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In vitro evaluation of 2-(chloroalkyloxy)naphthalene-1, 4-dione derivatives as anticaries agents

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ABSTRACT: Lawsone is a privileged antibacterial pharmacophore in natural and synthetic compounds. A structural modification that increases the lipophilicity may improve its antibacterial potency. This study investigated antimicrobial, antiglycolytic, and antibiofilm activities of synthetic 2-(chloroalkyloxy)naphthalene-1,4-dione derivatives (Compounds 1 and 2). Microdilution method and pH drop assay were used to evaluate the antibacterial and antiglycolytic activity of Compounds 1 and 2 against three bacterial strains: *Streptococcus mutans*, *Lacticaseibacillus casei*, and *Actinomyces naeslundii*. Crystal violet assay assessed the effect of Compounds 1 and 2 on *S. mutans* biofilm formation. The MTT assay was employed to assess Human Gingival Fibroblast (HGF-1) cell viability. According to minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values, *S. mutans* was more susceptible to Compounds 1 and 2 (MIC 0.78, 1.56 μg/ml, respectively) than *A. naeslundii* (MIC 6.25, 12.50 μg/ml, respectively), while, *L. casei* was resistant. Only the MBC of Compound 1 against *A. naeslundii* (100 μg/ml) was found. Both Compounds 1 and 2 significantly retarded the pH declined by *S. mutans*, but had no effect on the pH drop profile of *A. naeslundii*. Only Compound 1 significantly affected the pH drop profile of *L. casei*. At 12 h, all sub-MIC concentrations (1/2 to 1/8) of Compounds 1 and 2 significantly reduced *S. mutans* biofilm formation. However, at 24 h, only sub-MIC 1/2 concentration significantly reduced biofilm formation. Compound 1 exhibited dose-dependent cytotoxicity, with IC_{50} of 2.34 μg/ml. By structural modification of lawsone via an alkylation reaction on the 2-hydroxyl group, Compound 1 was more potent than Compound 2 in terms of inhibiting bacterial growth, biofilm formation, and acid production. Both compounds could be promisingly be a potential dental caries preventive agent.

KEYWORDS: lawsone derivatives, dental caries, antibacterial activity, acid production, dental biofilm

INTRODUCTION

Dental caries is considered as the most prevalent oral disease worldwide. It is a multifactorial disease, and virulent biofilm accumulated on the tooth surface is one of the main etiologic factors. *Streptococcus mutans* is widely recognized as a pathogen involved in the pathogenesis of dental caries. It plays an important role in the development of cariogenic biofilm [1, 2]. When exposed to fermentable carbohydrates, *S. mutans* and other acid-producing bacteria in the biofilm utilize sucrose and then produce organic acids as a by-product. The acids cause demineralization of dental hard tissue, eventually leading to cavitation [3, 4]. Besides *S. mutans*, other bacteria are also involved in the dental caries process, including *Lactobacillus* spp. and *Actinomyces* spp. [1, 3, 5]. Since cariogenic bacteria and biofilm are the main cause of dental caries, agents with antibacterial and antibiofilm properties are one of the strategies in dental caries prevention [6].

1,4-Naphthoquinone is regarded as a privileged pharmacophore for antibacterial activity in both naturally occurring and synthetic compounds. Lawsone (2-hydroxy-1,4-naphthoquinone) and its derivative, lawsone methyl ether (LME), demonstrated antifungal and antibacterial actions against many bacterial species, including the cariogenic bacteria *S. mutans* [7–9]. The 1,4-naphthoquinone moiety is essential for antibacterial activity. In the mitochondrial respiratory chain, it can react with oxygen molecules, causing the production of hazardous reactive oxygen species (ROS) that induce cell death. Additionally, bacteria's biomolecules can undergo the Michael addition reaction at position 3 of the 1,4-naphthoquinone ring, leading to their dysfunction [10, 11].

