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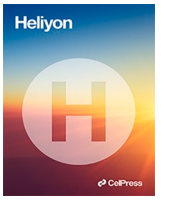

Research article

Effect of alkynyloxy derivatives of lawsone as an antifungal spray for acrylic denture base: An *in vitro* study

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ABSTRACT

Objective: The purpose of this study was to (i) synthesize and develop an alkynyloxy derivative of lawsone as an antifungal spray and (ii) assess the antifungal spray's effectiveness in reducing the viability of *Candida albicans* (*C. albicans*) on polymethylmethacrylate (PMMA) specimens.

Methods: Lawsone methyl ether (LME) and its derivative, 2-(prop-2-ynyloxy)naphthalene-1,4-dione (compound **1**) were synthesized and characterized. The synthetic compounds were screened for antimicrobial activities against *C. albicans* using the microtiter broth dilution method to determine the minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC). Compound **1** was further formulated as an antifungal spray in three concentrations (100, 200, and 400 μg/mL). *C. albicans* biofilms were developed for 48 h on PMMA specimens. The efficacy of using an antifungal spray for 1 and 3 min to remove biofilm was assessed using colony counting and scanning electron microscopy (SEM). Chlorhexidine gluconate (CHX), polident®, and distilled water were used as positive and negative control cleansing solutions, respectively.

Results: LME and compound **1** showed comparable inhibition against *C. albicans* with a MIC of 25 μg/mL and MFC of 50 μg/mL. For immediate treatment, *C. albicans* was not detected on PMMA specimens when expose to 2% CHX and compound **1** (100, 200, and 400 μg/mL) antifungal spray for 3 min. However, after recolonization, a small number of viable cells were observed in denture soaked in compound **1** antifungal spray for 3 min group. Following recolonization, polident® and distilled water had comparable viable cell counts of *C. albicans* to the no treatment group. Scanning electron microscope (SEM) images revealed that CHX, polident®, and compound **1** caused cell damage in various forms.

Conclusion: Denture spray containing synthetic alkynyloxy derivative of lawsone is a promising antifungal agent for *C. albicans* biofilm removal from the PMMA surface.

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1. Introduction

Polymethylmethacrylate (PMMA), the most commonly used material for denture base, is highly susceptible to microbial colonization, resulting in denture-associated infections, in which *Candida* species are primary microorganisms thought to play a significant role in denture stomatitis [1]. To address this issue, research has focused on ways to modify the PMMA antimicrobial properties through surface and chemical modifications, such as coating the denture base resin with antifungal agents and the incorporation of fillers or nanofillers into PMMA [2–5], while also emphasizing the importance of denture hygiene care [6–9]. However, the majority of published studies were conducted *in vitro*, with or without clinical simulation [5].

Denture hygiene care that can be performed mechanically, chemically, or through a combination of both is an important aspect of denture wearers' oral health maintenance [7–10]. Although brushing is the most used mechanical procedure for removing biofilm from the surfaces of patients' prostheses, its effectiveness in biofilm removal may be questioned as to the irregular shape of the dentures. Furthermore, most denture wearers are elderly and may have the knowledge, physical, or mental limitations that prevent them from performing oral hygiene care [11,12].

Dental biofilm typically forms shortly after tooth brushing and is regarded as a critical step in disease development. The complexity of biofilm formation highlights the importance of discovering new antibiofilm agents. Cleaning by chemical method entails immersing dentures in solutions containing solvents, detergents, antibacterial, and antifungal properties, and such solutions can be used alone or in conjunction with the mechanical or ultrasonic methods [7,10]. Microorganisms have been reported to be reduced by sodium hypochlorite (NaOCl), alkaline peroxides, and chlorhexidine gluconate (CHX). Unfortunately, chemical products can alter the irregularities, porosity, color, and even hardness of denture bases [13]. Furthermore, some products were effective in reducing the biofilm, but not able to prevent biofilm formation [6]. Importantly, their effects depend primarily their continued and proper use, according to the manufacturer's instruction. Given these challenges, novel oral products with antifungal activities that are practical for daily use are needed to control denture biofilms.

Napthoquinones (NQs) are natural pigments that are widely distributed in nature and have important biological activities [14,15]. Lawsone (2-hydroxyl-1, 4-naphthoquinone) and its synthetic derivatives, especially those containing nitrogen, have promising potential for the treatment of different diseases due to their antibacterial, antifungal, antiviral, and antitumor [15–18] activities. Lawsone methyl ether (LME) or 2-methoxy-1,4-naphthoquinone, an *O*-methyl derivative of lawsone, exhibited potent antifungal activity against several fungi including *Candida albicans* (*C. albicans*). The increased antifungal activity is due to its higher lipophilicity, which enhances cell membrane permeation [19]. LME-containing mouthwash has demonstrated satisfactory antifungal activity against oral *Candida* species both in *vivo* and in *vitro* [20]. Furthermore, an oral spray containing LME and α-mangostin has been reported as effective antimicrobial against common pathogens including *Streptococcus mutans* (*S. mutans*), *Porphyromonas gingivalis* (*P. gingivalis*), and *C. albicans* [21]. Modifying the 2-hydroxyl group of lawsone by incorporation of a lipophilic group e.g., alkynyloxy group could increase the lipophilicity of the compound and might make possible new anti-fungal agents with high potency.

In this study, therefore, alkynyloxy derivatives of lawsone have been synthesized and evaluated for their antifungal activity. We also formulate the antifungal spray and evaluate its efficacy in removing *C. albicans* biofilm from the PMMA surfaces.

