The antimicrobial activity of

Curcuma xanthorrhiza oil nanoemulsion

Mu-Yeol Cho

The Graduate School

Yonsei University

Department of Applied Life Science

The antimicrobial activity of *Curcuma xanthorrhiza* oil nanoemulsion

Directed by Professor Baek-Il Kim

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Mu-Yeol Cho

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This certifies that the Doctoral Dissertation of

Mu Yeol Cho is approved.

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Thesis Supervisor: Baek Il Kim

_____

Thesis Committee: Ho Keun Kwon

_____

Thesis Committee: Ki Ho Chung

_____

Thesis Committee: Hoi In Jung

_____

Thesis Committee: Choi Sung Hwan

The Graduate School

Yonsei University

December 2021

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Abstract

The antimicrobial activity of *Curcuma xanthorrhiza* oil nanoemulsion

Mu-Yeol Cho

Department of Applied Life Science

The Graduate School, Yonsei University

(Directed by Professor Baek-Il Kim)

Curcuma xantorrhiza oil (CXO) is a natural essential oil that has a significant antibacterial effect on dental bacteria. However, it is hydrophobic so it is difficult to solubilize. This study evaluated the conditions for CXO to form a nanoemulsion by nanoemulsification, a new solubilization method, and its antimicrobial effect.

This study was conducted in the form of three studies. The first study identified the ultrasonic time that indicated the smallest size of oil particles in CXO nanoemulsion. The second and third studies evaluated the antimicrobial effects of the CXO nanoemulsion on single-species and polymicrobial biofilms, respectively.

In the first study, CXO was nanoemulsified by both emulsification and ultrasonic processes at intervals of 5 minutes for up to 15 minutes. The turbidity, particle size, and particle size homogeneity in terms of polydispersity index, and stability against centrifugation and storage for 30 days of each nanoemulsion were evaluated. The CXO nanoemulsion, which was subjected to ultrasonic processing for 10 minutes, had the lowest turbidity, greatest transparency, lowest average particle size, and lowest polydispersity index, all of which were significantly different from those values for the CXO emulsion that was not subject to ultrasonic processing. The CXO nanoemulsion was stable against centrifugation and storage, whereas the CXO emulsion exhibited sedimentation after centrifugation and layer separation after long-term storage.

In the second study, the antibacterial effect of CXO nanoemulsion on planktonic *Streptococcus mutans* and *S. mutans* biofilms was evaluated. Sterile distilled water and Listerine were included as negative and positive control groups, respectively, and a CXO emulsion and a CXO nanoemulsion were used as experimental groups. Antibacterial effect was evaluated in terms of the number of colony-forming units present in *S. mutans* culture or on the biofilms after treatment, which reflects cell viability. Biofilms were additionally evaluated by determining the distribution of live and dead bacterial cells on confocal laser

scanning microscopy images. The CXO nanoemulsion was shown to completely kill all planktonic *S. mutans*. Additionally, a low number of colony-forming units was left on the biofilm treated with a CXO nanoemulsion, which was statistically significantly different from those treated with distilled water and Listerine. Confocal laser scanning microscopy images showed that the CXO nanoemulsion had the lowest average biomass, biofilm thickness, and inhibition of both live and dead bacterial cells.

In the third study, the antimicrobial effects of the CXO nanoemulsion were evaluated using a microcosm biofilm model, a polymicrobial biofilm model. The red fluorescence intensity of the biofilms was measured using quantitative light-induced fluorescence, the number of total and aciduric bacteria colony-forming units within the biofilms were measured, biomass analysis of the biofilms was conducted using the dry-weight method, and bacterial cell and extracellular polysaccharides analyses were performed using confocal laser scanning microscopy. The red fluorescence intensity of the biofilm treated with the CXO nanoemulsion was significantly lower than that of the biofilm treated with distilled water. In addition, the biofilm treated with CXO nanoemulsion had significantly lower total and aciduric bacterial colony-forming unit counts than the biofilm treated with distilled water. Dry-weight analysis showed that CXO nanoemulsion treatment resulted in a significantly lower biomass than distilled water or Listerine treatment. The dominance of bacterial cell and extracellular polysaccharides formation in the CXO nanoemulsion group was less observed among the other groups except for the chlorhexidine group.

These experiments showed that the CXO nanoemulsion was successfully solubilized

by nanoemulsification and that it had strong stability and antimicrobial effects. The CXO nanoemulsion had significant antibacterial and anti-biofilm effects on oral-related bacterial species on biofilm models that simulated real dental biofilm. Therefore, CXO nanoemulsion can be used as a new antibacterial agent as part of oral care.

Keywords: *curcuma xanthorrhiza* oil, nanoemulsion, *S. mutans*, microcosm biofilm, antimicrobial effect, antibiofilm effect.

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I. Introduction

Chemical therapies are often used to prevent plaque deposits that cause dental caries and periodontal disease. Mouthwashes and toothpastes, which are commonly used in these chemical therapies, are comprised of numerous synthetic compounds, including chlorhexidine (CHX), cetylpyridinium chloride, triclosan, and zinc citrate (Baehni and Takeuchi 2003). However, these synthetic compounds can only be administered in limited

dosages due to drug resistance and potential side effects (Lin, Voss, and Davidson 1991; Orvos et al. 2002; Wang et al. 2015). Natural products used in traditional medicine are employed as alternatives to avoid these side effects (Lahlou 2007). They are eco-friendly and possess biologically active compounds that have various pharmacological applications. They have, thus, garnered significant interest in recent times (Lahlou 2013; Robinson and Zhang 2011). The application of natural products as chemical therapies in dentistry can help improve the efficacy of synthetic materials and contribute to the development of new pharmacological resources for promoting oral health.

Curcuma xanthorrhiza is a plant in the ginger family, Zingiberacae, and is cultivated mainly in Southeast Asia and has been traditionally used as a medicinal plant. Extract of this plant has a strong antimicrobial effect against oral bacteria and biofilm formation (Hwang, Shim, and Pyun 2000; Kim et al. 2008). In a previous study, 0.1 mg/ml of the extract showed similar antimicrobial activity to 2 mg/ml of CHX on *Streptococcus mutans* biofilm and inhibited bacterial cell viability by 57.6% compared to 1% dimethyl sulfoxide (DMSO), the negative control (Kim et al. 2008). These effects are due to xanthorrhizol, the main component of the extract (Rukayadi and Hwang 2006a). The extract has antimicrobial effects against various oral bacterial species, including *Actinomyces viscosus*, *Candida albicans*, *Porphyromonas gingivalis*, *Lactobacillus acidophilus*, and *S. mutans* (Hwang, Shim, and Pyun 2000). Thus, *C. xanthorrhiza* extract has been included as an active ingredient in various oral products to suppress gingivitis and bad breath (Kim et al. 2005; Hong et al. 2005). Therefore *C. xanthorrhiza* extract can be utilized as a new active

ingredient in chemical treatment for oral care as a natural antimicrobial agent.

C. xanthorrhiza oil (CXO), one of *C. xanthorrhiza* extracts, is hydrophobic and therefore does not mix well with water. Thus, most studies have used DMSO as an organic solvent for solubilizing CXO (Kim et al. 2007; Lee et al. 2017). However, continuous exposure to DMSO causes apoptosis, which is particularly dangerous for young children (Hanslick et al. 2009). A novel solubilization method would allow for the safe clinical usage of CXO.

Emulsification is the process of preparing a mixture of oil and water using an emulsifier. Surfactants, which are emulsifiers commonly used in emulsification, stabilize and solubilize the emulsion by lowering the tension at the interface between the oil and water to help form oil particles (Mason et al. 2006). The sizes of the particles in the emulsion can be changed by a strong external stimulus (Modarres-Gheisari et al. 2019). Emulsions with particles with diameters < 100 nm are known as nanoemulsions (McClements 2012). This small particle size makes nanoemulsions highly biocompatible because of increased surface area and penetration activity (Hougeir and Kircik 2012). Nanoemulsions also have greater physical stability and antimicrobial effects than general emulsions (Quatrin et al. 2017; Solans et al. 2005). Due to these characteristics, nanoemulsions are applied in various ways, such as in drug delivery, foods, and cosmetics (Gupta et al. 2016b) and interest in them has been increasing recently (McClements 2012). Applying nanoemulsions in dentistry may facilitate the development of antimicrobial agents that contain natural essential oils with antimicrobial and anti-inflammatory

properties.

There are numerous nanoemulsification methods, such as microfluidization and high-pressure homogenization, but ultrasonic processing is the most effective and convenient method for reducing droplet size (Modarres-Gheisari et al. 2019). Soybean oil nanoemulsions prepared via ultrasonic processing had a stable droplet size of 29.6 nm and a strong antimicrobial effect against *Escherichia coli* (Ghosh, Mukherjee, and Chandrasekaran 2013). However, there have been no reports on the antimicrobial activity of CXO nanoemulsions prepared using ultrasonic processing. Therefore, it was hypothesized that CXO nanoemulsified via ultrasonic processing would likely show strong antimicrobial activity and stable solubization.

In dentistry, *S. mutans* is the oral bacteria that most often causes dental caries. Adhesive glucan, a metabolite of *S. mutans*, adheres to the surface of the teeth and aggregates various bacteria to form a biofilm, which results in dental caries (Gamboa, Estupiñan, and Galindo 2004), because it can withstand host defense mechanisms better than planktonic bacterial cells and is more resistant to antibiotics (Socransky and Haffajee 2002). Therefore, to evaluate the effectiveness of antimicrobial agents in the preclinical stage, it is necessary to investigate their effects on biofilm as well as planktonic bacteria themselves.