Lawsone exhibited low antibacterial potency. A structural modification that increased the lipophilicity of lawsone resulted in higher antibacterial potency. A previous study proposed the incorporation of a lipophilic group on the hydroxyl group at position 2

Fig. 1 Synthesis of the 2-(chloroalkyloxy)naphthalene-1,4-dione derivatives, Compounds 1 and 2.

of the lawsone structure, i.e., LME enhanced the absorption through the microbial cell membrane and thus increased its antimicrobial potency [8]. Therefore, we were interested in the modification of the 2-position of the lawsone structure by adding lipophilic groups and the evaluation of antimicrobial and anti-caries activities of the new derivatives for dental applications. In 2023, the O-alkynyl derivatives [12–14] and lawsone derivatives containing *N*-substituted 1,2,3-triazole were reported by our group [15].a

This research aimed to further examine how chloroalkyloxy substituents at the 2-position of 1,4-naphthoquinone system affecting its antibacterial activity against cariogenic bacteria. We also evaluated the impact of synthetic 1,4-naphthoquinone derivatives on *S. mutans* biofilm formation and bacterial acid generation.

MATERIALS AND METHODS

Starting materials were purchased from Merck AG (Darmstadt, Germany). Progress of the reaction was monitored by TLC on aluminium sheets coated with silica gel 60 F_{254} purchased from E. Merck using dichloromethane:hexane (1:1) as eluent. Melting points (mp, °C) were recorded using the Stuart SMP11 (Cole-Parmer, Staffordshire, UK). The samples were prepared as 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) spectroscopy was achieved on Bruker Ascend 500/Avance Neo (Massachusetts, USA) using $CDCl_3$ as a solvent. Chemical shifts (δ) are expressed in ppm relative to TMS. Multiplicity of the ^{1}H-NMR peaks 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) operating in positive mode.

Chemistry

Pathway for the synthesis of 2-(chloroalkyloxy) naphthalene-1,4-dione derivatives (Compounds 1 and 2) was illustrated in Fig. 1. The desired compounds were prepared via an alkylation reaction on the 2-hydroxyl group of lawsone in basic conditions with the presence of KI and a phase-transfer catalyst as described in previous work [16].

General method for the synthesis of 2-(chloroalkyloxy)naphthalene-1,4-dione derivatives (Compounds 1 and 2)

A mixture of lawsone (8, 0.87 g, 5 mmol), 1,3-dichloropropane (for the synthesis of Compound 1) or 1,4-dichlorobutane (for the synthesis of Compound 2) (50 mmol), anhydrous potassium carbonate (K_2CO_3) (1.40 g, 50 mmol), potassium iodide (KI) (0.34 g, 2 mmol), and benzyl-triethylammoniumchloride (TEBAC, 2.28 g, 10 mmol) in 30 ml acetonitrile was heated at 80 °C for 4 h. After cooling, the solid was filtered off, and the filtrate was evaporated under reduced pressure. The residue was purified by silica gel column chromatography using dichloromethane/hexane (1:1) as an eluent to give the desired product as a yellow solid. The compound was recrystallized in dichloromethane [16].

Predictive physicochemical parameters

The molecular weight (MW), consensus log P, number of hydrogen bond donors and acceptors, rotatable bonds, topological polar surface area (tPSA), and water solubility of the 2-(chloroalkyloxy) naphthalene-1,4-dione derivatives were calculated using the SwissADME program available at www.swissadme.ch.

Bacterial strains and growth conditions

S. mutans (DMST 41283), *L. casei* (TISTR 1463), and *A. naeslundii* (TISTR 2426) were used in this study. *S. mutans* and *A. naeslundii* were grown on Brain Heart Infusion (BHI) agar plates (HiMedia Laboratories, Mumbai, India), and *L. casei* was grown on deMan, Rogosa, Sharpe (MRS) agar plates (HiMedia Laboratories). All strains were grown at 37 °C in the presence of 5% CO_2. The same growth conditions were used throughout the study.