2. Materials and methods

2.1. Synthesis of 2-(prop-2-ynyloxy)naphthalene-1,4-dione (compound 1)

Potassium carbonate (79.5 mg, 0.58 mmol, Renkem, New Delhi, India) was added to a solution of lawsone (100 mg, 0.58 mmol, Sigma-Aldrich, Steinheim, Germany) in 4 mL *N*,*N*-dimethyl formamide (Sigma-Aldrich, Steinheim, Germany). The mixture was stirred at room temperature for 15 min. After addition of propargyl bromide (61.5 mg, 0.58 mmol, Sigma-Aldrich, Steinheim, Germany), the reaction mixture was further stirred for additional 72 h. When the reaction completed, distilled water (30 mL) was added to the reaction mixture and the product was extracted by ethyl acetate (3 × 30 mL, P.S. Science Chemical Co., Ltd., Songkhla, Thailand). The combined organic phases were dried over anhydrous sodium sulfate (Loba Chemie PVT. Ltd., Palghar, India) and concentrated under reduced pressure. The crude product was purified by silica gel (Ligand Scientific Co., Ltd., Quebec, Canada) column chromatography using dichloromethane: hexane (20:80, P.S. Science Chemical Co., Ltd., Songkhla, Thailand) as eluent and recrystallization from hexane/methanol (P.S. Science Chemical Co., Ltd., Songkhla, Thailand) to give the desired product as yellow solid (90 mg, 73% yield). Melting point of the compound was 149–151 °C (Mel-TEMP II laboratory devices, Massachusetts, USA).

Chemical structure and chemical reaction for preparing compound **1** are illustrated in Figs. 1 and 2**.** Chemical structure of the

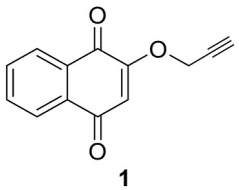

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Fig. 1. Chemical structure of 2-(prop-2-ynyloxy)naphthalene-1,4-dione General formula: $C_{13}H_8O_3$, MW 212.20, *c*LogP 1.93, Yellow solid, m. p. 149–151 °C.

synthesized compound was confirmed using infrared (IR, Perkin Elmer Spectrum One infrared spectrophotometer, Connecticut, USA), ^{1}H-nuclear magnetic resonance (NMR), ^{13}C-NMR (Bruker Ascend 500/Avance Neo NMR spectrometer, Bruker Corporation Massachusetts, USA), and high-resolution mass spectrometry (HR-MS, MAT 95XL mass spectrometer, Thermo Finnigan, Bremen, Germany).

2.2. Preparation of acrylic denture spray containing 100 μg/mL of compound 1

2.2.1. Preparation

Compound **1** (5 mg) was grinded with poloxamer 407 (1 g, P.C. Drug Center Co., Ltd, Bangkok, Thailand), saccharin (0.05 g, Vidhyasom Co., Ltd, Bangkok, Thailand), and menthol (0.025 g, Vidhyasom Co., Ltd, Bangkok, Thailand) in the mortar. Glycerin (5 mL, P.C. Drug Center Co., Ltd, Bangkok, Thailand) was gradually added into the mixture and the mixture was grinded until smooth paste was obtained. Distilled water (25 mL), ethanol (12.5 mL, P.C. Drug Center Co., Ltd, Bangkok, Thailand), paraben conc. 0.5 mL (P. C. Drug Center Co., Ltd, Bangkok, Thailand), and peppermint oil (1 drop, Vidhyasom Co., Ltd, Bangkok, Thailand) were added. The solution was transferred to a cylinder and volume adjusted to 50 mL with distilled water. The denture spray was obtained as a clear, homogenous, neutral (pH 7.32) solution with a fresh odor, mildly sweet flavor, and acrylic denture wettability. The solution was freshly prepared and protected from light. Content of compound **1** in the solution was determined using high-performance liquid chromatography (HPLC) before each biological evaluation.

2.2.2. Physical stability studies

The denture spray was subjected to physical stability studies, including centrifugation test and temperature cycling test. A centrifuge test was performed at 3000 round per minute (rpm) for 30 min to assess the stability of the acrylic denture spray (Centrifuge Z 206 A, Hermle Labortechnik GmbH, Wehingen, Germany). The temperature cycling test was carried out on the Memmert drying oven (Model 600, Memmert GmbH + Co. KG, Schwabach, Germany) by holding the tested samples at 50 ± 2 °C for 24 h before switching to 42 ± 2 °C for another 24 h. This procedure was repeated six times (i.e., 12 days). Color, odor, clarity, homogeneity, and pH (measured with Mettler Toledo S220 pH meter, Mettler Toledo, Ohio, USA) were used to assess the stability of the product before and after temperature cycling [22].

2.2.3. Content of compound 1 in the acrylic denture spray

HPLC procedure for determination of active ingredient in the denture spray formulation was modified from the protocol for simultaneous determination of three NQs in the leaves of *Impatiens balsamina* L. by reversed-phase high-performance liquid chromatography [23].

Standard preparation – 80, 60, 40, 20, and 10 μg/mL of the standard compound in mobile phase
Sample preparation – Nominally 40 μg/mL of compound **1** in mobile phase.

Chromatographic system – HPLC analysis was carried out using a Hitachi CM 5000 series equipped with photodiode-array detector (PDA) and autosampler (Hitachi High-Tech Corporation, Tokyo, Japan). Separation was achieved at 25 °C on Hypersil® BDS C18 column (250 × 4.6 mm, Thermo Fischer Scientific Inc., Massaschusetts, USA) using isocratic methanol - 2% aqueous acetic acid (55:45) solution at flow rate of 1 mL/min. The injection volume was 20 μL. Detection was carried out at 275 nm. Methanol (HPLC grade) and acetic acid (glacial, analytical grade) were purchased from ARI Labscan, Bangkok, Thailand.*Analysis* – A plot between peak response from standard solution and concentration was created as a standard curve. Concentration of compound **1** in the sample preparation was calculated from peak response from the sample preparation using standard curve equation.

$$\%\text{Labeled amount} = \frac{\text{Concentration of compound } \mathbf{1} \text{ in sample preparation} \times 100}{\text{Labeled concentration of compound } \mathbf{1}}$$

2.3. Strain and growth conditions

C. albicans (DMST 5815) used in this study, was purchased from the National Institute of Health, Department of Medical Sciences, Ministry of Public Health, Thailand. *C. albicans* was grown in Sabouraud Dextrose (SD: Himedia Laboratories Pvt. Ltd. Mumbai, India) at 37 °C. for 18 h (mid-log phase).

