Original Article

Antimicrobial and Anti-inflammatory Effects of α-Mangostin Soluble Film

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Received : 29-07-21
Revised : 07-10-21
Accepted : 10-10-21
Published : 08-04-22

Abstract

Objectives: Plant-derived compounds are a major source of medicinal agents. Common oral diseases, including dental caries, periodontal disease, and candidiasis, are caused by biofilms. The nature of biofilm formations is complex, emphasizing the importance of finding novel products that possess bioactivity against microbes associated with those oral infections. The aims of this study were to determine the antimicrobial activity and antibiofilm formation of α-mangostin (α-MG) soluble film. **Materials and Methods:** Antimicrobial assays against *Streptococcus mutans, Porphyromonas gingivalis*, and *Candida albicans* were performed by identifying the minimal growth inhibition concentration and the minimal bactericidal concentration. Time-killing kinetic studies against the organisms and inhibition of biofilm formation were determined by the broth microdilution method. Human gingival fibroblast cell line and macrophage RAW267.4 cells were cultured, and the cell viability was assessed by the MTT assay. The anti-inflammatory effect of the α-MG film was investigated by measuring the inhibition of nitric oxide production. **Results:** The α-MG film demonstrated antimicrobial activity against the oral pathogens tested. The formulation reduced microbial growth about 1–3 Log CFU/mL at 2–4 h and complete killing at 24 h. No significant difference in inhibiting the biofilm formation of those three microorganisms was noted. In addition, the film containing α-MG demonstrated anti-inflammatory activity through the inhibition of nitric oxide production in a dose-dependent manner. The formulation was safe and showed no cytotoxicity at therapeutic dose. **Conclusions:** The α-MG film is effective against *S. mutans, P. gingivalis*, and *C. albicans* without significant cytotoxicity *in vitro*. Thus, this new product may have potential advantage in preventing those common oral infections.

Keywords: *Antimicrobial activity, candidiasis, dental caries, mangosteen, mucoadhesive film, periodontitis*

Introduction

Dental caries, periodontal disease, and oral fungal infections are the three most common oral health problems caused by virulent biofilms.[1] It is well accepted that *Streptococcus mutans* (*S. mutans*) and *Porphyromonas gingivalis* (*P. gingivalis*) are keystone pathogens in the development of dental caries and periodontal disease, respectively,[2,3] whereas *Candida albicans* (*C. albicans*) is the most common species that causes oral candidiasis that is frequently observed in immunocompromised patients, including HIV-infected subjects and denture wearers.[4]

Mechanical therapy by toothbrushing with fluoride toothpaste and flossing along with chemical plaque controls by using mouthwash are the methods used in the management of dental caries and periodontal

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Access this article online

Quick Response Code:

Website: www.jispcd.org

DOI: 10.4103/jispcd.JISPCD_222_21

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How to cite this article: Tangsuksan P, Srichana T, Kettratad M, Nittayananta W. Antimicrobial and anti-inflammatory effects of α-mangostin soluble film. J Int Soc Prevent Communit Dent 2022;12:189-98.

diseases.[5] The treatment of oral candidiasis consists of various topical and systemic antifungal drugs, including polyenes and azoles. The lesions generally respond well to conventional therapies. However, such treatment usually causes reduction without a complete resolution of the infection, leading to drug resistance.[6,7]

At present, therapeutic approaches to control oral biofilm formation are still inadequate. It is well recognized that dental biofilm is a crucial step in the development of the diseases and it usually occurs shortly after toothbrushing. The complex nature of biofilm formations emphasizes the importance of finding novel agents that possess antibiofilm activity. Given these challenges, new oral products with anticaries, anti-periodontal, and antifungal activities along with antibiofilm formation are warranted.

Plants are valuable sources of novel bioactive compounds, as they produce a wide variety of secondary metabolites with biological properties against oral pathogens.[3] Mangosteen (*Garcinia mangostana* L.) is a widely cultivated fruit tree in Southeast Asian nations, including Thailand, Malaysia, Indonesia, The Philippines, and Vietnam. Mangosteen extract has been shown to demonstrate bactericidal activity against cariogenic bacteria.[8,9] Its pericarp has been used in traditional medicine to treat various infections caused by bacteria, virus, and fungus.[10,11] α-mangostin (α-MG) is a major xanthone derivative compound isolated from mangostin pericarp extract. Ngyyen *et al.*[12] reported that α-MG disrupted the development of *S. mutans* biofilms.

In addition, α-MG has been used as an antibacterial component in an adhesive paste to prevent dental caries[13] and added in a topical gel to treat chronic periodontitis.[14,15] Interestingly, mangosteen has been shown to mediate anti-inflammatory response in dental complications. For instance, it has been demonstrated to reduce inflammation related to gingivitis in rats.[16] A study by Kresnoadi *et al.*[17] further revealed that mangosteen pericarp extract could reduce the inflammation of post-tooth extraction in guinea pigs. A previous study demonstrated that oral spray containing α-MG is effective against oral pathogens.[10] These strands of evidence emphasize the use of mangosteen extract in preventing common oral diseases, including dental caries, periodontal disease, and oral candidiasis, and promoting oral hygiene.