Antibacterial activity of Compounds 1 and 2

MIC and MBC were determined using microdilution method performed in 96-well microtiter plates to assess the antibacterial activity of 2-(chloroalkyloxy) naphthalene-1,4-dione derivatives against three dental caries pathogens. The synthetic compounds were two-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. Bacterial suspension (approximately 10^5 CFU/ml) was added in each well. Chlorhexidine gluconate (CHX) and sterile broth solution were used as the positive and the negative controls for antibacterial activity, respectively. Microtiter plates were incubated, and MIC of individual samples was recorded after 24 h by the lowest concentration of compound that completely inhibited the growth of bacteria in wells (no increased turbidity). Then, the mixture in wells without bacterial growth

was collected and dropped on agar plates. MBC was recorded after 24 h incubation by the lowest concentration yielding no bacterial growth. The experiment was independently repeated three times.

Effect of Compounds 1 and 2 on bacterial acid production

Bacterial cultures were grown in broth until they reached mid-exponential phase of each strain. Bacteria cells were harvested 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. Cells were washed with a salt solution containing 50 mM KCl and 1 mM $MgCl_2$ before being resuspended in the same salt solution. Compounds 1 and 2 were separately added to obtain the final concentration of 100 μg/ml, while salt solution was added into 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 7.2–7.4 and monitored by pH meter (FiveEasy™ F20 pH/mV Meters, Mettler Toledo, Greifensee, Switzerland) every 10 min over a period of 120 min. The experiment was independently repeated three times.

Effect of Compounds 1 and 2 on *S. mutans* biofilm formation

S. mutans (10^5 CFU/ml) was placed in 96-well microtiter plates and incubated in different sub-MIC concentrations of Compounds 1 and 2 (1/2–1/8 MIC) in the presence of 5% (w/v) sucrose. Wells without lawsone derivative were served as controls. After the incubation periods of 12 and 24 h, the media and unbound cells were decanted. The microtiter plates were gently rinsed with distilled water to remove the remaining planktonic cells. The biofilms were stained with 0.1% crystal violet for 15 min at room temperature before being rinsed twice with distilled water. Ethanol was added, and the plates were placed on a shaker for 10 min to allow full release of the bound dye. The $absorbance_{595nm}$ of extracted dye in ethanol was read by spectrophotometer (Genesys 10s UV-VIS, Thermo Fisher Scientific, Waltham, MA, US). The experiment was independently repeated three times for each incubation period.

Cytotoxicity test

Cytotoxicity is a cellular reaction to toxic stimulants, resulting in a reduction of viable cells or inhibition of cell growth. We intended to perform the cytotoxicity test on a synthetic compound that exhibited the most promising antibacterial and anti-biofilm formation activities. Human Gingival Fibroblasts (HGF-1), which represent the human gingiva, were used in this study. The MTT assay was used to assess cell viability by measuring metabolic activity.

The synthetic compound was dissolved in DMSO to 2 mg/ml. Vehicle control (VC) was complete culture medium (DMEM supplemented with 2 mM L-glutamine and 10% FBS) containing 0.5% v/v DMSO. The compound was then diluted with complete medium to 10 μg/ml, and serially diluted with the vehicle control. Positive control (PC) was 1.5 mM organotin.

HGF-1 cells were cultured and incubated at 37 °C in a CO_2 incubator for 24 h. HGF-1 cells were then treated with eight concentrations of the tested compound sample for another 24 h. Results were averaged from three independent experiments. The half maximal inhibitory concentration (IC_{50}) was calculated using Four Parameter Logistic Curve.

Statistical analysis

The data of initial and terminal pH of three bacterial strains were expressed by mean ± standard deviation. Line graphs were used to show the changes over time of pH (pH drop profiles). Bar graph was used to display the optical density of the bound dye, which represented biofilm formation of *S. mutans*. Repeated measure ANOVA with pairwise *t*-test was used to determine the effect of compounds on pH changes. One-way ANOVA with LSD *post hoc* test was used to determine the significance of the difference between *S. mutans* biofilm formed with and without the compounds. The software STATA version 16.1 (StataCorp, College Station, TX, US) was used for data analysis.