For biofilm assays, SD broth was supplemented with 50 mM glucose. Overnight cultures (18 h) of *C. albicans* were harvested by refrigerated centrifugation (Labofuge 400R Heraeus, Kendro Laboratory Products Inc., Hanau, Germany) at 4 °C, 3000 g for 5 min,

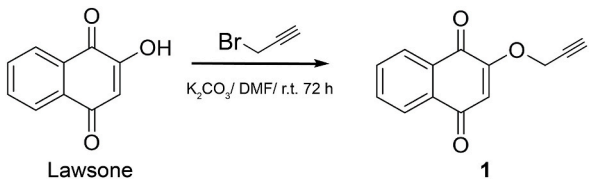

Fig. 2. Synthesis of 2-(prop-2-ynyloxy)naphthalene-1,4-dione.

washed twice in 0.1 M sterilized phosphate buffer (PBS, pH 7.2–7.4), and the cells were resuspended in fresh broth. Microbial suspensions were spectrophotometrically (Genesys 10s UV-VIS, Thermo Scientific, Thermo Fisher Scientific Inc., Massaschusetts, USA) standardized to a concentration of 1×10^7 cells/mL.

2.4. Microdilution and growth curve assay

In this study, *C. albicans* suspensions were standardized to a concentration of 1×10^5 cells/mL. The stock solutions were prepared by dissolving synthetic compounds (LME and compound **1**) in 20% dimethylsulphoxide and 4% ethanol to a final concentration of 400 μg/mL. The positive control was CHX (P.C. Drug center Co., Ltd, Bangkok, Thailand), clotrimazole and nystatin (Sigma-Aldrich, Steinheim, Germany) at a starting concentration of 1.92% (19,200 μg/mL), 200 μg/mL, and 128 μg/mL, respectively. The two-fold serial dilutions from the stock solutions of tested compounds were made, ranging from 100 to 6.25 μg/mL in 96-well microplates (SPL Life Science Co., Ltd, Gyeonggi-do, Korea). Then, 100 μL of *C. albicans* suspension was inoculated into each well. The microtiter plates of *C. albicans* were then incubated at 37 °C for 24 h in aerobic condition. The lowest concentration of each tested compound solution displaying no visible growth was recorded as the minimum inhibitory concentration (MIC). Twenty microliters were taken from the well obtained from the MIC value well and two wells above the MIC value and dropped on SD agar for *C. albicans.* After 24 h of incubation a minimum fungicidal concentration (MFC) was recorded as the lowest concentration yielding negative subcultures. The concentration of a sample that produces no colonies were considered the MFC value. Each experiment was repeated at least three times.

Similar to the microdilution assay, a volume of 100 μL of *C. albicans* suspension was inoculated in triplicate into 96-well microtiter plate. Synthetic compounds at MIC and sub-MIC was added. Three wells containing *C. albicans* suspension (growth control) and one well containing only media (background control) were included in this plate. The optical density of the suspensions in each well was measured at 530 nm every 2 h for 36 h with a microtiter plate reader (Multiskan Go, Thermo Scientific, Thermo Fisher Scientific Inc., Massachusetts, USA). Growth curves were then plotted.

2.5. Evaluation of the antifungal denture spray on removal of C. albicans biofilm

2.5.1. PMMA sample fabrication

One hundred and eighty specimens were obtained. Initially, a cylinder-shaped plastic pattern (diameter 10 mm × 5 mm) was fabricated. The plastic pattern was then invested in metal flasks with type IV dental stone. After the dental stone had set, the flasks were separated, and the patterns were removed, leaving cylindrical cavities. The molds were filled with heat polymerized acrylic resin (Vortex Rapid Simplified, Vertex-Dental B.V., Zeist, Netherlands), which was manipulated according to the manufacturer's instructions. The specimens were polymerized by boiling at 100 °C for 20 min in a polymerization unit (Wapo-Mat III, Wassermann, Hamburg, Germany). Subsequently, the specimens were deflasked and immersed in distilled water for 24 h to eliminate the residual monomer. The cylindrical acrylic resin was cut using a low-speed diamond saw (IsoMet 1000, Buchler, Lake Bluff, Illinois, USA) to obtain a thickness of a sliced specimen of 2 ± 0.1 mm. Both surfaces were polished in a horizontal polisher with 600 grit abrasive paper (Silicon carbide abrasive paper, TOA Paint (Thailand) Public Co., Ltd, Samutprakan, Thailand) to obtain a surface roughness between 0.3 and 0.4 μm. All specimens were sterilized with hydrogen peroxide gas at a temperature of 50 °C for 72 min. Specimens were randomly divided into 10 groups ($n = 3$) according to Table 3.

2.5.2. Biofilm assays

Biofilm assays were performed in triplicate in 3 independent experiments. Before developing the biofilm assays, each sterile acrylic resin specimen was coated with fetal bovine serum (FBS, Gibco, Life Technologies Ltd., Carlsbad, USA) at 37 °C and 75 rpm in a shaker incubator (Excella E24 Incubator Shaker Series, New Brunswick Scientific Co., Inc, Connecticut, USA) for 24 h. Specimens were then washed with PBS to remove any excess. Subsequently, each FBS-coated acrylic resin specimen was individually placed in each well of a 24-well culture plate (SPL Life Science Co., Ltd, Gyeonggi-do, Korea), and 1.5 mL of standard cell suspension (1×10^7 cells/mL) was prepared as mentioned above and added to each well containing a specimen. The plate was incubated for 90 min at 37 °C in a shaker incubator at 75 rpm to promote microorganism adherence to the specimen surfaces (adhesion phase). After the adhesion phase, the non-adherent was removed from the specimen by gently washing twice with 1.5 mL of PBS. To promote biofilm growth (biofilm phase), 1.5 mL of fresh broth was added to each well. The plates were covered and incubated at 37 °C at 75 rpm for 48 h under aerobic conditions.