As α-MG has been shown to possess various bioactivities, we hypothesized that a soluble film containing α-MG would provide antimicrobial activity against *S. mutans*, *P. gingivalis*, and *C. albicans* and may have effects on the formation of biofilm and inflammation. As such, this product could potentially prevent dental caries, periodontal disease, and oral candidiasis development. Therefore, in this *in vitro* study, α-MG soluble film was developed and tested for its cytotoxicity, antimicrobial activity, antibiofilm formation, and anti-inflammatory property.

Materials and Methods

This *in vitro* experimental study was performed for a duration of two years during 2018–2019 at laboratories of the Faculty of Dentistry, Thammasat University, and the Faculty of Pharmaceutical Sciences, Prince of Songkla University, Thailand.

Preparation of α-MG soluble film

Twenty percent α-MG derived from the pericarp of mangosteen extract (food grade) was purchased from a local company in Thailand (Chemipan, Bangkok, Thailand). A film containing active ingredients α-MG (5 μg/mL) was prepared by using hydroxypropyl methylcellulose (HPMC), as previously described.[18] The α-MG soluble film was sterilized by dry heat in an oven at 90°C for 130 min. The physicochemical properties of the formulation were recorded both before and after the stability study (Freeze-thaw 5 cycles).

Microbial growth condition and inoculum preparation

S. mutans (American type culture collection [ATCC] 25175) was cultured on brain heart infusion broth and incubated at 37°C for 18–24 h. *C. albicans* (ATCC 90028) was cultured in Sabouraud dextrose broth and incubated at 35°C for 24 h. *P. gingivalis* (ATCC 33277) was cultured in tryptic soy broth supplemented with hemin (5 μg/mL) and vitamin K_1 (1 μg/mL) and incubated under anaerobic conditions maintained in an anaerobic jar containing a gas-pak microbiology Anaerocult (Merck, Darmstadt, Germany) at 37°C for 48–72 h prior to use.

Antimicrobial activity assay

The minimal growth inhibition concentration

Samples of soluble film containing α-MG were tested against three organisms, *S. mutans, P.gingivalis*, and *C. albicans*, for their inhibitory activity using a broth microdilution method.[19] Briefly, 100 μL of the serially diluted samples (from 234 to 3.66 μg/mL) was added to each well plate; 10 μL of microbial suspension was added at the starting optical density at 10^5 CFU/mL to the sample well plates. The microtiter plates were incubated under the appropriate conditions for each microbial mentioned earlier. Each sample was conducted in four replicates. After the incubation period, 30 μL of a 0.02% of resazurin sodium salt dissolved in phosphate

buffer saline (PBS) was added to each well. The plates were further incubated for 3 h, and bacterial growth was indicated by the pink color. The minimal growth inhibition concentration (MIC) was determined as the lowest sample concentration at which no pink color (i.e., no bacterial growth) appeared.

The minimal bactericidal concentration

Minimal bactericidal concentration (MBC) was determined by subculturing the samples that have a value less than or equal to an MIC value on freshly prepared culture agar plates and incubated under the appropriate conditions for each organism. The highest dilution (lower concentration) showing no single bacterial colony was taken as the MBC value.

Time-kill assay

Time-killing kinetic studies against organisms were determined by the broth microdilution method.[19] The culture of organisms was diluted to 10^7 CFU/mL. The diluted microbial cultures were treated with the sample of α-MG soluble film (234 µg/mL). On 0, 1, 2, 4, 6, and 24 h incubation, the numbers of surviving cells were determined by the plate count method and the killing curves were constructed by plotting Log_{10} CFU/mL versus time. These experiments were repeated three times.

Inhibition of biofilm formation

The effects of α-MG soluble film on microbial biofilm formation were tested by the broth microdilution method.[19] Briefly, a concentration of the sample of α-MG soluble film (234 µg/mL) in culture media was added at a volume of 100 µL per well. Microbial cultures (10 µL) were added to the final concentration at 1×10^7 CFU/mL to the culture well, whereas the medium only was added as a blank control. After incubation of the microbial condition, the plates were washed twice with PBS to remove planktonic organisms and stained with 0.1% (w/v) crystal violet for 10 min. The plates were rinsed two times with water to remove excess, dried, and finally air-dried for 30 min. After air-drying, the biofilms were extracted using 95% ethanol and quantified using a microplate reader by measuring absorbance at 570 nm.