More than 700 bacteria, including *S. mutans*, inhabit the oral cavity (Marsh and Zaura 2017). These bacteria interact, coexist, and form biofilms. These polymicrobial biofilms

have stronger antimicrobial resistance and higher pathogenicity compared to bacteria in the planktonic state and single-species biofilms (Kara, Luppens, and ten Cate 2006). To accurately evaluate antimicrobial effect, experiments using polymicrobial biofilm models that simulate the actual oral environment should be used.

The microcosm biofilm model is a polymicrobial biofilm model that uses human saliva as an inoculum (Kim et al. 2020). It is possible to reproduce and evaluate the real oral microbial ecosystem using this model in a controlled environment. However, no study has yet evaluated the antibacterial effect of CXO nanoemulsion in such a polymicrobial biofilm model. Therefore, in this study, the antimicrobial effect of CXO nanoemulsion was evaluated using a microcosm biofilm model.

This study was conducted to determine the antimicrobial effectiveness of CXO nanoemulsion prepared using ultrasonic nanoemulsification against oral bacteria. The detailed objectives of this study were as follows. The first study was conducted to determine the optimal ultrasonic processing duration. The second study was conducted to evaluate antimicrobial and antibiofilm effects of CXO nanoemulsion against *S. mutans* bacteria and *S. mutans* biofilm. The third study was conducted to evaluate antimicrobial and antibiofilm effects of CXO nanoemulsion against microcosm biofilm.

The null hypotheses for the experiments were as follows. For the first study, it was hypothesized that the CXO emulsion's turbidity, particle size, distribution of particle sizes, pH, and stability would not change according to ultrasonic processing time. For the second

study, it was hypothesized that the antibacterial effect of CXO nanoemulsion against planktonic *S. mtuans* and *S. mutans* biofilm would not be different from that of the negative control group. For the third study, it was hypothesized that the antimicrobial effect of CXO nanoemulsion against microcosm biofilm would not be different from that of the negative control group.

II. Materials and methods

2.1. Ultrasonic processing time (Study I)

2.1.1. Nanoemulsion preparation

Nanoemulsions were prepared using CXO (S & D CO., Ltd., Korea), non-ionic surfactant Tween 80 (T80; Daejung, Korea), and distilled water (DW). Initially, CXO was mixed 1:1 with T80 and diluted with DW to produce coarse emulsions with a final CXO concentration of 1% (v/v). These coarse emulsions were subjected to an ultrasonic process for 0, 5, 10, and 15 min in a sonicator (Digital Sonifier 450, Branson, USA) with a maximum power of 400 W. Each cycle in the process consisted of 30 s intervals with an amplitude of 20%. Because continuous high-energy ultrasonic processes generate heat, the emulsions were placed in an ice bath to minimize temperature differences. All procedures were conducted at room temperature.

2.1.2. Nanoemulsion characterization

Nanoemulsion turbidity was assessed via optical density (OD) analysis at a wavelength of 570 nm using a 680 XR microplate reader (Mitsuibishi Inc., Tokyo, Japan) and performed in triplicate. Nanoemulsion droplet size and polydispersity were determined using an ELS 1000zs particle size analyzer (Otsuka Electronics, Japan). Polydispersity was calculated according to polydispersity index (PDI), indicating the stability and homogeneity of the droplet size in the nanoemulsions. Droplet size was measured using

dynamic light scattering after nanoemulsions were diluted to 1/30 (v/v) with DW to minimize multiple scattering effects. The pH of each nanoemulsion was measured at room temperature using an Orion 4-Star pH meter (Thermo Scientific, USA). Physical stability was tested after centrifuging samples for at 10,000 rpm for 30 min (Kandadi et al. 2011). The nanoemulsions were visually evaluated after storage for one month at room temperature to determine the stability.

2.1.3. *C. xanthorrhiza* oil profile

High-performance liquid chromatography (HPLC) was performed to determine the components of CXO. The HPLC model was Agilent 1200 series and the column was Zorbax C18 (Table 1). dH_2O (0.2% H_3PO_4) and MeOH were used as mobile phases and UV detectors for wavelengths of 224 and 280 nm were used. Xanthorrhizol was used as a standard substance and its components were compared with those of CXO.

Table 1. HPLC analysis conditions.

Parameters	**Conditions**		
Model	Agilent 1200 series		
Column	Zorbax C18 (5 μm, 4.6 x 150 mm)		
Temperature	40 ℃		
Flow rate	1.0 ml/min		
Injection	10 μl		
Mobile phase	A: dH_2O (0.2% H_3PO_4)		
	B: MeOH		
Detector	224, 280 nm		
Gradient	Time (min)	A* (%)	B* (%)
	0	25	75
	14	25	75
	16	2	98
	30	2	98
	32	25	75
	40	25	75

*A and B indicate dH_2O and MeOH, respectively.

2.2. Antibacterial activity of *C. xanthorrhiza* oil nanoemulsion on *S. mutans* (Study II)

2.2.1. Bacterial strain

The ATCC 25175 strain of *S. mutans* (Korea Research Institute of Bioscience and Biotechnology, Seoul, South Korea) was inoculated in a brain-heart infusion broth (BHI) (Becton, Dickinson and Company, USA) and incubated for 24 h in an incubator (Thermo Fisher Scientific, USA) in a 10% CO_2 environment at 37 ℃. The culture was diluted to a 1:1 ratio using 80% glycerin (Duksan, South Korea) and stored in a deep freezer at -80 ℃.

2.2.2. Planktonic *S. mutans* test

The single-plate method (Thomas et al. 2015) was modified and used to evaluate whether the CXO nanoemulsion was solubilized and exhibited antimicrobial activity against planktonic *S. mutans* bacteria. Briefly, 20 u l of *S. mutans* adjusted to 10^7 CFUs/ml was inoculated to 180 ul of DW as a negative control, Cool Mint Listerine (Johnson & Johnson, USA) as a positive control, and CXO emulsion and nanoemulsion as experimental groups. Bacterial solutions were treated with the treatment solutions for 1 min, then serially diluted to 10^{-5}, and 20 ul of each dilution solution was inoculated on BHI agar plates. The numbers of CFUs were calculated after the plates were incubated for 48 h at 37℃ in an atmosphere of 10% CO_2. These procedures were repeated three times.

2.2.3. *S. mutans* biofilm test

Nanoemulsion that was sonicated for 10 min was used as the treatment solution to evaluate antibiofilm activity because it had the smallest droplet size and PDI and had good stability. Unsonicated emulsion was used to compare the antibiofilm effects of the emulsion and nanoemulsion. DW and Listerine were used as the negative and positive controls, respectively. The biofilm model of Guggenheim et al. (Guggenheim et al. 2004) was modified to form the *S. mutans* biofilm (Fig. 1). Sterile hydroxyapatite (HA) disks were immersed in 1.5 ml of artificial saliva for 4 h in a 24-well cell culture plate to induce the formation of the acquired pellicle. The artificial saliva contained 2.2 g/l of gastric mucin, 0.381 g/l of NaCl, 0.213 g/l of $CaCl_2 \cdot 2H_2O$, 0.738 g/l of KH_2PO_4, 1.11 g/l of KCl (pH 6.8). HA disks coated with acquired pellicle were then deposited in a 1.6 ml mixture of 800 μl of artificial saliva and growth medium and inoculated with 200 μl of *S. mutans* adjusted to 10^7 CFUs/ml. The plate containing the disks was incubated in growth medium containing 36.4 g/l of Todd Hewitt broth, 8 g/l of yeast extract, 15 g/l of sucrose at 37℃ in an atmosphere of 10% CO_2.

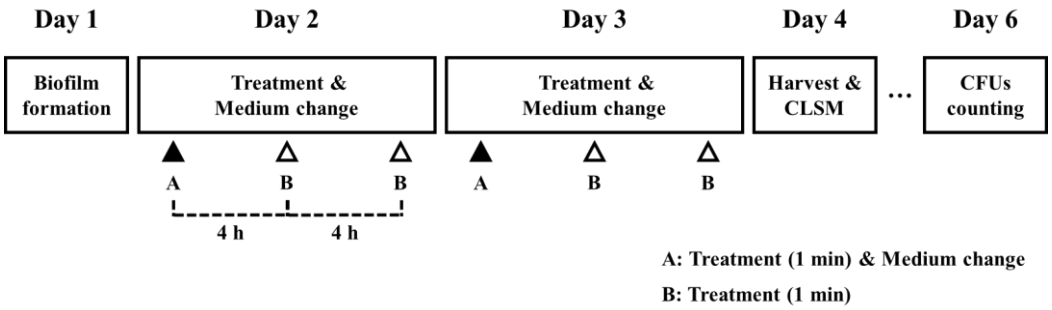

Figure 1. Flow diagram of the procedures testing the solutions' effectiveness against *S. mutans* biofilm.

After 16 h of inoculation, biofilms formed on the surfaces of the HA disks and were treated with 1.5 ml of the treatment solutions for 1 min and then washed three times with 1.5 ml of DW to remove excess treatment solution. To better simulate the oral cavity, treatment was performed three times per day for 1 min at 4 h intervals for 2 days. The treatment was repeated at 16, 20, 24, 40, 44, and 48 h after inoculation and cultured for a total of 64 h. At 16 and 40 h after inoculation, the saliva-medium mixture was refreshed.

To measure the cell viability of *S. mutans* within the biofilm formed on the surface of the HA disks, the disks were washed with DW to remove loosely attached bacteria. The disks were then placed in a tube containing 1 ml of DW and sonicated to induce bacteria within the biofilm to float from the surface of the biofilm. The suspended bacteria were diluted in multiple stages and inoculated in BHI agar. CFUs were counted after incubation for 48 h at 37℃ in an atmosphere of 10% CO_2. This method was repeated 10 times.