After the biofilm growth for 48 h, the plates were removed from the incubator, and the wells were gently washed twice with PBS. Each specimen was then treated with the assigned control treatment agents (negative control, distilled water for 3 min, 2% CHX for 3 min, or polident® denture cleanser for 3 min). Polident® (Stafford-Miller Ireland Ltd, Waterford, Ireland) ingredients are sodium bicarbonate, citric acid, potassium caroate (potassium monopersulfate), sodium carbonate, sodium carbonate peroxide, tetra acetyl ethylene diamine (TAED), sodium benzoate, PEG-180, and sodium lauryl sulfate, VP/VA copolymer, aroma, cellulose gum, CI 42090, CI 73015, CI 19140.

Antifungal spray formulation containing 100 μg/mL, 200 μg/mL, or 400 μg/mL of synthetic compound **1** were used for 1 or 3 min. Then, the specimens were washed twice in 1.5 mL of PBS following the treatment. After washing, the specimens were transferred to a tube containing 5 mL of PBS and vortexed using a vortex mixer (Genie 2, Scientific Industries, Scientific Industries Inc., Massachusetts, USA) for 1 min to disrupt the biofilm cell aggregates. The resultant suspension containing the detached biofilm cells was 10-fold diluted and dropped (20 μL × 5) onto SD agar. After incubation, colony counts of each Petri dish were quantified. The obtained

microbial count data were expressed as log (CFU)/mL.

For recolonization groups, after being treated with each synthetic compound and washing, the specimens were transferred to SD broth supplemented with 50 mM glucose and incubated at 37 °C for 18 h. The non-adherence biofilms were removed by washing twice in 1.5 mL of PBS. Residual biofilms were then disrupted by vertexing for 1 min in PBS. The solutions were serially diluted in PBS and dropped (20 μL × 5) onto SD agar. The plates were incubated for 24 h at 37 °C.

2.5.3. Scanning electron microscopic (SEM) assessment

Specimens with attached biofilms were rinsed with sterile PBS and placed in 2.5% glutaraldehyde for 2 h at 4 °C and subsequently dehydrated in a series of ethanol washes (50%, 70%, 80%, 90%, 95%, 100%, and 100% for 25 min) and critical-point dried before sputter coating with gold. Next, specimens were mounted on aluminum stubs and coated with gold. The residual biofilm features were observed using a scanning electron microscope (SU3900, Hitachi, Tokyo, Japan) at 100×, 1000×, and 5000× in a high-vacuum mode at 20 kV.

2.6. Statistical analysis

The statistical test was performed by using STATA version 13.1 (StataCorp, College Station, Texas). The significant level was set at 0.05. The assumption of normal distribution of the data was tested using Shapiro-Wilk test, and the homogeneity of variance was tested using the Levene statistical test. The efficacy of the tested agents in removing *C. albicans* biofilm was compared by the Kruskal-Wallis test, followed by Mann-Withney *U* test with Bonferroni correction.

3. Results

3.1. Characterization of compound 1

Compound **1** (Fig. 1) was synthesized from lawsone in high yield and its chemical structure was confirmed using IR, ^{1}H-NMR, ^{13}C-NMR, and HR-MS.

IR (cm^{-1}, neat): 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, 75.44, 78.20, 111.61, 126.24, 126.75, 131.05, 131.89, 133.48, 134.38, 158.05, 179.83, 184.71; **HR-MS** (*m/z*, $[M+1]^+$): 213.0531 (calcd for $C_{13}H_8O_3$, 212.0473).

3.2. Physical stability study

In centrifugation and temperature cycling tests, the denture spray demonstrated good physical stability. The solution remained homogenous after centrifugation at 3000 rpm for 30 min. The formulation remained clear, colorless, homogenous, and neutral with slightly reduced pH value after 6 temperature cycles (initial pH = 7.30, terminal pH = 7.19).

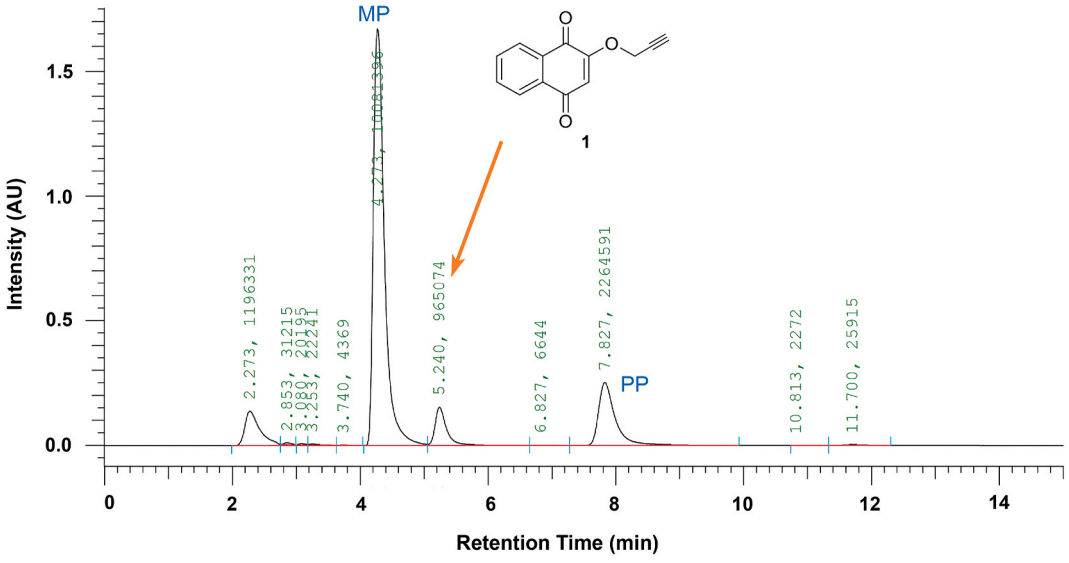

Fig. 3. HPLC chromatogram of the denture spray (MP: methyl paraben, PP: propyl paraben).