Cell culture conditions

Human gingival fibroblast cell line

Human gingival fibroblast cell lines were provided by the Faculty of Dentistry, Prince of Songkla University, Thailand. They were cultured in Dulbecco's Modified Eagle Medium (DMEM, Gibco®, USA) containing 10% fetal bovine serum (FBS, Gibco®, USA) and antibiotics (100 U/mL penicillin/streptomycin, Gibco®, USA) under 5% CO_2 at 37°C. The media were changed every alternate day. When the cells reached confluence, they were harvested using 0.25% trypsin-EDTA (Gibco®, USA), followed by the addition of fresh culture medium to create a new single-cell suspension for further incubation.

Mouse monocyte/macrophage cell line

Mouse monocyte/macrophage cell line (RAW264.7, ATCC TIB-71, USA) was cultured in DMEM (Gibco®, USA) supplemented with 10% FBS (Gibco®, USA), 100 U/mL penicillin/streptomycin (Gibco®, USA). Cells were incubated at 37ºC in a 5% CO_2 incubator, and the media were changed every two days. They were harvested by gentle rocking, followed by the addition of fresh culture medium to create a new single-cell suspension for further incubation.

Cell viability assay

Human gingival fibroblast cells and RAW264.7 at a concentration of 1×10^5 cell/mL were seeded in a 96-well plate and incubated at 37ºC under 5% CO_2 for 24 h. After incubation, the sample of various concentrations, that is, 3.66, 7.32, 14.63, 29.20, 58.50, 117, and 234 µg/mL in fresh medium, was added to the culture plates, respectively. Cells without sample served as a negative control. After incubation for 24 h, methylthiazol tetrazolium (MTT) assay was performed to evaluate cell activity. Briefly, the cells were treated with 50 µL of fresh media along with 50 µL of MTT solution and incubated at 37ºC under 5% CO_2 for 4 h. Thereafter, the media containing MTT were removed and 100 µL of dimethyl sulfoxide was added. The absorbance was determined by a microplate reader (Biohit 830, Finland) at a wavelength of 570 nm. The percentage of cell proliferation was calculated and compared with a negative control.

Measurement of nitric oxide production

RAW267.4 cells were seeded at a concentration of 1×10^6 cell/mL into 96-well plates in complete medium. After 24 h of incubation, 100 µL of the sample of α-MG soluble film at various concentrations (1.83, 3.66, 7.32, 14.63, and 29.20 µg/mL) or 1 µg/mL of the lipopolysaccharide (LPS) from *Escherichia coli* (Sigma-Aldrich) as a positive control in fresh medium was added to each well. Wells with media only served as negative controls. The Griess reaction assay was then employed to determine nitric oxide (NO) content in the cell supernatants. Briefly, 100 µL of Griess reagent (1% sulfanilamide [Sigma-Aldrich]) in 2.5% phosphoric acid and 0.1% *N*-(1-naphthyl) ethylenediamine dihydrochloride (Sigma-Aldrich) was mixed with an equal volume of culture media. In this assay, a pink solution indicates a positive result, whereas a

yellow solution indicates a negative result. Based on a standard curve of $NaNO_2$, the quantity of NO was determined by measuring the absorbance at 450 nm using a microplate reader.

In vitro anti-inflammatory study

RAW264.7 cell line was seeded in 96-well plates with 1×10^5 cells/well and allowed to adhere for 1 h at 37°C in a humidified atmosphere containing 5% CO_2. After that, the medium was replaced with a fresh medium containing 1 μg/mL of LPS together with the test samples of α-MG soluble film at various concentrations (1.83, 3.66, 7.32, 14.63, and 29.20 μg/mL) and was then incubated for 24 h. The Griess reaction assay was then employed to determine NO content in the cell supernatants. Briefly, 100 μL of Griess reagent was mixed with an equal volume of culture media. The quantity of NO was determined by measuring the absorbance at 450 nm using a microplate reader. The % inhibition was calculated based on the following equation values;

$$\text{NO inhibition (\%)} = ((A - B) / (A - C)) \times 100$$

A: LPS (+), sample (−)

B: LPS (+), sample (+)

C: LPS (−), sample (−)

Statistical analysis

Statistical analysis was performed using Microsoft Excel (One Microsoft Way Redmond, WA, USA). Comparisons between untreated and treated groups were investigated by Student's *t*-tests. The data are expressed as mean ± SD (standard deviation). The statistically significant differences were considered when $P < 0.05$.