Confocal laser scanning microscopy (CLSM) analysis was performed to structurally and quantitatively analyze the biofilm. The *S. mutans* biofilm incubated for 64 h was stained using a LIVE/DEAD BacLight Bacterial Viability Kit (Molecular Probes, Eugene, USA), following the manufacturer's instructions. The kit consisted of the nucleic acid-based fluorescent dyes SYTO 9 and propidium iodide. SYTO 9 penetrates all bacterial membranes and stains the cells green while propidium iodide only penetrates cells with damaged membranes and stains the cells red, with the combination of the two causing red fluoresce in the cells. To rule out selection bias, the region of interest on the biofilm was randomly selected by a third party unrelated to this study. Using an LSM 700 camera (Carl

Zeiss, Jena, Germany), z-axis stacked images were obtained with Zen software (Carl Zeiss). These images were analyzed via the COMSTAT plug-in (Lyngby, Denmark) in ImageJ with 64-bit Java 1.8.0_112 image analysis software (National Institute of Health, USA) software to quantify the live and dead cell biomasses and biofilm thickness.

2.3. Antimicrobial activity of *C. xanthorrhiza* oil nanoemulsion on microcosm biofilm (Study III)

2.3.1. Formation of dental microcosm biofilm

Ethical approval for collecting human saliva was granted by the Ethics Committee of Yonsei Dental Hospital, South Korea (Institutional Review Board No. 02-2021-0095). Saliva samples were collected from a healthy adult donor who had not performed any oral hygiene activities within the preceding 24 h, had not taken antibiotics within the last three months, or engaged in any cariogenic activities or exhibited signs of periodontal disease within the last three months. The collected saliva samples were filtered through sterilized glass wool (Duksan Chemicals, Ansan, South Korea) and then diluted in sterile glycerol (Duksan Pure Chemicals Co., Ltd., Ansan, South Korea) to prepare stock solutions with a final concentration of 30%. The stock solutions were stored in a deep freezer at -80 ℃ and the same batch of frozen saliva was used as an inoculum for each set of experiments. Microcosm biofilms were formed on HA disks. Each disk in a 24-well cell culture plate was inoculated with 1 ml stock saliva and incubated at 37 ℃ and an atmosphere of 10% CO_2 for 4 h to induce the bacterial acquire pellicle. The pellicle formed on the disk was photographed using a quantitative light-induced fluorescence-digital (QLF) Biluminator camera (Inspektor Research Systems BV, Amsterdam, Netherlands) to be used as a baseline image. QLF cameras are digital SLR cameras with QLF technology and can produce both white light and fluorescence images. The QLF white light images were captured with a 1/60 s shutter speed, 7.1 aperture value, and 1000 ISO speed while fluorescence images

were captured with a 1/60 s shutter speed, 7.1 aperture value, and 1600 ISO speed while ensuring that the distance between light sources and biofilms was consistent. C3 v. 1.16 (Inspektor Research Systems BV) automatically captured and stored all digital images on a PC. After images were captured of samples, the samples were immersed into new wells that included growth medium (Wong and Sissions 2001) that contained 0.2% sucrose and basal medium mucin growth medium and incubated in anaerobic conditions in an atmosphere of 80% N_2, 10% CO_2, and 10% H_2 for up to 4 days (Fig. 2).

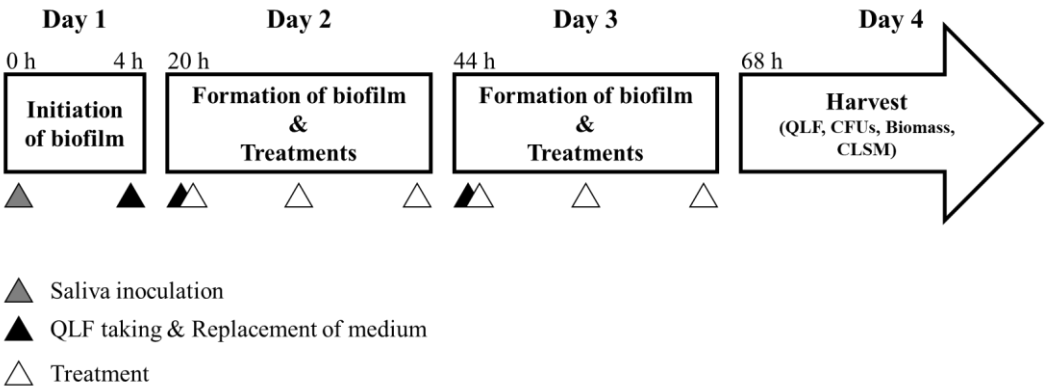

Figure 2. Flow diagram for testing the antimicrobial effectiveness of the solutions when treating dental microcosm biofilm.

2.3.2. Treatments

The treatment solutions were CXO emulsion and nanoemulsion as the experimental treatments, 0.1% chlorhexidine digluconate (CHX) and Listerine as the positive controls groups, and DW as the negative control. The biofilms were first treated 20 h after saliva inoculation. Treatments were performed 3 times per day for 1 min at 4 h intervals for 2 days. Then 2 ml of each solution was put into a new 24-well cell culture plate and HA disks were immersed in each solution for 1 min, simulating the use of mouthwash. After each treatment, the disks were rinsed for 1 min with cysteine peptone water (CPW) (Deng, Buijs, and Ten Cate 2004) to remove excess treatment solution before being returned to the growth medium.

2.3.3. Red fluorescence of dental microcosm biofilm

A QLF was used to observe red fluorescence of microcosm biofilm formed on the HA disk and to capture microcosm biofilm images daily on days 1–4. To compare the red fluorescence intensities of the images, regions of interest were identified in the images. The red value of every pixel within that region was obtained using ImageJ with 64-bit Java 1.8.0_112 image analysis software (National Institute of Health, USA). A total of 10 samples were analyzed for each group. All analyzes were performed by the same examiner.

2.3.4. Cell viability in biofilms

The number of CFUs of total and aciduric bacteria were measured to analyze their viability in the biofilm after treatment with the antibacterial solutions. After biofilm had been allowed to form for 4 days, the HA disks were washed in 2 ml of CPW to remove loosely attached bacteria and transferred to new tubes containing 2 ml of CPW. In order to detach and disperse the biofilm formed on the HA disks, the disks were placed in ultrasonic baths and vortexed for 1 m. Bacterial suspensions were serially diluted and plated on a 5% tryptic soy blood and BHI agar plates adjusted to pH 4.8. The agar plates were incubated for 72 h at 37℃ under anaerobic conditions. Total and aciduric bacteria viabilities were evaluated by measuring the average CFU value on the tryptic soy blood agar plate and a pH 4.8 BHI agar plate for 10 samples for each group.

2.3.5. Dry-weight analysis

Biofilm biomass was analyzed using dry weight analysis as performed by Lemos et al. (Lemos et al. 2010) with some modifications. In brief, biofilm was allowed to mature for 4 days on HA disks, washed in 2 ml CPW, and transferred to a sterile tube containing 2 ml CPW. The tube was placed in an ultrasonic bath for 1 minute to detach the biofilm formed on the disks. The disks were removed from the tubes and the tubes were centrifuged at 5,500 g at 4℃ for 10 min. The supernatant was carefully removed, 2 ml of DW was added, and resuspension was performed by vortexing. The supernatant removal process after centrifugation was repeated. The formed pellets were compared by measuring their 1 ml

dry weight after resuspension in 2 ml DW. A total of 10 samples from each group were analyzed.

2.3.6. CLSM analysis

CLSM analysis was performed to evaluate the inhibitory effect of the treatment substance on bacterial cells and bacterial extracellular polysaccharide (EPS) within the biofilm. In order to fluorescently stain the EPS produced during biofilm formation (Koo et al. 2010), 647/668 nm Alexa Fluor 647-dextran conjugate (Molecular Probes) was included in basal medium mucin growth medium at a concentration of 1 μM. After antibacterial treatment and biofilm formation for 4 days, fluorescent staining of bacterial cells was performed using SYTO9 for 15 minutes. Confocal images were acquired using an LSM700 camera (Carl Zeiss, Jena, Germany) and three sites were randomly taken from each sample. Images divided by the z-axis were obtained using Zen software (Carl Zeiss) that were used to quantify the mass, thickness, and EPS of the biofilms using the COMSTAT plugin (Lyngby, Denmark) of the ImageJ software (Heydorn et al. 2000). A total of three samples from each group were used for CLSM analysis.

2.4. Statistical analysis

All statistical analyses were performed using the PASW Statistics package v. 25.0 (SPSS, IBM Corporation, USA). None of the data followed a normal distribution, so Kruskal-Wallis analyses were performed to analyze whether there were significant differences in the nanoemulsions' particle sizes and PDI and OD values according to ultrasonic processing time and whether the treatment solutions had significantly different antibacterial effects. Significant differences between groups were analyzed *post hoc* using the Mann-Whitney U test. Statistical significance was set as α = (0.05 / number of comparison groups).

III. Results

3.1. Characterization of nanoemulsions (Study I)

3.1.1. Transparency

Nanoemulsions were more transparent than emulsions (Fig. 3). Visual transparency evaluation showed that the ultrasonic treatment time written on the back of the container was clearly observed through the nanoemulsions but no number could be observed through the emulsions.