3.3. Content of compound 1 in the acrylic denture spray

Content of compound **1** in the acrylic denture spray solution was determined using HPLC analysis with protocol described previously. The calibration curve was established from the authentic compound **1** at the concentration range of 10–80 μg/mL, with a linear equation of Y = 43412X – 14614 (r^2 = 0.9999). Peaks of the denture spray components were well resolved as shown in the chromatogram (Fig. 3). Percent labeled amount of compound **1** in the solution can be determined as shown in Table 1. In each antimicrobial determination, percent labeled amount of compound **1** in the solution was controlled to be within the range of 90.0–110.0% labeled amount.

3.4. Antifungal properties

All tested agents showed antifungal activity against *C. albicans.* The MIC and MFC of LME and compound **1** are equivalent (25 μg/mL and 50 μg/mL, respectively). Their MFC/MIC ratio was 2, demonstrating their fungicidal efficacy (Table 2). As shown in the growth curves, the presence of LME and compound **1** at MIC caused the suppression of the growth of *C. albicans,* corresponding to microdilution assay (Fig. 4).

3.5. Efficacy of antifungal denture spray containing compound 1 in C.albicans biofilm removal

For immediate treatment, all three concentrations of compound **1** antifungal denture spray used for either 1 or 3 min and 2% CHX were more effective in the *C. albicans* biofilm removal than polident® ($P = 0.004$) and distilled water. The viable cells were either not detected or had a lower number (0–3.1 Log CFU/mL).

Following the recolonization, *C. albicans* were detected in all three concentrations of the compound **1** antifungal spray used for either 1 or 3 min (0–3.68 Log CFU/mL). However, a number of viable cells was reduced compared with polident® (5.48–5.97 Log CFU/mL) ($P < 0.001$) and distilled water (5.30–5.97 log CFU/mL). At the same concentration of compound **1**, longer exposure times tend to improve the efficacy of *C. albicans* removal, however, the majority of tested groups (1 *vs.* 3 min) did not show statistical significance (Fig. 5).

The SEM images of *C. albicans* treated with the compound **1** antifungal spray showed a variety of cell damage. Fig. 6 (A-H) shows the various morphological changes of yeast forms of *C. albicans*, where wrinkles, shrinkage, and some deep craters were noted on the cell surfaces in all cleansing groups: 2% CHX (Fig. 6; A), compound **1** (Fig. 6; B-G), and Polident® (Fig. 6; H), whereas there was no discernible change in distilled water group (Fig. 6; I) and untreated group (Fig. 6; J). The residual *C. albicans* on the PMMA surface was lower in the compound **1** antifungal denture spray that was applied for 3 min (Fig. 6; C, E, G) than when it was applied for 1 min (Fig. 6; B, D, F), and there was more clear evidence of cell destruction when used with higher concentration and longer exposure time (Fig. 6; G).

4. Discussion

In our attempt to find new potent 1,4-napthoquinoe analogs, an alkynyloxy derivative of lawsone, compound **1**, was successfully designed and synthesized in this study. With a MIC of 25 g/mL against *C. albicans* and the ability to reduce viable cell counts on PMMA specimen, compound **1** is a promising candidate for antifungal spray for acrylic dentures.

LME and compound **1** are both derivatives of NQs, the most common type of quinone found in nature. A previous review suggested strong scientific evidence that NQs and their structural diversity have antifungal activity against *Candida* species [14]. Beside antifungal activity, previous studies have also reported other promising biological activities including antibacterial and antiparasitic of NQs [15–18]. NQs' diverse activities offer them a promising armory to combat microbial pathogens, including multi-drug resistance (MDR) bacteria and the ESKAPE (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae, Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* spp.) group. In bacteria, NQs may exhibit their function in the following ways: (i) plasmid curing, (ii) inhibiting efflux pumps (EPs), (iii) generating reactive oxygen species (ROS), and (iv) the inhibition of topoisomerase activity [15].

Different mechanisms of antifungal action of NQs have been previously described [15,17,19]. The antifungal potency of synthetic NQs against fungi has been reported as altering cell-membrane permeability [15]. LME, an *O*-methyl derivative of lawsone, exhibited potent antifungal activity against several fungi including *Candida albicans.* The potent antifungal activity of LME is based on its higher lipophilicity which enhanced cell membrane permeation [19]. From the SwissADME program, considered from Log P value (Data not shown), introduction of the more lipophilic substituents to the 2-hydroxyl group of lawsone resulted in the more lipophilic derivatives. Lipophilicity ranges from lawsone < LME < compound **1.** However, in the present study the increased lipophilicity of compound **1** did not significantly improve antifungal susceptibility. In addition, several *O*-alkyl- and *O*-acyl derivatives of lawsone and other NQs

Table 1
Percent labeled amount of compound **1** in the denture spray.

Formulation of compound 1	% Labeled amount
100 μg/mL	104.8
200 μg/mL	104.4
400 μg/mL	106.2

Table 2
Antifungal activity of synthetic lawsone derivatives and positive controls against *C.albicans*.

Compound	*C.albicans* (DMST 5815)		
	MIC (μg/mL)	MFC (μg/mL)	MFC/MIC
LME	25	50	2
Compound **1**	25	50	2
CHX	4.88	4.88	1
Clotrimazole	3.125	50	16
Nystatin	2	4	2

LME: Lawsone methyl ether; CHX: Chlorhexidine gluconate.
MIC: Minimal inhibition concentration; MFC: Minimal fungicidal concentration.
The MFC/MIC ratio $\leq$4 indicates fungicidal activity.

Table 3
Log (CFU/mL) values of *C.albicans* obtained for the control and experimental groups.