Table 1: Physicochemical properties of α-mangostin (α-MG) soluble film after preparation and under stability study (freeze-thaw 5 cycles)

Properties	α-MG soluble film	
	After preparation	After freeze-thaw
Physical appearance	Yellow brown film	Dark brown film
pH	6.27	6.28
% content of active compound	7.3	7.3

Results

Physical properties and antimicrobial activities of α-MG soluble film

α-MG soluble film was formulated. The physical properties, pH, and the stability of the film after five freeze thaw cycles are shown in Table 1.The film turned from yellow brown to dark brown with a stable pH around 6.3. The α-MG content stayed constant after the freeze–thaw process in comparison with that after the preparation; therefore it proved to be chemically stable. No differences in antimicrobial activity were detected against *S. mutans, P. gingivalis*, and *C. albicans* [Table 2]. According to results from the time-kill assay, the film showed reducing effects on growth of the organisms about 1–3 Log CFU/mL at 2–4 h and complete killing at 24 h. In contrast, the control did not demonstrate any inhibitory effects even at 24 h [Figure 1]. Based on the results from biofilm assay, the α-MG soluble film demonstrated no significant difference in inhibitory effects against *S. mutans*, *P. gingivalis*, and *C. albicans* [Figure 2].

Cytotoxicity of α-MG soluble film

Cytotoxicity of α-MG soluble film was determined in the murine macrophage cell line (RAW264.7 cells) and human gingival fibroblast using the MTT assay. The film was not toxic to the cells, with 100% survival at the maximum concentration of 29.20 μg/mL concentrations [Figure 3]. Toxicity was observed at the concentration of ≥ 58.50 μg/mL in both RAW264.7 macrophage cell lines and human gingival fibroblasts.

Anti-inflammatory activities of α-MG soluble film

In order to assess anti-inflammatory activity of the α-MG soluble film, the percent inhibition of NO was determined. The anti-inflammatory activity of the film was evaluated in RAW264.7 cells. The α-MG soluble

Table 2: Antimicrobial activity of α-mangostin (α-MG) soluble film against common oral microbes

Sample tested	*S. mutans* ATCC 25175		*P. gingivalis* ATCC 33277		*C. albicans* ATCC 90028		
	MIC (μg/mL)	MBC (μg/mL)	MIC (μg/mL)	MBC (μg/mL)	MIC	MFC (μg/mL)	MFC (μg/mL)
α-MG	117	234	117	234	117	234	234
Nystatin	–	–	–	–	0.78	0.78	0.78
Chlorhexidine	<0.78		<0.78			–	–

ATCC = American type culture collection, MIC = minimum inhibitory concentration, MFC = minimum fungicidal concentration; MBC = minimum bactericidal concentration, *S. mutans* = *Streptococcus mutans*, *P. gingivalis* = *Porphyromonas gingivalis*, *C. albicans* = *Candida albicans*