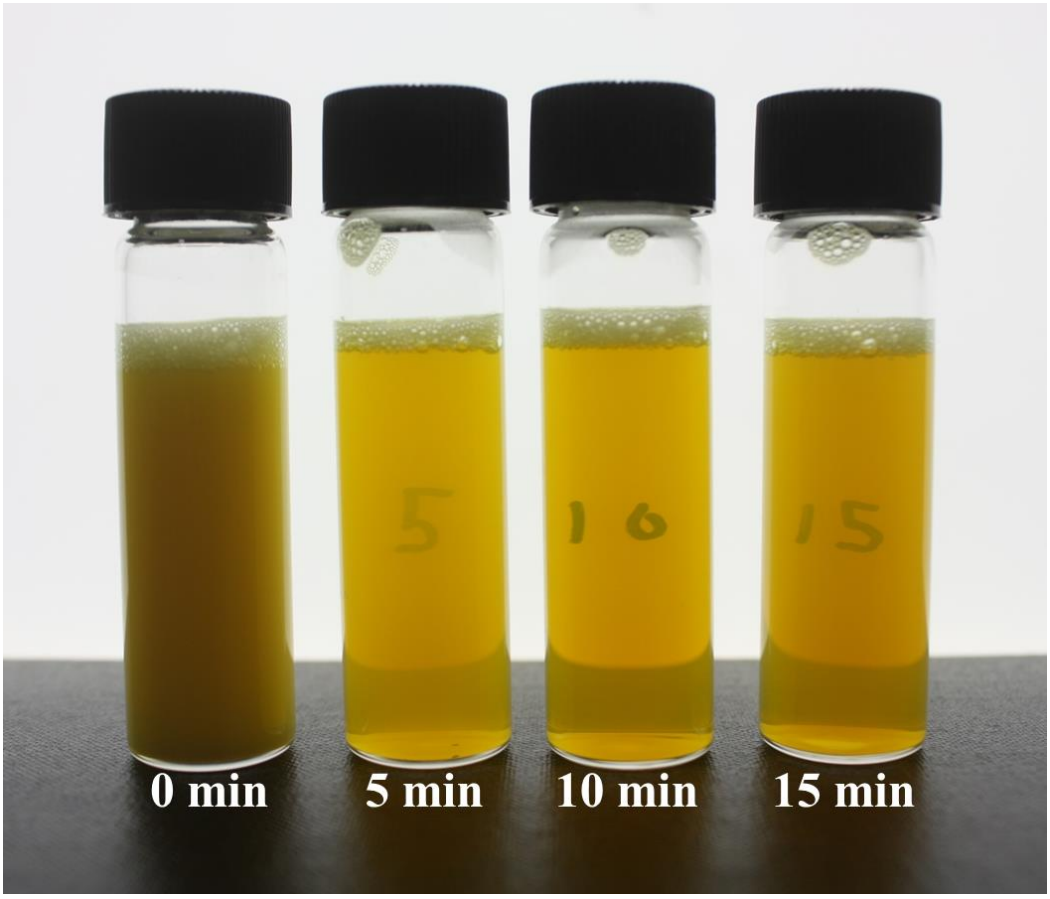

Figure 3. Representative image of *C. xanthorrhiza* oil emulsion and nanoemulsions. The time values represent sonication duration.

3.1.2. Optical density (OD)

Quantitative evaluation of the turbidity of the solutions by absorbance showed that the nanoemulsion sonicated for 10 min had the lowest turbidity, which was 86% lower than that of the emulsion (Fig. 4). The absorbance of the nanoemulsions sonicated for 5 and 15 min were 72% and 80% lower than the emulsion, respectively.

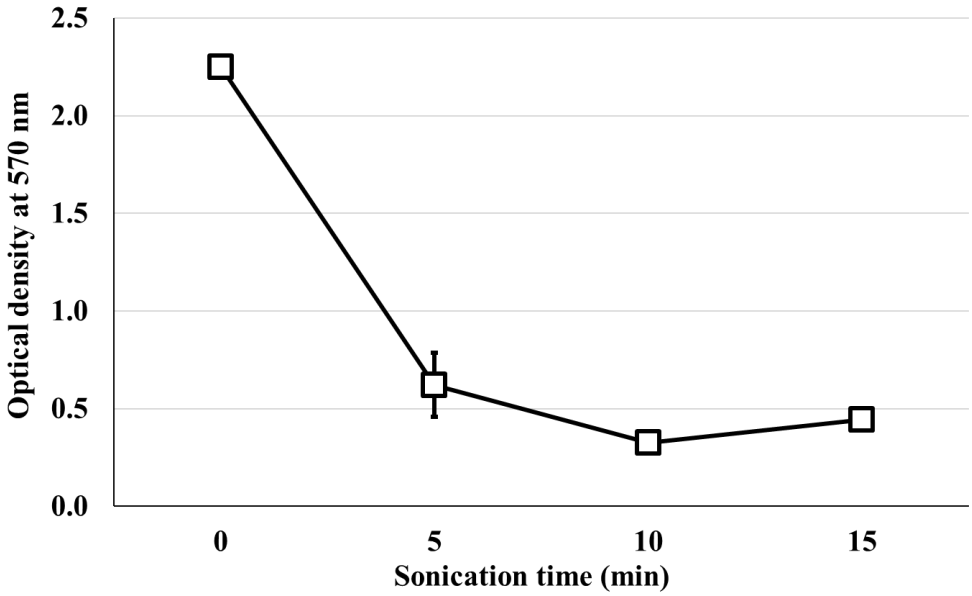

Figure 4. Mean absorbance characteristics of CXO emulsions according to sonication duration. Error bars indicate standard deviations (n = 5).

3.1.3. Droplet diameter and polydispersity

Average particle size analysis (Table 2) showed that the nanoemulsion sonicated for 10 min exhibited was had a 75% smaller average particle size than the emulsion ($p = 0.008$). The nanoemulsions sonicated for 5 and 15 min also exhibited statistically significant differences from the emulsion ($p = 0.008$ and $p = 0.008$, respectively). There was no significant difference in average particle size between the nanoemulsions by sonication duration.

The PDI test, which evaluated particle polydispersity, showed that the nanoemulsion sonicated for 10 min had the lowest PDI value that was significantly different from those of the emulsion and nanoemulsion sonicated for 5 min ($p = 0.008$ and $p = 0.008$, respectively). The PDI value of the nanoemulsion sonicated for 5 min was not significantly different from that of the emulsion ($p = 0.222$).

Table 2. Characterization of CXO emulsions by sonication times.

Sonication time (min)	Droplet diameter (nm)	Polydispersity (PDI)
0	236.9 (9.1)[a]	0.36 (0.06)[a]
5	69.0 (1.7)[b]	0.27 (0.01)[a]
10	58.3 (3.1)[b]	0.16 (0.02)[b]
15	62.1 (2.0)[b]	0.17 (0.02)[b]

All values are expressed as mean (S.E.) (n = 3). Different letters indicate statistically significant differences between the groups (Kruskal-Wallis test with *post hoc* Mann-Whitney U tests).

3.1.4. pH value

The pH values of the nanoemulsions and the emulsion were 4.94–5.00. The nanoemulsions did not have significantly different pH values (Table 3).

Table 3. pH values of CXO emulsions by sonication times.

Sonication time (min)	**pH**
0	4.94 (0.02)
5	5.00 (0.03)
10	4.98 (0.01)
15	4.95 (0.02)

All values are expressed as mean (S.E.) (n = 3).

3.1.5. Stability

None of the nanoemulsions' stabilities changed after centrifugation, but precipitate was observed in the emulsion (Fig. 5). After one month of storage at room temperature, all nanoemulsions remained stable while the emulsion exhibited layer separation after 24 h (Fig. 6).

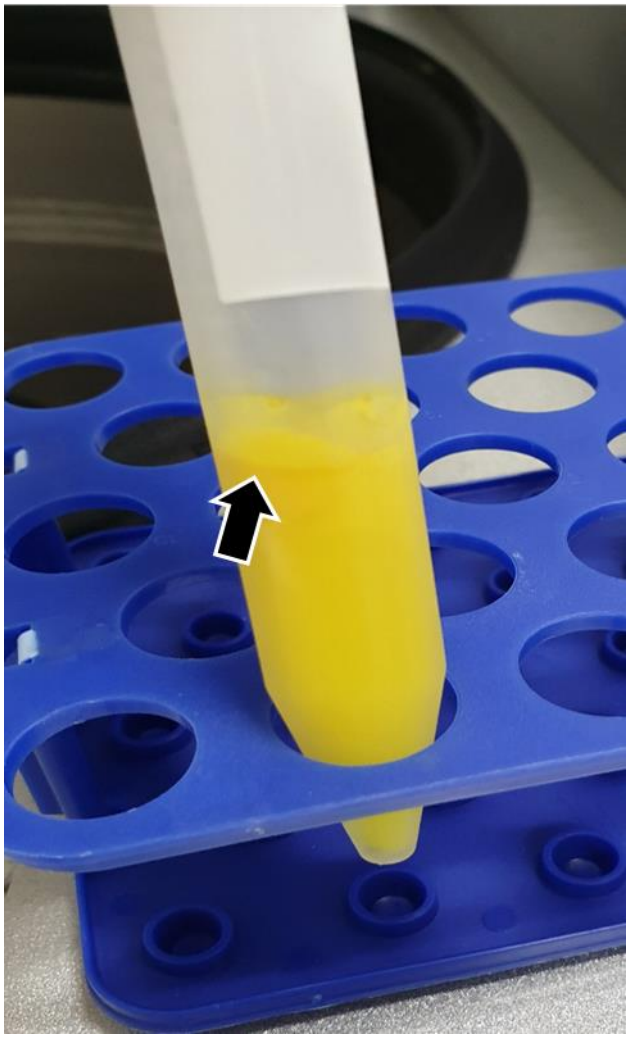

Figure 5. Precipitate (black arrow) formed in the emulsion after centrifuging.