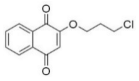
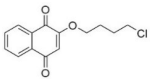
RESULTS AND DISCUSSION

1,4-Naphthoquinones, whether natural or synthetic, are compounds that possess a diverse range of biological activities. New 1,4-naphthoquinone derivatives with various modifications of the chemical structure were synthesized, and their biological effects were evaluated [17]. Recently, synthetic lawsone derivatives containing *N*-substituted 1,2,3-triazole were investigated for their effects on dental caries pathogens; and consequently, their potential use in dental caries prevention was suggested because of the antibacterial activity and the ability to inhibit the biofilm formation of *S. mutans* [15]. In the present study, 1,4-naphthoquinones with chloroalkyloxy substituents at 2-position were synthesized. The purpose of the substitution was to increase the compound's lipophilicity.

Synthesis and predictive physicochemical parameters of Compounds 1 and 2

Two 2-(chloroalkyloxy)naphthalene-1,4-dione derivatives, Compounds 1 and 2, were synthesized with high yields of 81% and 79%, respectively. The chemical structures of the compounds were confirmed by IR spectroscopy, ^{1}H-NMR, ^{13}C-NMR spectroscopy, and high-resolution mass spectrometry. Melting points of the Compounds were also recorded. Appearance and

Table 1 Physicochemical descriptors, ADME properties, and MIC/MBC values of synthetic lawsone derivatives.

Cpd	Chemical structure	MW	Log P	HBD	HBA	tPSA (Å^2)	Rotatable bonds	Water solubility	MIC/MBC value (μg/ml)		
									S. mutans	*L. casei*	*A. naeslundii*
Lawsone		174.15	1.21	1	3	54.37	0	Soluble	–	–	–
LME		188.18	1.43	0	3	43.37	1	Soluble	1.56/ >100	50/ >100	6.25/100
1		250.68	2.32	0	3	43.37	4	Moderately soluble	0.78/ >100	100/ >100	6.25/100
2		264.70	2.65	0	3	43.37	5	Moderately soluble	1.56/ >100	>100/ >100	12.50/ >100
CHX	–	–	–	–	–	–	–	–	1.95/ 15.60	15.60/ 31.25	1.95/ 3.90

Cpd, Compound; MW, molecular weight; log P, Predicted octanol/water partition coefficient log P; HBD, H-Bond Donors; HBA, H-Bond Acceptors; tPSA, topological polar surface area; MIC, Minimum inhibitory concentration; MBC, Minimum bactericidal concentration; CHX, Chlorhexidine gluconate; 1, Compound 1; and 2, Compound 2.

characterization data of the Compounds corresponded with previously reported data [16]. The anticipated physicochemical parameters of the two derivatives from the SwissADME calculator were shown in Table 1. Both Compound 1 (cLog P = 2.32) and Compound 2 (cLog P = 2.65) possessed higher lipophilicity than lawsone (cLog P = 1.21) and LME (cLog P = 1.43).

Antibacterial activity of Compounds 1 and 2

MIC and MBC values of Compounds 1 and 2 against *S. mutans*, *L. casei*, and *A. naeslundii* were shown in Table 1. MICs of Compound 1 against the three strains were lower than those of Compound 2, indicating the higher antibacterial potency of Compound 1 compared with CompoundCompound 2. *S. mutans* and *A. naeslundii* were sensitive to both Compounds, while *L. casei* was resistant. For MBC against the three strains, only the MBC of Compounds 1 against *A. naeslundii* was found, which was 100 μg/ml. The activity of Compound 2 against *S. mutans* was comparable to CHX, while Compound 1 was more potent.

The antibacterial effect of Compound 1 against *S. mutans* was more potent compared with LME, while the effects of Compound 2 against the three tested strains were not improved. Previous studies suggested that polar hydroxyl group in the lawsone structure hindered its penetration through the bacterial plasma membrane, and the incorporation of lipophilic moiety at 2-position of lawsone structure promoted the absorption through microbial cell membrane and increased its antimicrobial potency [8, 10]. This study demonstrated that the increased lipophilicity of functional group at 2-position of 1,4-naphthoquinone resulted in an enhanced antibacterial activity against particular bacterial species, and the activity did not accordingly improve with lipophilicity. Therefore, other physicochemical parameters including higher molecular weight, poorer water solubility, and steric side chain which were affected by the structural modification (along with increased lipophilicity) might reduce the molecule's ability to pass through the cell membrane or to bind with the target enzymes.