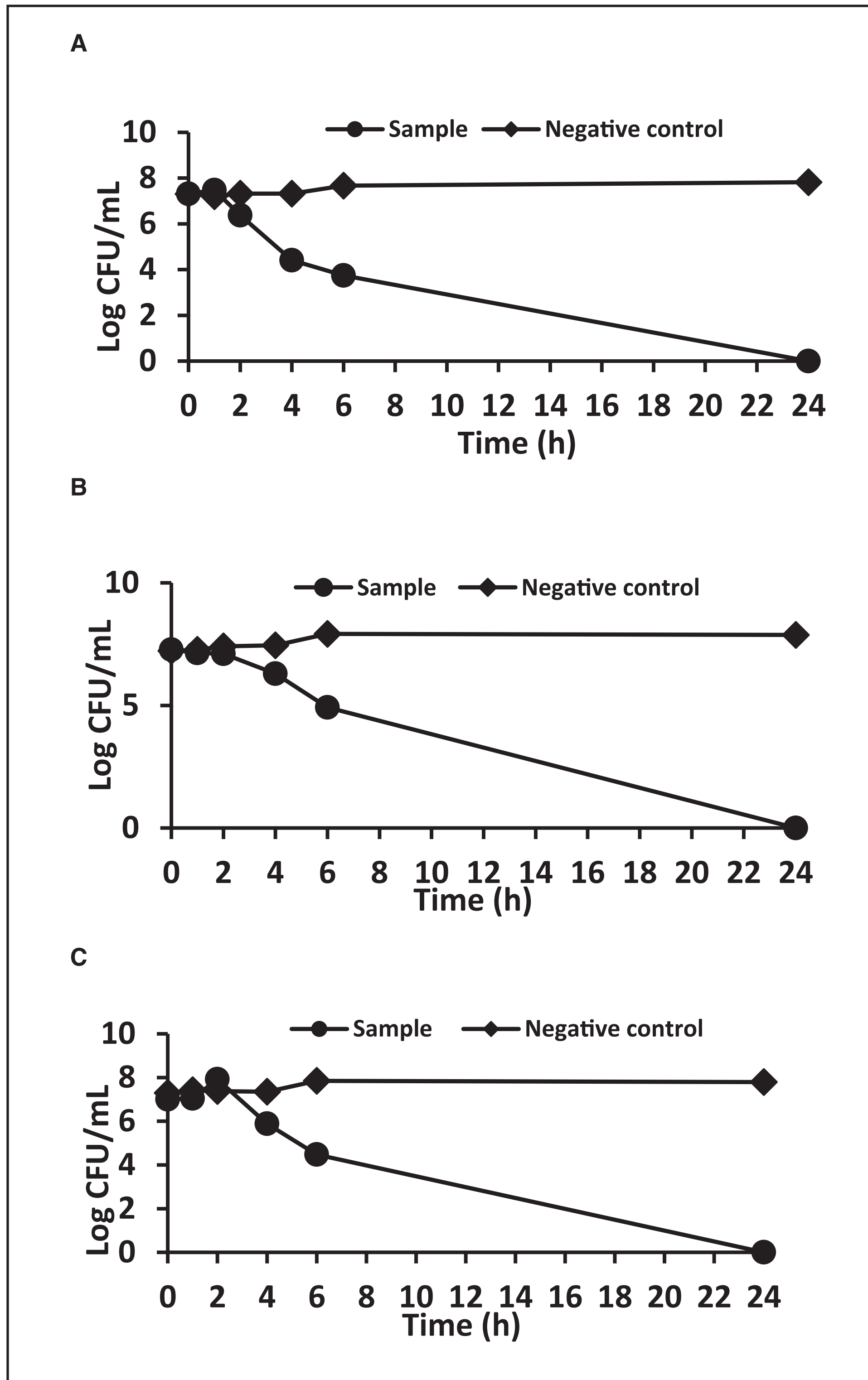

Figure 1: Time-kill assay of (A) *Streptococcus mutans* ATCC25175, (B) *Porphyromonas gingivalis* ATCC33277, and (C) *Candida albicans* ATCC90028 after treatment with the formulation of α-mangostin (α-MG) soluble film at various times (mean ± SD, *n* = 4). ATCC = American type culture collection

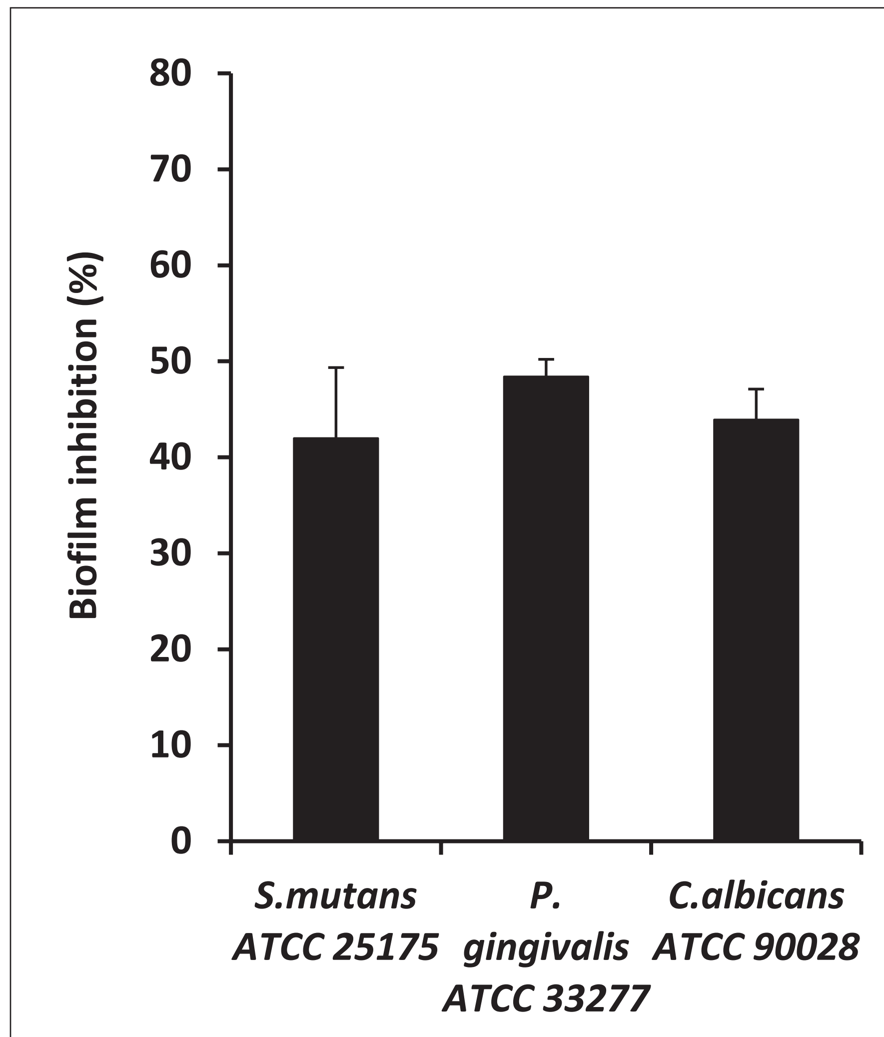

Figure 2: Antibiofilm formation of *Streptococcus mutans* ATCC25175, *Porphyromonas gingivalis* ATCC33277, and *Candida albicans* ATCC90028 after treatment with the formulation of α-mangostin (α-MG) soluble film (mean ± SD, $n = 4$). ATCC = American type culture collection

film demonstrated the inhibition of NO in a dose-dependent manner [Figure 4].