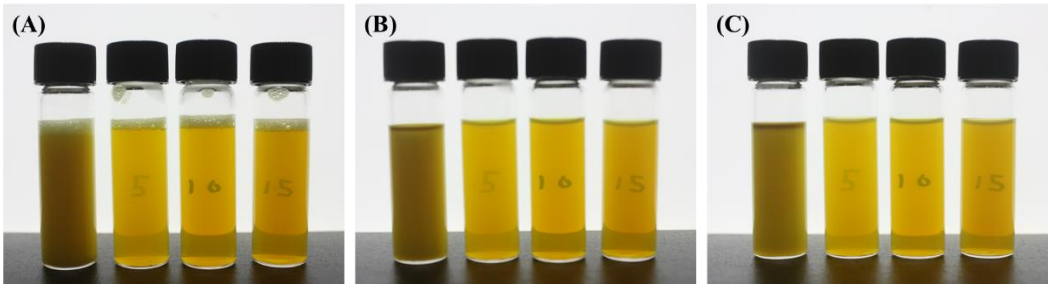

Figure 6. Images of the emulsion and nanoemulsions (A) just after, (B) 1 day after, and (C) 30 days after preparation.

3.1.6. HPLC analysis

HPLC analysis identified CXO's active ingredients (Fig. 7). Both 224 nm and 280 nm analyses showed the highest peak point at about 12 min and additional peak points at other time points. HPLC analysis of xanthorrhizol (Fig. 8) showed that its highest peak point was at 12 min, which was the same as CXO. These results indicate that xanthorrhizol was present in a high ratio in CXO. Measurement of the area of the xanthorrhizol peak point relative to the total area under the CXO curve indicated that xanthorrhizol accounted for approximately 47% of CXO.

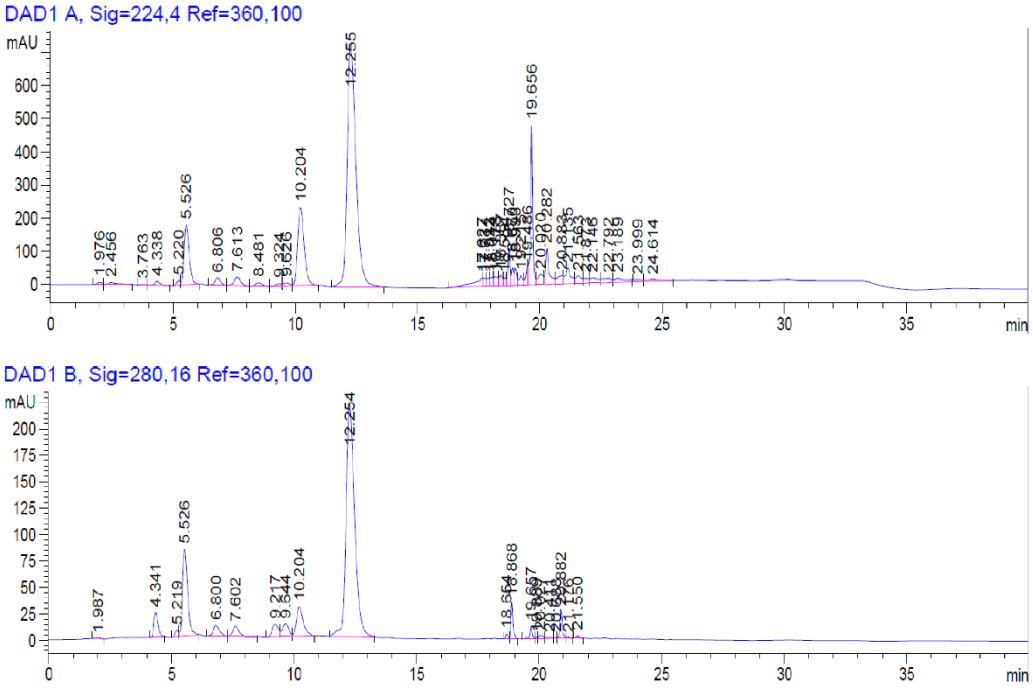

Figure 7. HPLC results for CXO. The results for detection at (upper) 224 nm and (lower) 280 nm.

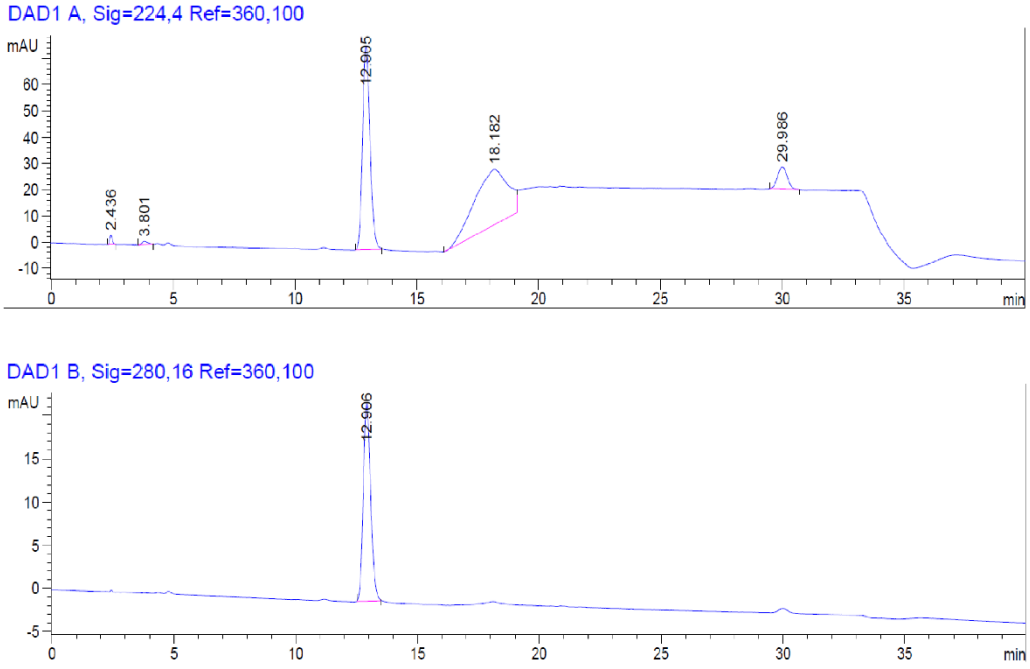

Figure 8. HPLC results for xanthorrhizol. The results for detection at (upper) 224 nm and (lower) 280 nm.

3.2. Antibacterial activity of *C. xanthorrhiza* oil nanoemulsions on *S. mutans* (Study II)

3.2.1. Planktonic test

DW as the negative control exhibited a value of 5.79 ± 0.16 log (CFUs/ml), but Listerine and the CXO emulsion and nanoemulsions completely killed planktonic *S. mutans* bacteria (Fig. 9).

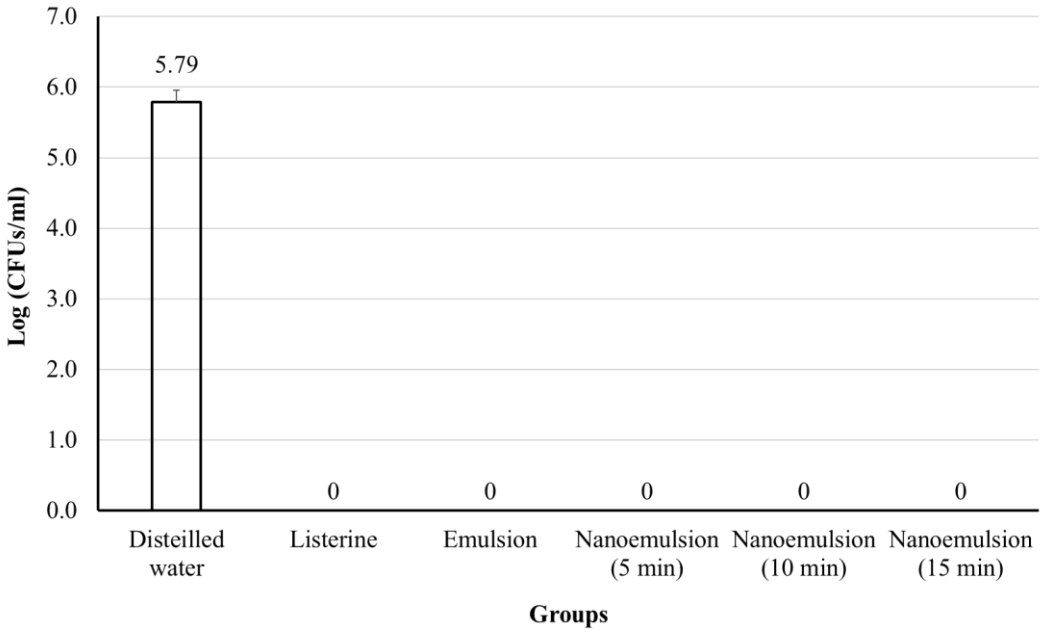

Figure 9. Antibacterial effect of DW, Listerine, emulsion, and nanoemulsions sonicated for 5, 10, and 15 min on *S. mutans* planktonic cells.

3.2.2. Biofilm test

3.2.2.1. Cell viability analysis

In the antibacterial test conducted on *S. mutans* biofilm, cell viability in the biofilm treated with the nanoemulsion sonicated for 10 min had the lowest value of log 5.72 CFU/ml, which was statistically significantly different from DW's value of log 7.83 ($p < 0.001$ and Listerine's value of log 7.52 ($p < 0.001$) but not statistically significantly different from the emulsion's value of log 5.73 CFU/ml ($p = 0.74$) (Fig. 3). The interquartile range (IQR) of the log values was then calculated to determine whether the solutions had a uniform antimicrobial effect. The IQR of the emulsion was the highest at 1.22, followed by the nanoemulsion at 0.84, Listerine at 0.41, and DW at 0.35. The emulsion's IQR was 1.5 times that of the nanoemulsion and 3.4 times that of DW.