Agents	Exposure time (minutes)	Viable counts (log CFU/mL)					
		Immediate			Recolonization		
		Min	Median	Max	Min	Median	Max
Antifungal spray (Compound **1** = 100 μg/mL)	1	0.00	0.00	2.70	0.00	2.00	3.68
	3	0.00	0.00	0.00	0.00	0.00	2.85
Antifungal spray (Compound **1** = 200 μg/mL)	1	0.00	0.00	3.11	0.00	2.78	3.51
	3	0.00	0.00	0.00	0.00	0.00	2.00
Antifungal spray (Compound **1** = 400 μg/mL)	1	0.00	0.00	2.30	0.00	2.00	3.61
	3	0.00	0.00	0.00	0.00	0.00	2.00
Polident®	3	2.00	3.36	4.08	5.48	5.79	5.97
2% CHX	3	0.00	0.00	0.00	0.00	0.00	0.00
Distilled water	3	3.85	4.87	5.29	5.30	5.58	5.97
Negative control	N/A	5.69	5.73	5.81	5.12	5.65	6.03

Biofilm assays were performed in triplicate in 3 independent experiments (n = 9).
CFU/ml: colony forming unit per milliliter; Min: minimum; Max: maximum; CHX: Chlorhexidine gluconate.

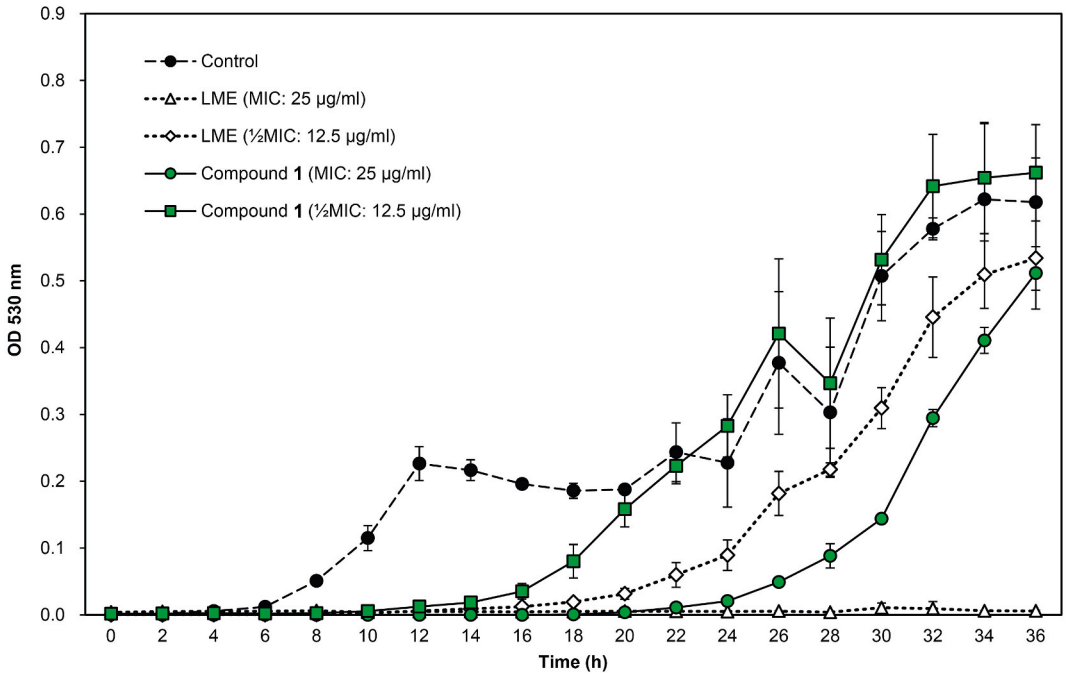

Fig. 4. Growth curve evaluation with MIC and sub-MIC concentration of LME and compound **1** on *C. albicans*.

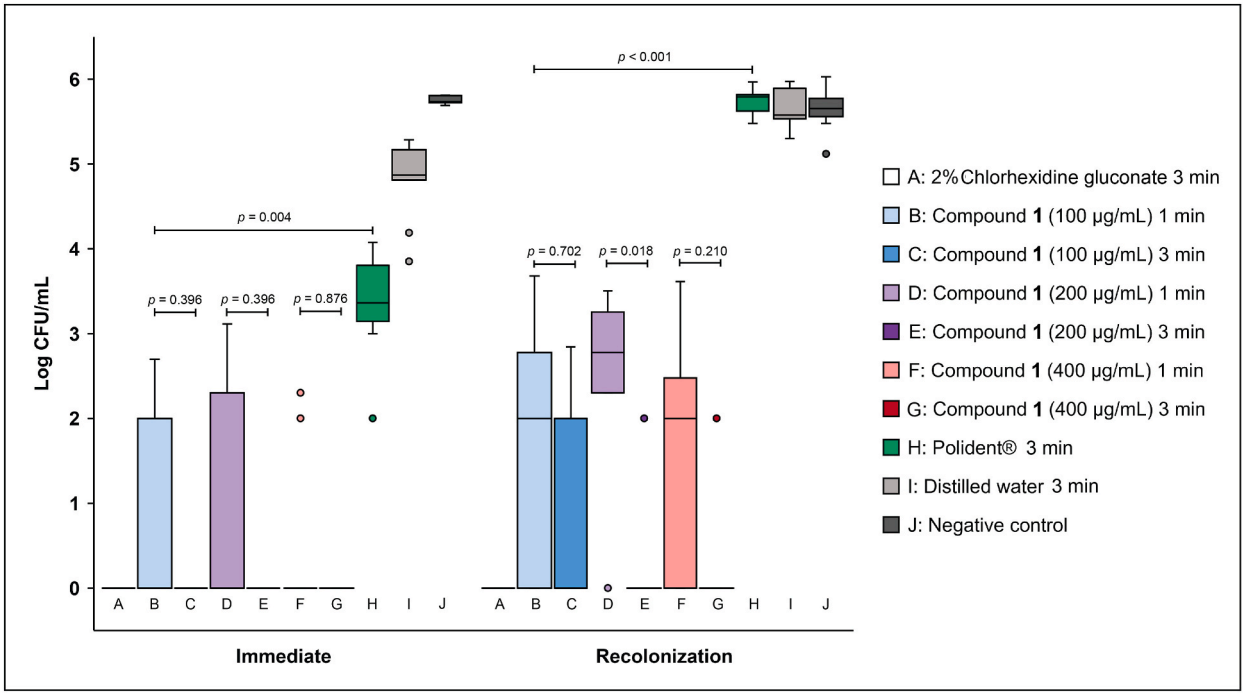

Fig. 5. Box-plot diagram of the distribution of cell viability (Log CFU/mL) of *C. albicans* after exposed to tested agents. The efficacy of the tested agents in removing *C. albican* biofilm was compared by the Kruskal-Wallis test, followed by Mann-Withney *U* test with Bonferroni correction to test the effect of exposure time (1 *vs.* 3 min) of each concentration of antifungal spray formulation.