The evaluation of antibacterial activity by MIC/MBC determination demonstrated that Compounds 1 and 2 exhibited strong antibacterial activity against *S. mutans* and *A. naeslundii* while the effect on *L. casei* was weak. The results were consistent with what reported in a previous study investigating antibacterial activity of synthetic lawsone derivatives containing *N*-substituted 1,2,3-triazole against the same three strains [15]. Since the antibacterial activity of 1,4-naphthoquinone arises from quinone moiety, mainly via redox cycling at the mitochondrial respiratory chain in the presence of O_2 resulting in ROS generation [10, 11, 18]; therefore, the oxygen requirement of bacteria is one determinant of the sensitivity of each strain to the Compounds [8]. *L. casei* is an aerotolerant anaerobe, and that could be the reason for its resistance to 1,4-naphthoquinones [19]. *S. mutans* and *A. naeslundii* are facultative anaerobes, consuming oxygen resulting in ROS generation and subsequently causing toxicity to the cells [20, 21].

Effect of Compounds 1 and 2 on bacterial acid production

The pH drop profiles of the three bacterial strains were shown in Fig. 2. Compound 1 ($p < 0.001$) and Compound 2 significantly retarded the pH declined by *S. mutans* ($p = 0.027$). Only compound 1 significantly affected the pH drop profile of *L. casei* ($p = 0.022$). Both Compounds had no significant effect on pH drop profile of *A. naeslundii* (Table 2).

Acid production of cariogenic bacteria is the cause of the demineralization of tooth surface and subsequently dental caries formation. The effect of Compounds 1 and 2 on glycolytic acid production of the three strains was indirectly evaluated by monitoring the pH changes over time of each strain in the presence of the synthetic compounds and compared with the control group. Compounds 1 and 2 significantly affected pH change across time of *S. mutans*, and only Compound 1 had significant effect on *L. casei*. The pH drop profiles of *S. mutans* and *L. casei* in the presence of Compound 1 were noteworthy that the initial rates of pH drop were slower than those of the other groups. At 10 min, the pH values of both strains with Compound 2 and control group dropped below 5.5, which is the critical pH of enamel (the point where enamel starts to dissolve) [22]. On the other hand, at the same time point in the presence of Compound 1, the pH values of both strains were still above 5.5. In the oral cavity, plaque pH decreases below critical pH in a few minutes after exposure to fermentable carbohydrate and recovers to normal soon after exposure to saliva [23]. If a compound can slow down the initial pH drop of dental plaque and extend the time to reach critical pH, it may imply that the compound can shorten the duration of tooth demineralization and also inhibit dental caries process. However, it cannot be concluded that the compound has any inhibitory effect on acidogenicity of dental caries pathogens. For the present study, the results of pH drop assay were influenced by many factors including (i) the antibacterial activity of Compounds 1 and 2, (ii) the ability of the Compounds to inhibit the bacterial glycolytic metabolism, and (iii) the Compounds' buffer capacity [24]. Further studies are needed to investigate the mechanism of inhibitory effect on pH reduction, for example, examining the effect of the Compounds on a glycolytic enzyme lactate dehydrogenase or the phosphoenolpyruvate-phosphotransferase system (PEP-PTS).

Effect of Compounds 1 and 2 on *S. mutans* biofilm formation

Sub-MIC concentrations of Compounds 1 and 2 markedly reduced biofilm formation of *S. mutans* (Fig. 3). At 12 h, there were significant differences in the biofilm formed in all tested groups (both Compounds at the concentration of 1/2–1/8 MIC) compared with the control groups. However, at 24 h, both Compounds 1 and 2 at the concentrations lower than 1/2 MIC did not significantly reduce *S. mutans* biofilm formation.

