sufficient to conclude that compounds **1** and **2** suppresses *S. mutans*-induced pH reduction *in vitro*.

EFFECTS OF SYNTHETIC 1,4-NAPHTHOQUINONE DERIVATIVES ON BIOFILM FORMATION

Sub-MIC concentrations of lawsone derivatives reduced the biofilm formation of *S. mutans*, and the percentage of inhibition decreased with decreasing concentration. However, the percentage of inhibition was not different among all compounds at each time point (Figure 4). With the concentrations of 1/2 MIC, lawsone derivatives inhibited 12-hour biofilm formation by 93.31-99.53%. The inhibitory effect on 24-hour biofilm formation decreased to 67.43-91.38%.

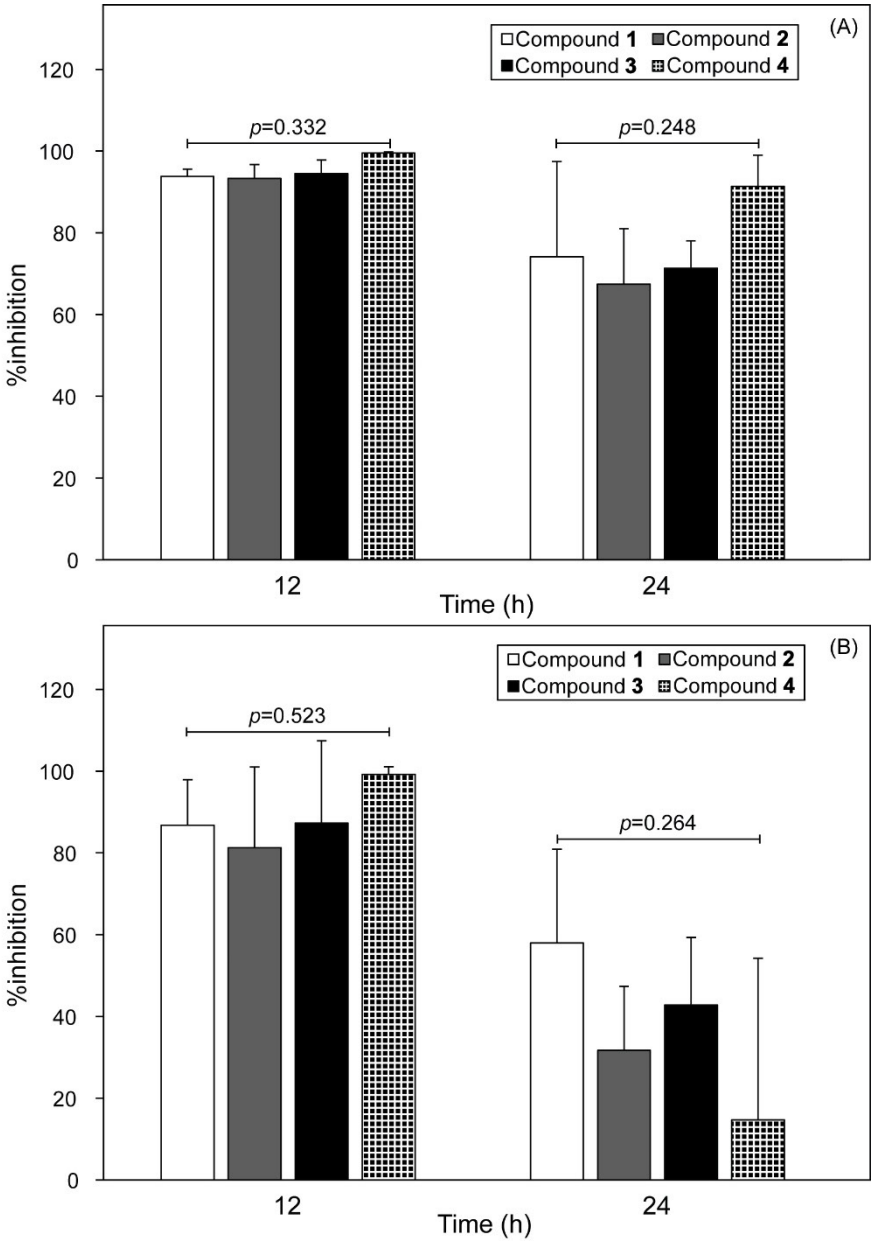

FIGURE 4. Percentage inhibition of 12-h and 24-h *S. mutans* biofilm formation by synthetic 1,4-naphthoquinone derivatives; (A) concentrations of 1/2 MIC, and (B) concentrations of 1/4 MIC