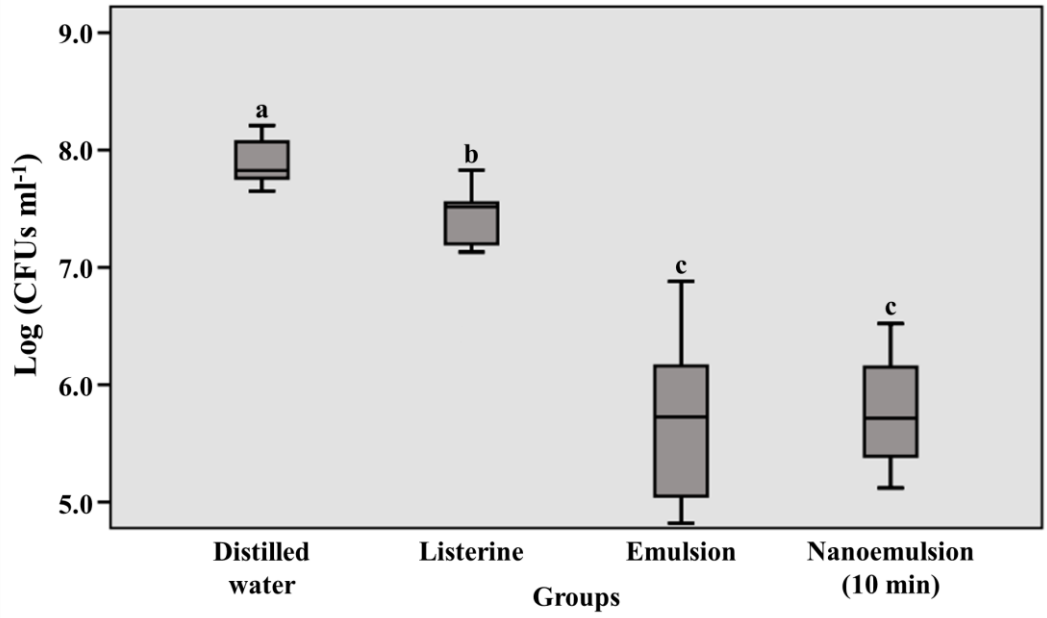

Figure 10. Effects on the viability of *S. mutans* cells within biofilms. The boxes show the first quartile (lower line), median (middle line), and third quartile (upper line). Letters above the box whisker plots indicate statistically significant differences between the groups (Kruskal-Wallis tests with *post hoc* Mann-Whitney U tests, $p < 0.001$, $n = 10$).

3.2.2.2. CLSM live and dead cell analysis

CLSM images of biofilm treated with DW showed that there were more live cells than dead cells whereas those of biofilm treated with Listerine showed similar numbers of live and dead cells (Fig. 11). In the biofilms treated with the emulsion and nanoemulsion, dead cells were more abundant than live cells. The biofilm treated with the nanoemulsion had a relatively small amount of total biomass (Fig. 12). In addition, CLSM images showed that the live-dead cell ratio in the biomass treated with nanoemulsion was 50% less than that in the biomass treated with DW but only 20% less than that of the biomass treated with Listerine (Table 4). The biofilm treated with DW was thickest, followed in order of decreasing thickness by Listerine, the emulsion, and the nanoemulsion.

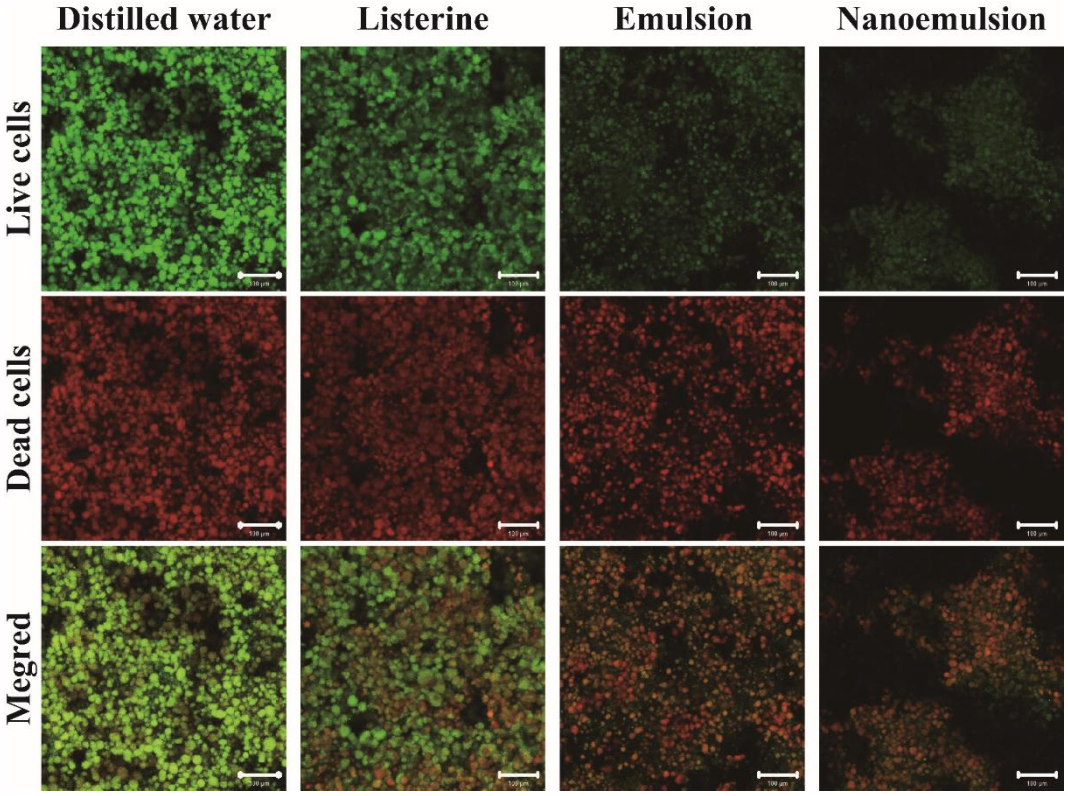

Figure 11. Top view of CLSM images of live and dead cells in *S. mutans* biofilms.

Scale bars correspond to 100 μm.

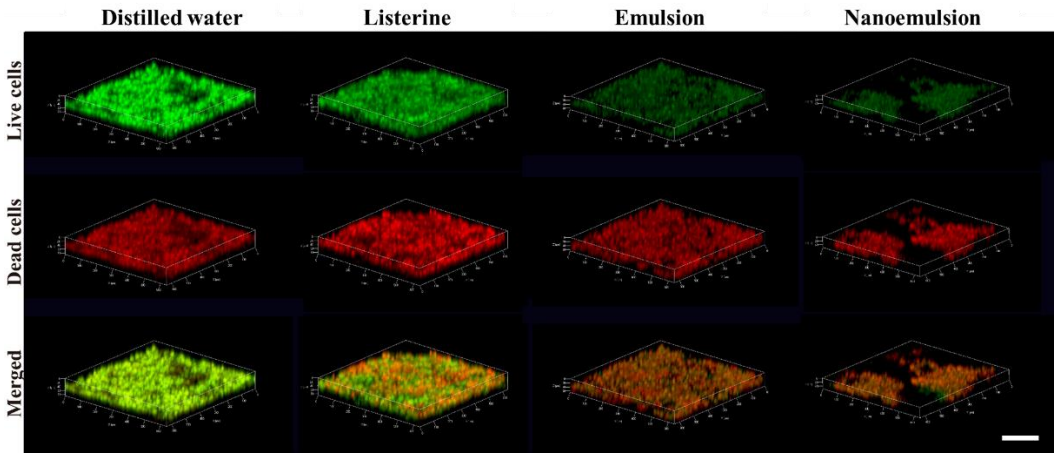

Figure 12. 3D structures of merged z-stacks in CLSM images of live and dead cells within *S. mutans* biofilms. The scale bar corresponds to 200 μm.

Table 4. Results of quantitative analysis of biomass within *S. mutans* biofilm by treatment solution.

	Biomass ($\mu m^3/\mu m^2$)			**Thickness (μm)**		
	Live cells	**Dead cells**	**Live/ Dead**	**Live cells**	**Dead cells**	**Mean**
Distilled water	62.5 (17.5)	51.2 (14,8)	1.2	76.6 (9.6)	74.0 (8.6)	75.3 (9.1)
Listerine	35.2 (3.7)	37.5 (5.0)	1.0	62.7 (4.7)	62.9 (0.8)	62.8 (2.6)
Emulsion	19.5 (5.6)	32.0 (7.9)	0.6	44.8 (0.9)	54.4 (1.3)	49.6 (1.1)
Nanoemulsion	12.4 (6.2)	19.1 (8.0)	0.6	35.1 (6.4)	42.2 (6.8)	38.6 (6.6)

Data are presented as the mean (S.D.).

3.3. Antimicrobial activity of *C. xanthorrhiza* oil nanoemulsion on dental microcosm biofilm (Study III)

3.3.1. Red fluorescence

The red fluorescence of the microcosm biofilms formed on the HA disk for 4 days and then treated with the tested solutions was observed using QLF fluorescence imaging (Fig. 13). The red fluorescence of the nanoemulsion-treated biofilm was less than that of the DW-treated biofilm. The emulsion-biofilm had weaker red fluorescence than the DW-treated biofilm but stronger red fluorescence than that of the Listerine and the nanoemulsion. The CHX-treated biofilm, which was one of the positive controls, had green fluorescence instead of red fluorescence.