The accumulation of cariogenic biofilm is one of the crucial factors in the dental caries process. *S. mutans* plays an important role in biofilm formation. It is not only an early colonizer but also produces glucosyltransferase enzymes (GTFs), which is one of its virulence factors in the pathogenesis of dental caries [6, 25]. GTFs are involved in the formation of cariogenic biofilm as they provide to the bacterium an ability to synthesize adhesive extracellular polysaccharides (EPS) matrix of biofilm and promote bacterial adherence. In this study, we determined the effect of Compounds I and 2 on biofilm formation of *S. mutans* by the crystal violet assay. Our results showed that sub-MIC concentrations of both Compounds significantly reduced biofilm formed by *S. mutans* at both 12 h and 24 h incubation time points and more effective at 12 h than 24 h. If these Compounds are applied as an anticaries agent by incorporation into an oral care product, which is used daily and simultaneously with oral hygiene routine such as toothpaste and mouthwash, 12 h duration of action is considered long enough. In addition, applying the agent twice a day is considered practical. Nevertheless, the biofilm method used in this study was only an indirect method to estimate the total adhered biomass of both live and dead cells, as well as the biofilm matrix [26]. In order to investigate the exact mechanism of biofilm reduction including polyspecies biofilms [27], other assays are needed, for instance, sucrose-independent and sucrose-dependent adherence determination, measurement of GTF activity, observation of structure and morphology of biofilm by scanning electron microscope (SEM), and analysis of whole-cell proteomes from the biofilm.

Cytotoxicity

A tested compound is considered non-cytotoxic when the cell viability at the highest concentration is $\geqslant 70\%$. In this study, the cytotoxicity test for Compound 1 was conducted. The Compound 1 showed a dose-dependent cytotoxicity in HGF-1 cells and was not toxic at low concentrations (< 1.25 µg/ml) with the calculated IC_{50} of 2.34 µg/ml (Fig. 4).

Apart from the investigation of anticaries properties of synthetic 1,4-naphthoquinone derivatives, the safety to normal cells was also assessed using HGF-1. Cytotoxicity test was performed on Compound 1 because it exhibited better antibacterial activity and pH drop inhibition. The present study showed that Compound 1 was non-cytotoxic (percentage of cell survival higher than 70%) at the concentration lower than 1.25 µg/ml (Fig. 4). Although a previous study reported that LME oral spray was not cytotoxic at the concentration lower than 6.25 µg/ml [28], the