exerted antiproliferative activity against the yeast *Saccharomyces cerevisiae* via generating of ROS and subsequent mitochondrial function disruption [17]. Based on NQs structure, quinones can be cytotoxic through several mechanism including ROS [24]. Increased intracellular ROS content in *Candida* species can result in oxidation of intracellular lipids, protein, and nucleic acids, which can lead to problems such as (i) DNA damage; (ii) protein function alteration; (iii) mitochondrial activity disruption; (iv) enzymatic inhibition; and (v) membrane damage [15,24]. Because of their antifungal and bacterial properties, NQs are promising candidates for the development of antifungal sprays for acrylic dentures.

In the present study, LME and compound **1** were synthesized at high yields. The synthesized compounds were purified, and their chemical structure was confirmed using IR, ^{1}H-NMR, ^{13}C-NMR, and HR-MS. To ensure the amount of the active ingredient in the formulation for biofilm assay experiments, the content of compound **1** in the denture spray was measured using HPLC. The denture spray from compound **1** was developed as a simple solution. Due to its low water solubility, Poloxamer 407 and ethanol were added to enable its solubilization. Glycerin was added for increased denture base wettability. Saccharin was added in the formulation to give a better taste. Organoleptic property was improved by addition of flavoring agents such as menthol, and peppermint oil to give fresh and clean sensation. Small amount of paraben concentrated was added as a preservative.

For the denture spray, centrifugation and temperature cycling tests revealed that the mixture was physically stable. The formulation, however, was discovered to be photosensitive, and it was recommended that it be protected from light during storage and all testing settings. Photodegradation of 1,4-naphthoquinones such as menadione (vitamin K) has been reported. In this case, the underlying photodegradation mechanism in this example was proposed as photo-induced addition of oxygen, culminating in the creation of an epoxide [25].

The efficacy of the compound **1** antifungal denture spray on the removal of *C. albicans* biofilms was evaluated at three concentrations (100, 200, and 400 µg/mL) and two exposure times (1 and 3 min). The antifungal spray in any concentration and 3 min exposure time provided more satisfactory results for immediate treatment compared to polident® or distilled water. There was a no viable *C. albicans* left on the PMMA specimens similar to 3 min of immersion in 2% CHX. When those cleaned PMMA specimens were cultured for 18 h in a culture medium supplemented with glucose, we observed *C. albicans* biofilm recolonization in all tested groups, except for 2% CHX 3 min. However, utilizing the compound **1** antifungal denture spray for either 1 or 3 min showed reduced number of viable *C. albicans* for biofilm recolonization more than polident®, distilled water, or negative control. In addition, employing the compound **1** formulation for 3 min at the higher concentration (200 and 400 µg/mL) was more effective in reducing number of viable *C. albicans* for biofilm recolonization than lower concentration (100 µg/mL). This suggests that concentration and exposure time are critical factors for the efficacy of the antifungal spray on *C. albican* biofilm removal. Similar to an *in vitro* using citric acid demonstrated that citric acid denture cleansers reduce cell viability but did not prevent biofilm recolonization within 48 h [26].

In this investigation, our study results suggested that the compound **1** formation was more effective than polident® for immediate therapy and recolonization. This could be because the biofilms were disturbed with polident®, but they were not completely removed.