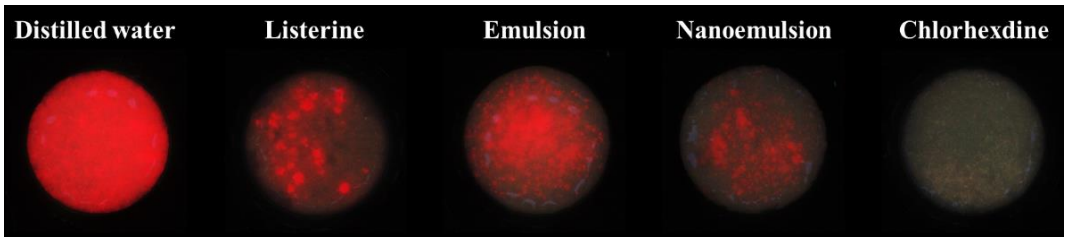

Figure 13. Red fluorescence images of microcosm biofilms that formed on HA disks for 4 days by treatment solution.

3.3.2. Red fluorescence by maturation time

To observe how microcosm biofilm color changed by maturation period, white light QLF images were analyzed (Fig. 14). The biofilms were brown and the color was the most on day 4 of biofilm formation. The change in the fluorescence color of the biofilms by maturation period was also analyzed (Fig. 15). On day 1, no fluorescence was observed in any group. On day 2, all of the groups' biofilms had green fluorescence. On day 3, red fluorescence began to be observed in all groups, but the strongest and most widely distributed fluorescence was observed in the DW group. On day 4, the strongest red fluorescence was still observed in the DW group, followed in order of decreasing magnitude by the emulsion group; the Listerine and nanoemulsion groups, which had similar levels of red fluorescence; and the CHX group.

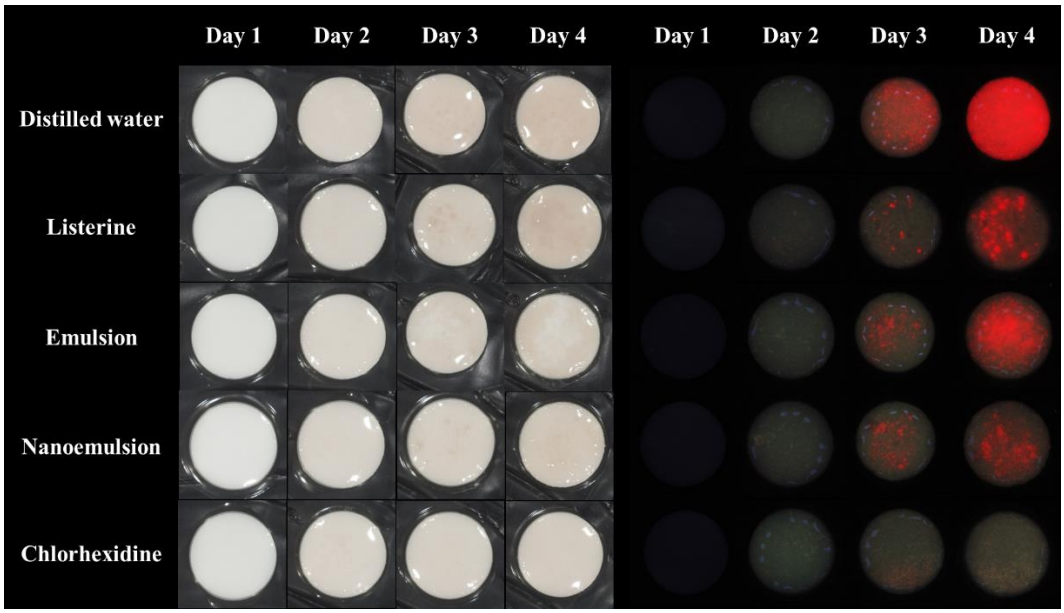

Figure 14. Quantitative light-induced fluorescence images of microcosm biofilms that formed on HA disks over the course of 4 days.

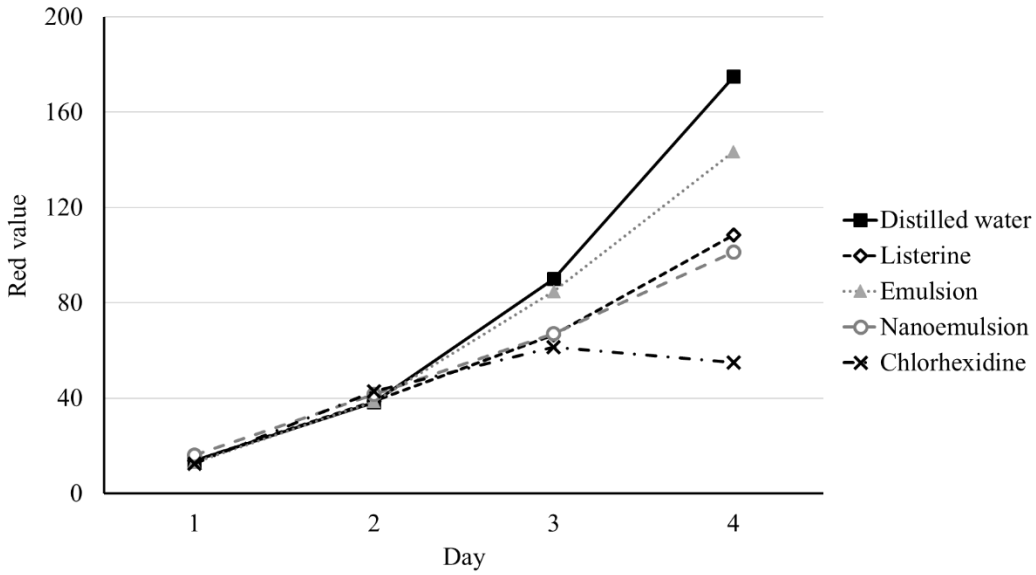

Figure 15. Changes in red fluorescence of microcosm biofilms treated with treatment solutions over time.

3.3.3. Red value and cell viability

The red fluorescence intensity of the microcosm biofilm after 4 days and the viabilities of total and aciduric bacteria were compared analyzed (Table 5). The red fluorescence intensity of the nanoemulsion-treated biofilm was significantly lower than that of the DW-treated biofilm ($p < 0.001$). The red fluorescence intensity of the emulsion- and DW-treated biofilms were not significantly different ($p = 0.17$). The Listerine and CHX groups, which were the positive control groups, had significantly lower fluorescence values than DW group ($p < 0.001$ and $p < 0.001$, respectively). The CHX group had the lowest fluorescence intensity.

In the cell viability test for total bacteria, the bacteria had significantly lower viability values when treated with the nanoemulsion than when treated with DW or Listerine ($p < 0.001$ and $p < 0.001$, respectively). The CHX group had the lowest total bacteria viability. Aciduric bacteria viability was only lower in the nanoemulsion group than in the DW group ($p = 0.01$). It was also significantly lower in the nanoemulsion group than in the Listerine group, which was a positive control ($p = 0.01$). All aciduric bacteria in the CHX group were killed, so it was not included in the statistical analysis.

Table 5. Mean (S.D.) red fluorescence values and cell viabilities for microcosm biofilms after 4 days of maturation and treatment.

Groups	Red value*	Total bacteria (log CFUs/ml)	Aciduric bacteria (log CFUs/ml)
Distilled water	175.0 (27.1)[a]	8.32 (0.17)[a]	7.58 (0.24)[a]
Listerine	108.6 (25.4)[b]	8.06 (0.09)[b]	7.49 (0.16)[a]
Emulsion	143.4 (51.1)[a, b]	7.93 (0.27)[b, c]	7.44 (0.13)[a, b]
Nanoemulsion	101.2 (24.8)[b]	7.71 (0.23)[c]	7.28 (0.19)[b]
Chlorhexidine	55.0 (12.8)[c]	6.00 (2.05)[d]	0.00 (0.00)

Different letters within the same column indicate significant differences between groups (Kruskal-Wallis test with *post hoc* Mann-Whitney U tests, $n = 10$).
CFUs mean colony-forming units.
* Red value indicates intensity of red fluorescence from microcosm biofilm on day 4.

3.3.4. Biomass

The antibiofilm effect of the nanoemulsion was evaluated using the dry weight of the biofilm after 4 days of maturation and treatment (Fig. 16). The biomass of the biofilm treated with the nanoemulsion was significantly lower than those of the biofilms treated with DW, the negative control, and Listerine, the positive control ($p < 0.001$ and $p < 0.001$, respectively). However, the emulsion and Listerine groups' biomasses were not significantly different from the DW group's biomass ($p = 0.28$ and $p = 0.12$, respectively). The CHX group had the lowest biomass and was significantly different from that of the nanoemulsion's biomass ($p = 0.003$).

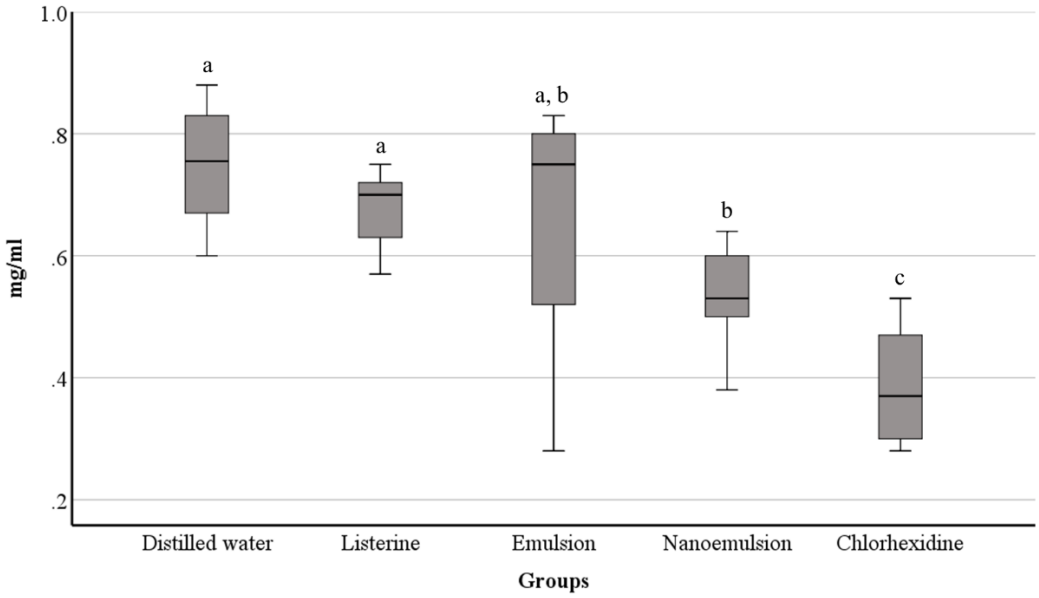

Figure 16. Biomass determined by dry weight. Different letters above the error bars indicate statistically significant differences between the groups as determined by Kruskal-Wallis tests with *post hoc* Mann-Whitney U tests ($p < 0.01$) ($n = 10$).