In this study, lawsone derivatives markedly inhibited the biofilm formation of *S. mutans*. The concentration of 1/2 MIC of lawsone derivatives reduced 12-h *S. mutans* biofilm formation by more than 90% (Figure 4(A)). A previous study also reported the antibiofilm activity of LME in oral spray against *S. mutans* (Nittayananta et al. 2018). Moreover, we found that the inhibitory effect of lawsone derivatives on *S. mutans* biofilm formation was time- and dose-dependent. There are several stages in the formation of a biofilm. Our study was designed to test the effect of lawsone derivatives on different stages of biofilm formation, which are the initial attachment phase, active accumulated phase, and plateau accumulated phase, at 6-, 12-, and 24-hour intervals. The effect on 12- and 24-hour biofilm formation demonstrated that the inhibitory effect of lawsone derivatives on *S. mutans* biofilm formation declined with time. However, the effect of 1/2 MIC lawsone derivatives on 24-h biofilm formation was still satisfying (Figure 4(B)). The effect on 6-h biofilm formation was not seen in our study because of the limitation of the method that we adopted for biofilm measurement. Crystal violet assay is the most frequent biofilm quantification technique used in a microtiter plate (Azeredo et al. 2017). This chemical method indirectly measures biofilm biomass by adsorption of a dye. It is a versatile method with high-throughput capability. However, this method lacks sensitivity. This may explain the failure to measure 6-h biofilm in this study, which is only the initial stage of biofilm formation. The possible mechanisms of biofilm formation inhibition include bacterial growth inhibition, promotion of bacterial aggregation, which results in reduced bacterial adherence, and inhibition of glucosyltransferase activity, an essential enzyme of *S. mutans* for the synthesis of extracellular polysaccharides, enhancing bacterial adherence and biofilm cohesiveness.

Conclusions

This *in vitro* study demonstrated that synthetic lawsone derivatives have an inhibitory effect on the growth of *S. mutans*, *L. casei,* and *A. naeslundii*, which are the primary causative agents of dental caries. Moreover, these compounds also inhibit pH reduction and biofilm formation of *S. mutans*. However, this study is only the beginning step of the investigation for the potential use of lawsone derivatives in caries research. Yet, this effect suggests that lawsone derivatives are promising compounds that could be developed as a novel prophylactic, therapeutic agent for dental caries. To develop lawsone derivatives, future studies are recommended to explore their mechanisms. Moreover, there may be a possibility to make further structural modifications to afford lawsone derivatives with enhanced anticaries properties.

Acknowledgements

This research was supported by the National Science, Research, and Innovation Fund (NSRF) (Grant No. DEN6405026S), Faculty of Pharmaceutical Sciences (Grant No. PHA6304038S), and Graduate Research Fund, Prince of Songkla University. The authors would like to thank the staff at the Dental Research Unit and Office of Scientific Instrument and Testing (OSIT) for their assistance in conducting the lab testing process.

References

Ahn, S.J., Wen, Z.T. & Burne, R.A. 2007. Effects of oxygen on virulence traits of *Streptococcus mutans*. *Journal of Bacteriology* 189(23): 8519-8527.

Alaki, S.M., Burt, B.A. & Garetz, S.L. 2009. The association between antibiotics usage in early childhood and early childhood caries. *Pediatric Dentistry* 31(1): 31-37.

Anaissi-Afonso, L., Oramas-Royo, S., Ayra-Plasencia, J., Martín-Rodríguez, P., García-Luis, J., Lorenzo-Castrillejo, I., Fernández-Pérez, L., Estévez-Braun, A. & Machín, F. 2018. Lawsone, Juglone, and β-Lapachone derivatives with enhanced mitochondrial-based toxicity. *ACS Chemical Biology* 13(8): 1950-1957.

Azeredo, J., Azevedo, N.F., Briandet, R., Cerca, N., Coenye, T., Costa, A.R., Desvaux, M., di Bonaventura, G., Hébraud, M., Jaglic, Z., Kačániova, M., Knøchel, S., Lourenço, A., Mergulhão, F., Meyer, R.L., Nychas, G., Simões, M., Tresse, O. & Stenberg, C. 2017. Critical review on biofilm methods. *Critical Reviews in Microbiology* 43(3): 313-351.

Caufield, P.W., Schön, C.N., Saraithong, P., Li, Y. & Argimón, S. 2015. Oral lactobacilli and dental caries: A model for niche adaptation in humans. *Journal of Dental Research* 94(9 Suppl): 110S-8S.

Dieterle, M.E., Fina Martin, J., Durán, R., Nemirovsky, S.I., Sanchez Rivas, C., Bowman, C., Russell, D., Hatfull, G.F., Cambillau, C. & Piuri, M. 2016. Characterization of prophages containing "evolved" Dit/Tal modules in the genome of *Lactobacillus casei* BL23. *Applied Microbiology and Biotechnology* 100(21): 9201-9215.

Han, S., Abiko, Y., Washio, J., Luo, Y., Zhang, L. & Takahashi, N. 2021. Green tea-derived epigallocatechin gallate inhibits acid production and promotes the aggregation of *Streptococcus mutans* and non-mutans streptococci. *Caries Research* 55(3): 205-214.