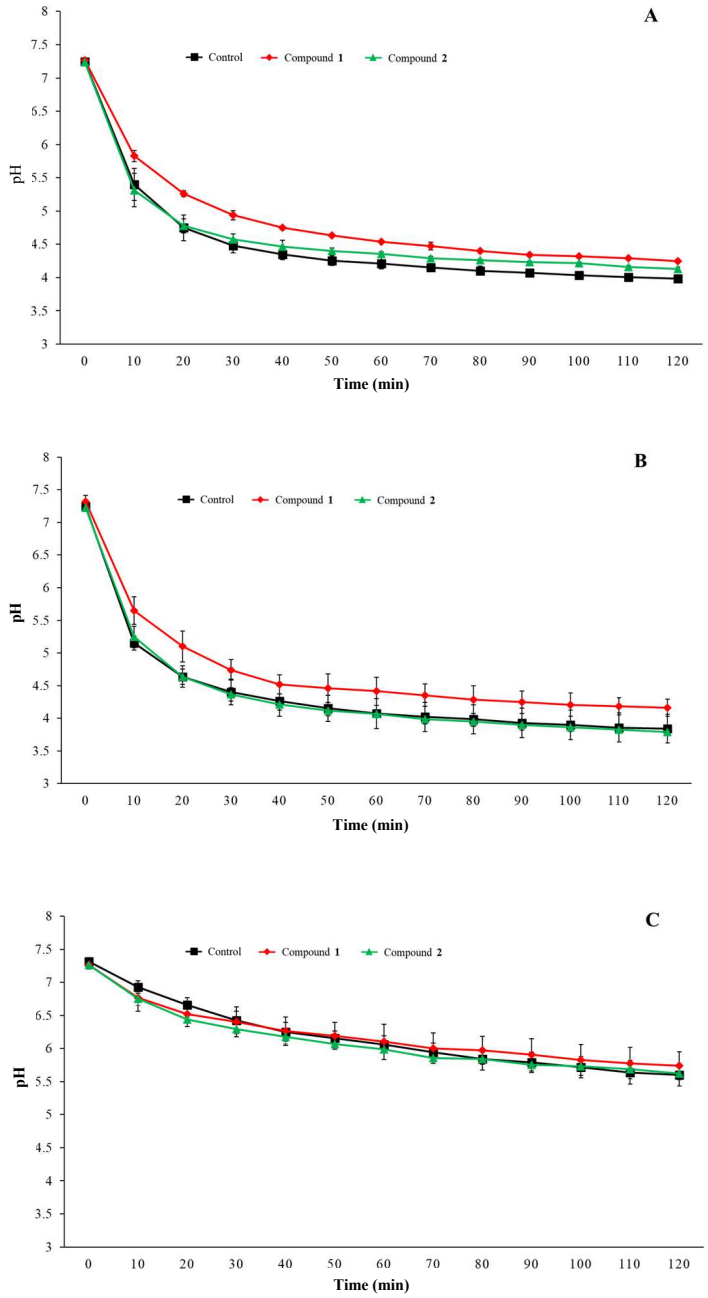


Fig. 2 pH drop profiles of bacterial strains with the presence of Compounds 1 and 2, at the concentration of 100 µg/ml, and the control group consisting of harvested bacterial cells suspended in a salt solution with 1% glucose without adding Compounds 1 or 2. (A) *S. mutans*; (B) *L. casei*; and (C) *A. naeslundii*.

Table 2 Initial pH and terminal pH of bacterial suspensions with the presence of 1% glucose and lawsone derivatives.

	S. mutans		*L. casei*		*A. naeslundii*	
	Initial pH	Terminal pH	Initial pH	Terminal pH	Initial pH	Terminal pH
Control	7.24 ± 0.039	3.98 ± 0.039[a]	7.25 ± 0.037	3.84 ± 0.218[a]	7.31 ± 0.059	5.60 ± 0.167[a]
Compound 1	7.26 ± 0.015	4.25 ± 0.010[b]	7.32 ± 0.096	4.16 ± 0.131[b]	7.27 ± 0.057	5.74 ± 0.212[a]
Compound 2	7.24 ± 0.046	4.14 ± 0.026[c]	7.23 ± 0.046	3.79 ± 0.050[a]	7.26 ± 0.061	5.62 ± 0.032[a]

[a–c] Different letters in the same column indicate statistically significant difference ($p < 0.05$) of the pH across time.