Discussion

This *in vitro* study demonstrated that a-MG soluble film possessed antimicrobial activity against common oral pathogens, including *S. mutans*, *P. gingivalis*, and *C. albicans*. The film also demonstrated the anti-inflammatory effects and inhibited biofilm formation *in vitro* without cytotoxicity at a therapeutic dose.

In this study, HPMC was used to enhance adhesion of the film to the mucosa and/or tooth structure, which, in turn, may help to increases the contact time of α-MG with those oral structures. The uses of HPMC as a thickening agent and a bioadhesive are well documented.[20] This polymer is one of the most common hydrophilic carriers used in controlled oral drug delivery systems. It is produced by the synthetic modification of naturally occurring polymer cellulose and is safe for human use.

Although most oral products available in the market are effective against particular pathogens, the α-MG soluble film in this study demonstrated antimicrobial activity against *S. mutans, P. gingivalis*, and *C. albicans*. The inhibition of biofilm formation was also observed for *C. albicans, S. mutans*, and *P. gingivalis.* Moreover, anti-inflammatory activity of the film was noted and was found to be increased in a dose-dependent manner.

The α-MG soluble film in the present study inhibited the biofilm formation of *S. mutans, P. gingivalis*, and *C. albicans*. These findings are consistent with previous studies that reported that α-MG acts as an antimicrobial agent against *S. mutans*, and a biofilm-forming and

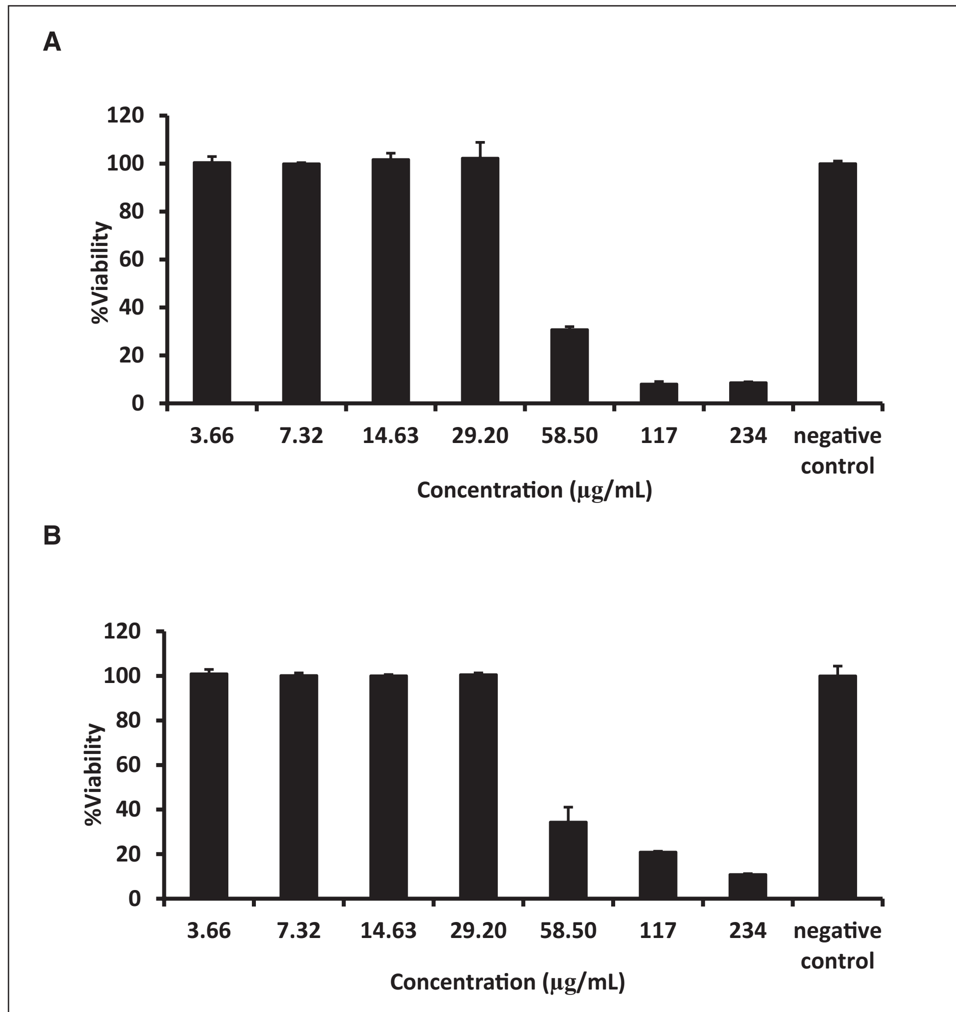

Figure 3: Cytotoxicity of α-mangostin (α-MG, 5 mg/mL) soluble film on (A) RAW264.7 macrophage cell lines, and (B) human gingival fibroblast determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Data are presented as mean ± SEM ($n = 4$)