Howell, A., Jordan, H.V., Georg, L.K. & Pine, L. 1965. *Odontomyces viscosus*, gen. nov., spec. nov., a filamentous microorganism isolated from periodontal plaque in hamsters. *Sabouraudia* 4(2): 65-68.

Jeon, J.G., Rosalen, P.L., Falsetta, M.L. & Koo, H. 2011. Natural products in caries research: Current (limited) knowledge, challenges and future perspective. *Caries Research* 45(3): 243-263.

Kawashima, J., Nakajo, K., Washio, J., Mayanagi, G., Shimauchi, H. & Takahashi, N. 2013. Fluoride-sensitivity of growth and acid production of oral *Actinomyces*: Comparison with oral *Streptococcus*. *Microbiology and Immunology* 57(12): 797-804.

Levison, M.E. 2004. Pharmacodynamics of antimicrobial drugs. *Infectious Disease Clinics of North America* 18(3): 451-465.

Li, J., Helmerhorst, E.J., Leone, C.W., Troxler, R.F., Yaskell, T., Haffajee, A.D., Socransky, S.S., & Oppenheim, F.G. 2004. Identification of early microbial colonizers in human dental biofilm. *Journal of Applied Microbiology* 97(6): 1311-1318.

Lopez, L.I.L., Flores, S.D.N., Belmares, S.Y.S. & Galindo, A.S. 2014. Naphthoquinones: Biological properties and synthesis of lawsone and derivatives - A structured review. *Vitae* 21(3): 248-258.

Nittayananta, W., Limsuwan, S., Srichana, T., Sae-Wong, C. & Amnuaikit, T. 2018. Oral spray containing plant-derived compounds is effective against common oral pathogens. *Archives of Oral Biology* 90: 80-85.

Panichayupakaranant, P. & Reanmongkol, W. 2008. Evaluation of chemical stability and skin irritation of lawsone methyl ether in oral base. *Pharmaceutical Biology* 40(6): 429-432.

Panichayupakaranant, P., Septama, A.W. & Sinviratpong, A. 2019. Synergistic activity of lawsone methyl ether in combination with some antibiotics and artocarpin against methicillin-resistant *Staphylococcus aureus*, *Candida albicans*, and *Trychophyton rubrum*. *Chinese Herbal Medicines* 11(3): 321-325.

Qiu, W., Zhou, Y., Li, Z., Huang, T., Xiao, Y., Cheng, L., Peng, X., Zhang, L. & Ren, B. 2020. Application of antibiotics/antimicrobial agents on dental caries. *Biomed Research International* 2020: 5658212.

Sakunphueak, A. & Panichayupakaranant, P. 2012. Comparison of antimicrobial activities of naphthoquinones from *Impatiens balsamina*. *Natural Product Research* 26(12): 1119-1124.

Simón-Soro, A. & Mira, A. 2015. Solving the etiology of dental caries. *Trends in Microbiology* 23(2): 76-82.

Taff, H.T., Nett, J.E. & Andes, D.R. 2012. Comparative analysis of candida biofilm quantitation assays. *Medical Mycology* 50(2): 214-218.

Takahashi, N. & Nyvad, B. 2016. Ecological hypothesis of dentin and root Caries. *Caries Research* 50(4): 422-431.

Tanner, A., Kressirer, C.A., Rothmiller, S., Johansson, I. & Chalmers, N.I. 2018. The caries microbiome: Implications for reversing dysbiosis. *Advances in Dental Research* 29(1): 78-85.

ten Cate, J.M. & Zaura, E. 2012. The numerous microbial species in oral biofilms: How could antibacterial therapy be effective? *Advances in Dental Research* 24(2): 108-111.

van der Hoeven, J.S. & van den Kieboom, C.W.A. 1990. Oxygen-dependent lactate utilization by *Actinomyces viscosus* and *Actinomyces naeslundii*. *Oral Microbiology and Immunology* 5(4): 223-225.

Vohra, F., Akram, Z., Safii, S.H., Vaithilingam, R.D., Ghanem, A., Sergis, K. & Javed, F. 2016. Role of antimicrobial photodynamic therapy in the treatment of aggressive periodontitis: A systematic review. *Photodiagnosis and Photodynamic Therapy* 13: 139-147.

Wilkins, J.C., Homer, K.A. & Beighton, D. 2002. Analysis of *Streptococcus mutans* proteins modulated by culture under acidic conditions. *Applied and Environmental Microbiology* 68(5): 2382-2390.

Yang, X., Summerhurst, D.K., Koval, S.F., Ficker, C., Smith, M.L. & Bernards, M.A. 2001. Isolation of an antimicrobial compound from *Impatiens balsamina* L. using bioassay-guided fractionation. *Phytotherapy Research* 15(8): 676-680.

Zotta, T., Parente, E. & Ricciardi, A. 2017. Aerobic metabolism in the genus Lactobacillus: Impact on stress response and potential applications in the food industry. *Journal of Applied Microbiology* 122(4): 857-869.

*Corresponding author; email: supawadee.n@psu.ac.th