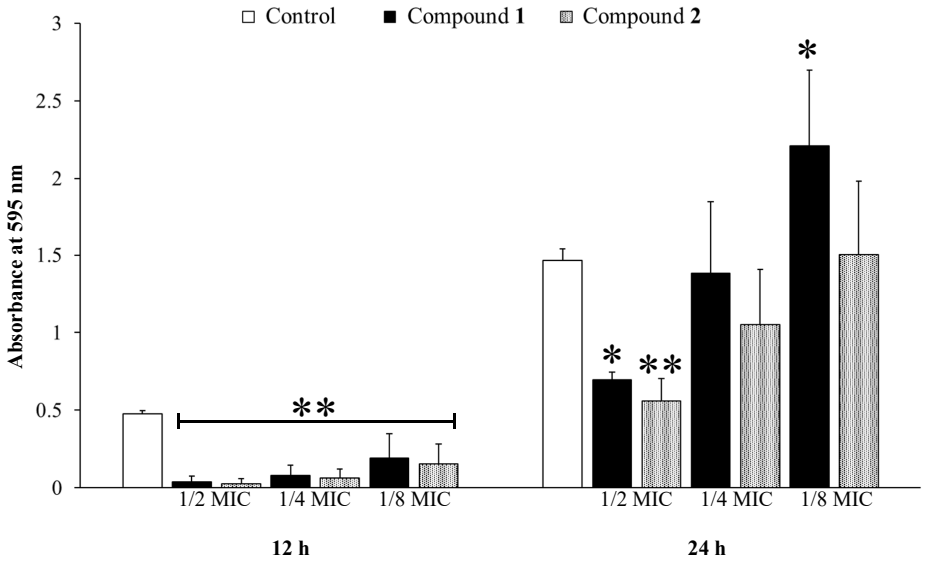


Fig. 3 Effect of Compounds 1 and 2 on *S. mutans* biofilm formation at 12 h and 24 h. The control group consisted of *S. mutans* with 5% sucrose in 96-well plates without adding the synthetic Compounds 1 or 2. $^{*}p < 0.05$, $^{**}p < 0.01$, significantly different from the control group at each time point.

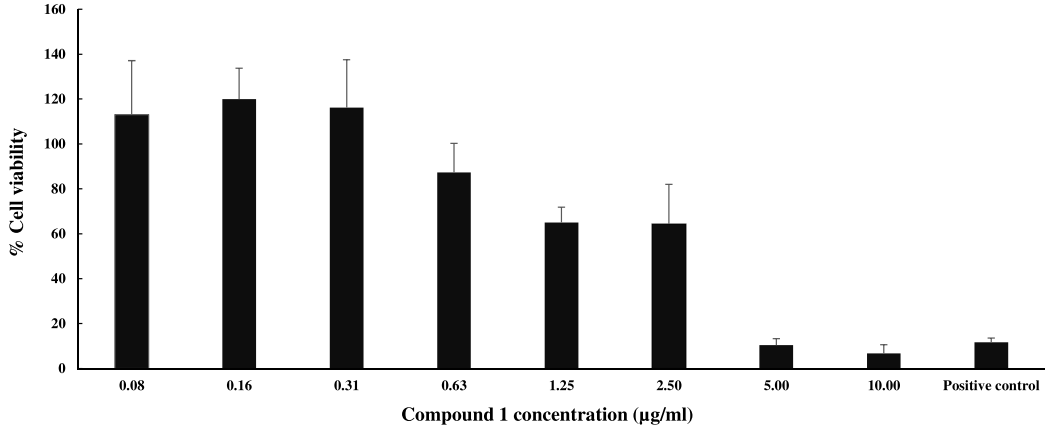


Fig. 4 Percentage of cell viability at different concentrations of Compound 1.

comparison of cytotoxicity between Compound 1 and LME could not be made because different cell lines were used in the tests. Regardless of its relatively low maximum non-cytotoxic concentration, Compound 1 was still considered safe and beneficial for clinical uses as an anticaries agent because the MIC against *S. mutans* and the concentration needed for the inhibition of *S. mutans* biofilm formation were lower than 1.25 µg/ml.

Even though fluoride has been extensively used in various preparations, dental caries is still highly prevalent because the current applications of fluoride can neither provide total protection against caries, nor effectively solve biological cause of the disease [29]. The present *in vitro* study demonstrated desirable properties of 2-(chloroalkyloxy)naphthalene-1,4-dione derivatives, Compounds 1 and 2, which were: (i) a potent antibacterial activity against *S. mutans* and *A. naeslundii*, (ii) an ability to inhibit pH reduction of *S. mutans* and *L. casei*, and (iii) an ability to inhibit *S. mutans* biofilm formation. Hence, the two Compounds could be considered as promising agents and developed as adjunctive treatment or combined with fluoride for the better dental caries prevention, and next phases of *in vitro* study are needed to explore deeper in the biological activities and mechanisms of action. Experimental designs using multispecies biofilm are required, followed by *in vivo* studies. Moreover, there might be possibilities to further modify the structure of 1,4-naphthoquinone and obtain new

compounds with better anticaries properties.

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