acid-producing cariogenic organism[12] and against planktonic cells of oral *Candida*.[21] In addition, gels containing α-MG have been shown to improve periodontal health, possibly by the targeting of periodontal pathogens.[22]

In the present study, fungicidal and bactericidal mechanisms were assessed and were shown to be concentration dependent. A previous study reported that oral spray containing α-MG showed antifungal activity against *C. albicans* with MIC and MFC of 23.4 µg/mL, and it also had some antibacterial activity against *S. mutans* with MIC and MBC of 31.2 and 62.5 µg/mL, respectively.[10] Another study by Juntavee *et al.*[9] reported bactericidal activity against *S. mutans* of mangosteen extract with an MIC of 0.25 mg/mL. The time-kill kinetics revealed that 1 mg/mL of the extract reduced *S. mutans* by 50% and dropped to zero within 5 s and 60 s, respectively. Using time-kill assays, the α-MG soluble film (234 µg/mL) in the present study reduced growth of the microorganisms about 1–3 Log CFU/mL at 2–4 h and complete killing at 24 h; however, the oral spray containing α-MG in our previous study reduced the growth of the microorganism about 1–2 Log CFU/mL at 1–3 h, and the killing effects were complete at 24 h.[10] These findings suggest that different formulations of the oral products may affect the antimicrobial activity of α-MG.

In this study, antibiofilm formation of the α-MG soluble film against *S. mutans, P. gingivalis*, and *C. albicans* was observed. These may be due to multiple mechanisms, including the reduction of acid production by disrupting the membranes of the organisms.[23] A previous study by Nguyen *et al.*[12] revealed that α-MG compromises the ability of *S. mutans* to develop biofilms. Thus, the α-MG film in the present study might serve as an effective oral product for controlling biofilm formation.