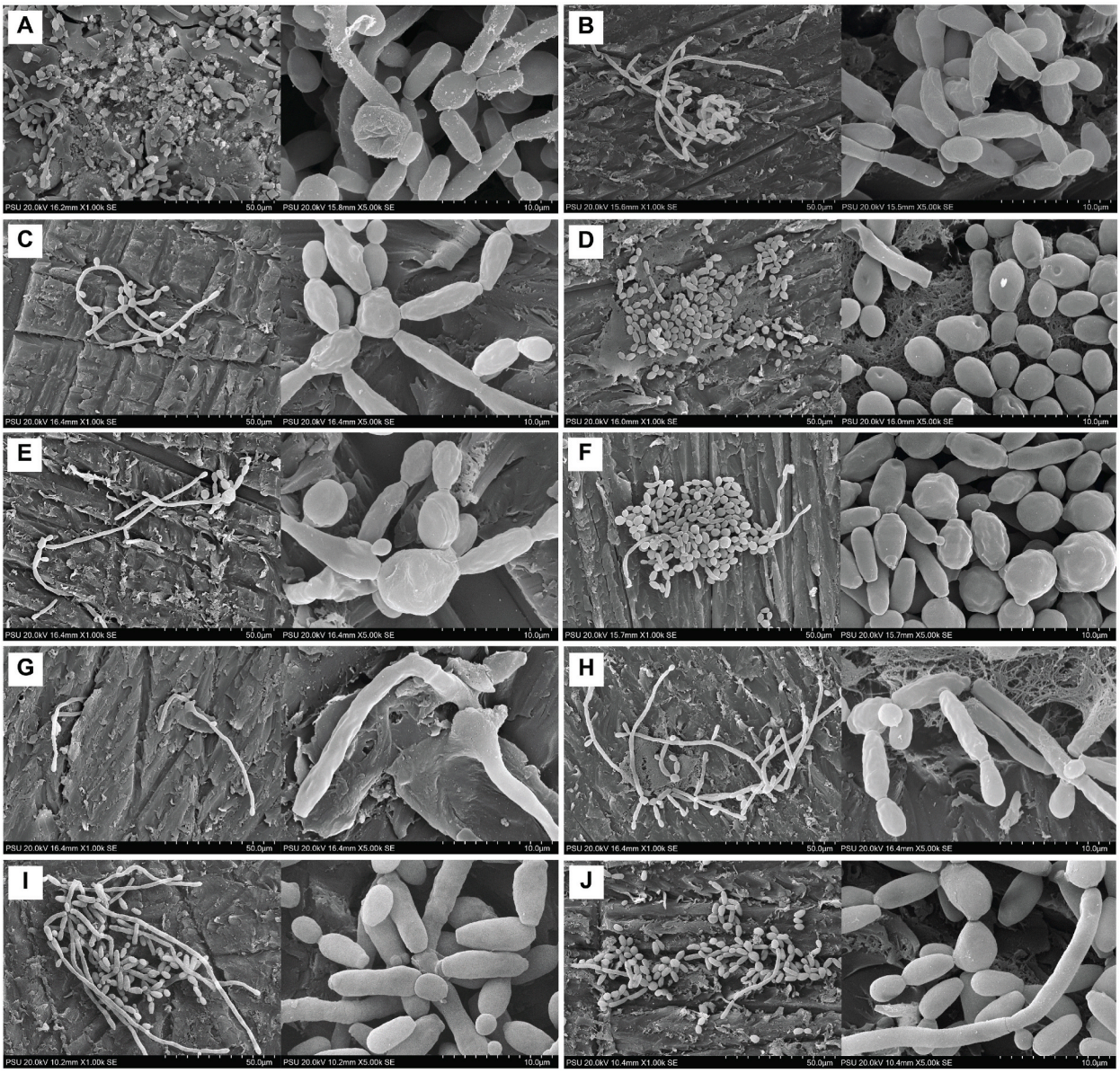

Fig. 6. Representative scanning electron microscopy images (1000× and 5000×) of *C. albicans* biofilm formed on PMMA surfaces according to different treatments. **A**, 2% CHX 3 min; **B–C**, Compound **1** formulation (100 μg/mL) 1 and 3 min; **D-E**, Compound **1** formulation (200 μg/mL) 1 and 3 min; **F-G**, Compound **1** formulation (400 μg/mL) 1 and 3 min; **H**, Polident® 3 min; **I**, Distilled water 3 min; **J**, the untreated groups was used as the negative control.

It has been anticipated that polident® used in conjunction with a mechanical approach, such as brushing, may show a more consistent effect on biofilm removal. The mechanical force from spraying may remove loosely adhered biofilm on PMMA specimens, increasing the efficacy of the compound **1** formulation.

Although, mechanical method is the most commonly used and effective procedure for reducing and removing biofilm formation. Using chemical agent as an adjunctive may be essential for the elderly or one who has mental or physical disability to helping them maintaining adequate denture hygiene [11,12]. Among the chemical cleansing agent, a previous clinical study reported that sodium hypochlorite 0.25% was the most effective disinfection protocol for denture stomatitis patients [27]. Chemical products, on the other hand, can change the irregularities, porosity, color, and even hardness of denture bases [13]. In addition to being bactericidal or fungicidal, an ideal denture hygiene method should have no effect on the color stability, surface roughness, and dimensional stability of the denture base and artificial teeth for long-term use [10]. Further study on a long-term use of the antifungal spray on physical properties of the PMMA, therefore, is necessary.

The methods employed in this study are straightforward and possess some strengths. To quantify the antifungal activity of each

agent tested, the growth curve assay at MIC and sub-MIC values was performed in conjunction with the microdilution assay. This enables us to observe the dynamic interaction (effect of time and concentration) between the tested agents and *C. albicans*. In addition, to simulate the clinical use of antifungal spray and the behaviors of *C. albicans* during daily treatment, cell viability was evaluated for immediate non-adherence biofilm removal and after 18 h recolonization. This could demonstrate whether the tested agents can completely remove the biofilm and prevent its recolonization. As a limitation of the study, the efficacy of synthetic compound **1** was based on *in vitro* monospecies biofilm of *C. albicans* and only a single laboratory strain of *C. albicans* was evaluated. The susceptibility of different *Candida* strains to antifungal agents may vary between strains. In future studies, the antifungal activity of compound **1** against different *C. albicans* strains and clinical isolates from candida-associated denture stomatitis patients would be evaluated. Furthermore, the acrylic disc specimen does not account for the complex topology of denture structures. The acrylic denture biofilm is a much more complex and diversified biofilm. The results of the present study should be interpreted with care as the mixed-species biofilm was not evaluated in this study. Bacteria have been shown to considerably impact and modify candida development and biofilm formation through time. In addition, a previous study indicated that the blastopore/hyphae index increased with maturing of the mono-and multispecies biofilms, suggesting an increased amount of *C. albicans* hyphae in mature biofilms on the surface of denture base materials [28]. This highlights the importance of regular oral hygiene in denture wearers because hyphae in biofilms increase the likelihood of *C. albicans* tissue invasion and suggest further research using a multispecies biofilm model [28,29]. Lastly, the different formulations should be also considered and tested on clinical samples and those resistant to *Candida* spp.

5. Conclusion

Within the limitation of this study, the microdilution and growth curve assays clearly illustrated that synthetic alkynyloxy derivative of lawsone showed favorable inhibitory activity against *C. albicans*. The biofilm removal can be achieved via the application of the antifungal denture spray containing compound **1**.

Author contribution statement

Supawadee Naorungroj and Luelak Lomlim: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Jutharat Manuschai, Pichayaporn Ratti, and Jiraporn Kara: Performed the experiments; Analyzed and interpreted the data.

Athip Sakunphueak and Pharkphoom Panichayupakaranant: Conceived and designed the experiments.

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Data availability statement

Data will be made available on request.

Declaration of interest's statement

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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