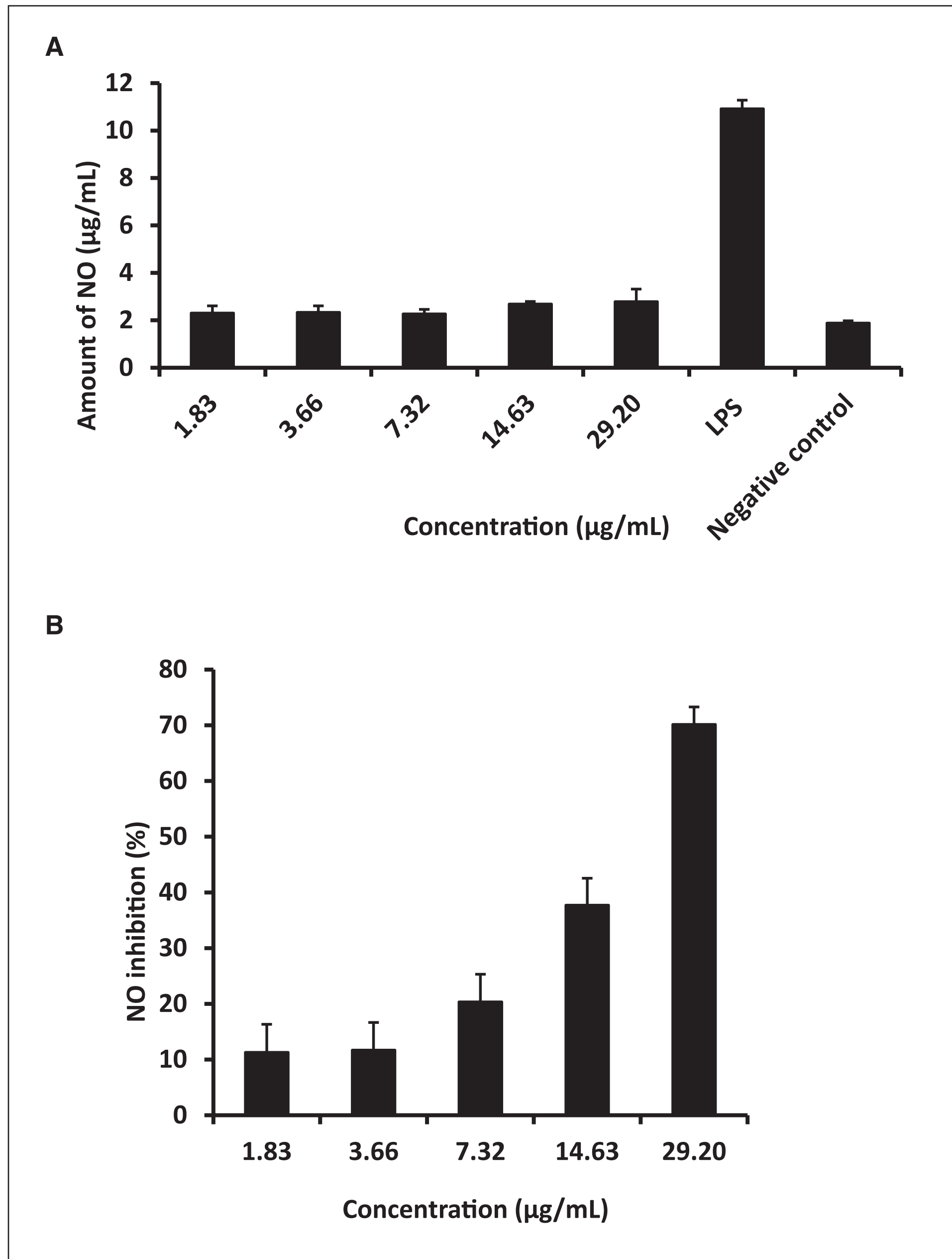

Figure 4: Anti-inflammatory activity of α-mangostin (α-MG) soluble film. (A) Amount of nitric oxide production and (B) percent inhibition of nitric oxide production in RAW264.7 macrophage cell lines after treatment with α-mangostin (α-MG, 5 mg/mL) soluble film. Data are presented as mean ± SEM ($n = 4$)

The present study revealed that the anti-inflammatory activity of the α-MG soluble film was increased in a dose-dependent manner. A previous study reported that the percent inhibition of NO consistently declined with a high concentration of the oral spray containing α-MG.[10] This may be because the oral spray at a high concentration seemed to be toxic to the cells exhibiting anti-inflammatory activity. This may lead to cell death, which would also demonstrate decreased production of NO by those cells. However, the present study revealed that the production of NO was stable even when the concentration of the tested sample of the α-MG soluble film was increased [Figure 4]. Again, these findings suggest that different formulations of the oral products may result in different anti-inflammatory activity of α-MG.

It is well accepted that natural products are excellent sources for new bioactive compounds that can inhibit the key microorganisms associated with the biofilm formation.[24] Among these, α-MG shows promising results with antimicrobial activity both *in vitro* and *in vivo*.[8-10] The present study demonstrated that the α-MG

soluble film possesses antimicrobial, antibiofilm, and anti-inflammatory activities without cytotoxic effects. Thus, this film could be developed as a novel product for the prevention of common oral diseases such as dental caries, periodontal disease, and oral candidiasis, or as an adjunctive treatment.

A limitation of this study was that it was only conducted *in vitro* and thus could not be generalized to clinical settings. Further studies should include randomized clinical trials of the α-MG soluble film to determine its activity *in vivo*, because saliva, pH, and other environmental factors may influence the activity of the film. The present study still lacks information regarding adhesion of the film to tooth surfaces. Antimicrobial activities of the α-MG soluble film on clinical isolates of the microorganisms should be investigated. Testing of the activities on multispecies of dental biofilms should also be carried out in order to mimic the oral cavity *in vivo.* Additional studies on antimicrobial activities and application testing by adapting the film to tooth and mucosal surfaces should be conducted. Further, mechanisms involved in antimicrobial activity and anti-inflammatory effects of the α-MG soluble film should be determined in future studies. Other properties such as wound healing, anticancer, and antioxidant activities of the film should also be further investigated.

Conclusions

The α-MG soluble film has antimicrobial, antibiofilm, and anti-inflammatory activities without cytotoxic effects. Thus, the film could be developed as a novel product for preventing common oral diseases, including dental caries, periodontal disease, and oral candidiasis, or as an adjunctive treatment to their conventional therapy.

Acknowledgments

The authors thank Ms. Titpawan Nakpeng for her technical support.

Financial support and sponsorship

This study was supported by Thammasat University Research Fund, Contract No. TUGG 43/2562.

Conflicts of interest

The authors declare that there is no conflict of interests regarding the publication of this article.

Authors' contributions

PT and WN designed the study. PT and TS performed the study and collected data. TS and WN analyzed and interpreted the data. WN drafted the article. TS, MK, and WN revised the article. All authors approved the final version of the article and agree to be accountable for all aspects of the work.

Ethical policy and institutional review board statement

This study was approved by the Institutional Biosafety Committee of Thammasat University No. 130/2561.

Patient declaration of consent

Not applicable.

Data availability statement

The datasets generated and/or analyzed during the current study are not publicly available, but they are available from the corresponding author on reasonable request.

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