3.3.5. Cell and EPS CLSM analysis

CLSM analysis was performed to evaluate the inhibitory effect of the nanoemulsion on the bacterial cell and EPS in microcosm biofilm (Fig. 17). Bacterial cell and EPS formation for all groups were lower than for those of the DW group. The bacterial cell and EPS distributions were dramatically lower in the nanoemulsion and CHX groups than in the other groups.

Biofilm biomass and thickness were quantified based on CLSM images (Table 6). The nanoemulsion-treated biofilm had lower biomass and thickness than DW, Listerine, and the emulsion groups. Listerine had lower biomass and thickness than the DW group but higher values than the nanoemulsion group. The emulsion group had higher S.D. and EPS values than the DW group.

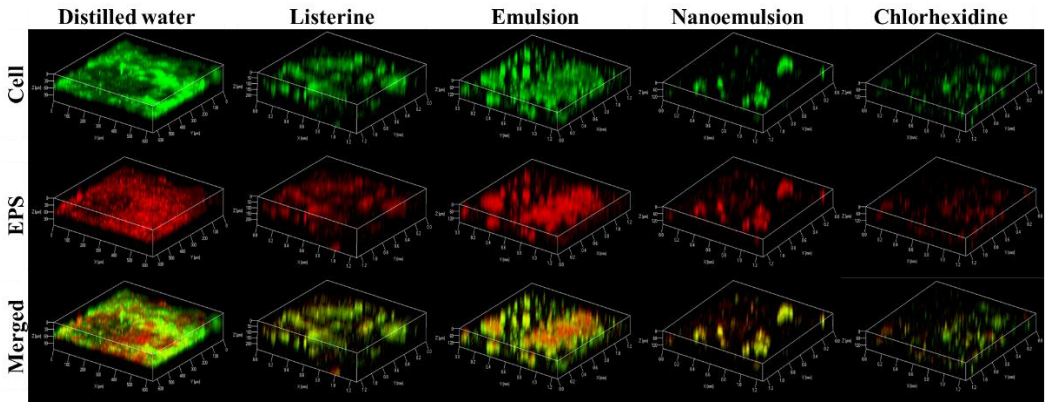

Figure 17. CLSM images of bacterial cells and EPS in dental microcosm biofilms treated with antimicrobial agents.

Table 6. Biomasses and thicknesses of dental microcosm biofilm treated with treatment solutions as determined by COMSTAT analysis.

	Biomass ($\mu m^3/\mu m^2$)		**Thickness (μm)**	
	Bacterial cells	**EPS**	**Bacterial cells**	**EPS**
Distilled water	55.3 (27.4)	12.3 (4.3)	72.5 (38.0)	23.2 (10.2)
Listerine	42.4 (19.0)	12.0 (4.1)	60.0 (34.4)	16.3 (7.9)
Emulsion	35.9 (15.8)	12.5 (8.7)	63.6 (43.4)	23.7 (21.1)
Nanoemulsion	7.7 (1.7)	9.2 (2.1)	8.8 (2.1)	14.1 (5.0)
Chlorhexidine	1.1 (0.9)	6.6 (3.7)	1.3 (1.0)	12.2 (4.5)

Data are presented as the mean (S.D.).

IV. Discussion

This study's results showed that the optimal CXO nanoemulsion can be nanoemulsified by 10 minutes of ultrasonic treatment. Sonciation duration significantly affected particle size and polydispersity. CXO nanoemulsions were more stable than the CXO emulsion. Then it was evaluated whether the CXO nanoemulsion exhibited antibacterial and anti-biofilm effects on oral bacteria and the results showed that it had significantly stronger antibacterial and anti-biofilm effects on planktonic *S. mutans*, *S. mutans* biofilm, and microcosm biofilm than DW, the negative control. In addition, CXO was found to contain a significant amount of xanthorrhizol, indicating that xanthorrhizol is CXO's effective antibacterial ingredient. This study was the first study to show that CXO can be nanoemulsified and it is meaningful in that it evaluated CXO nanoemulsion's antimicrobial effects in various ways.

In the first study, the physicochemical characteristics of CXO nanoemulsions, namely their turbidity, droplet size, PDI, pH, and stability, were analyzed by sonication times. A previous study on regarding the emulsification of eucalyptus oil via sonication reported that emulsion turbidity, droplet size, and PDI were negatively correlated with sonication time (Sugumar et al. 2014), which indicates that the force of ultrasonic emulsification reduced the size of the oil droplets. PDI is a measure of the stability and homogeneity of droplet sizes in an emulsion, so smaller values result in a smaller range of droplet sizes (Sun et al. 2012). However, in this study, turbidity, droplet size, and PDI were higher after

sonication for 15 min than for 10 min. This result may have been a product of the fact that droplet size increased due to the fact that acoustic pressure changed with sonication time (Modarres-Gheisari et al. 2019). A previous study also reported that sonication time must be set according to treatment conditions and materials because droplets can coalesce over time (Consoli et al. 2017). In this study, sonication for 10 min was determined to be optimal for producing CXO nanoemulsion and so was used for the antimicrobial experiments. There were no significant differences in nanoemulsion pH. They did not significantly vary by sonication duration with a range of 4.92–5.0, but they did have higher pH than that of Listerine at 4.0 (Soares et al. 2012). Similarly, a previous study also showed eucalyptus oil nanoemulsion pH did not vary by ultrasonic process (Sugumar et al. 2014).

Physical stability is also an important characteristic for mouthwashes. Centrifugation was employed to assess stability (Kandadi et al. 2011). Creaming was observed in the emulsion immediately after centrifugation, but the sonicated nanoemulsions remained stable, indicating that sonicated nanoemulsions are more suitable for use as antimicrobial agents than un-sonicated emulsions. In the static stability test, the emulsion and nanoemulsions were stored at room temperature for one month after preparation. The emulsion showed separation after 24 h, but the nanoemulsions remained stable and did not show any layer separation for one month as a result of their surfactant and emulsifier content. In the emulsion, the creaming phenomenon occurred due to particle coalescence, ultimately resulting in phase separation (Gupta et al. 2016a). These results indicate that the large oil droplets formed in emulsions are highly hydrophobic and unstable under high

stress or long-term storage. However, the smaller oil droplets in the nanoemulsions were stable for a long period of time because their diffusion rate was higher than gravity settling or creaming due to Brownian motion (Solans et al. 2005). Thus, the nanoemulsions would remain stable for a long period of time.

HPLC analysis showed that xanthorrhizol was the most common CXO component. The xanthorrhizol content varies by extraction method, but generally *C. xanthorrhiza* extract contains the highest concentration of xanthorrhizol. A previous study showed that CXO extracted through hydrostillation contains 46.3% xanthorrhizol (Cheah et al. 2009), which was similar to this study's result of 47%. CXO was also shown to contain several other substances, among which curcumin was the second-most abundant active substance, accounting for 1–2% of the CXO's content (Ruslay et al. 2007). Curcumin, which belongs to the phenol group, also has strong antibacterial effects against oral bacterial strains and biofilms (Hu, Huang, and Chen 2013). Therefore, the CXO nanoemulsions' antibacterial effects were likely a product of these active ingredients.

CXO has antimicrobial effects (Hentschel, Eglau, and Hahn 1996), largely due to the –OH group and the hydrocarbon chain of xanthorrhizol, which is an active component in CXO (Kim et al. 2008; Lee et al. 2008). A previous study evaluating the antimicrobial effect of xanthorrhizol on planktonic *S. mutans* reported a 5 log reduction after 1 min of exposure to xanthorrhizol at a 5 μg/ml concentration (Hwang et al. 2000). In addition, the nanoemulsions' effects on planktonic *S. mutans* were similar to those of xanthorrhizol, resulting in a 5.8 log reduction. Xanthorrhizol is a pure substance obtained via a complex

purification process, so it is more difficult to manufacture and more expensive than CXO. Therefore, the CXO nanoemulsion tested in this study could be used to cost-effectively produce a strong oral antibacterial agent.

In the evaluation of the *S. mutans* biofilm model, which simulated the use of mouthwash, both the CXO nanoemulsion and emulsion significantly reduced cell viability in the biofilm more than DW and Listerine. A previous study that solubilized *C. xanthorrhiza* extract using DMSO reported a 0.4 log reduction in cell viability in *S. mutans* biofilm after 5 min of exposure (Kim et al. 2008). CXO nanoemulsion exposure produced a much stronger antimicrobial effect with a 2.1 log reduction, indicating that nanoemulsification can achieve a strong antimicrobial effect without purification or the use of organic solvents.

C. xanthorrhiza extract damages the peptidoglycan layer of *S. mutans* bacteria cells within the biofilm (Kim et al. 2008), allowing it to penetrate the biofilm and destroy the bacteria within it. The nanoemulsions and emulsion evaluated in this study also exhibited antibacterial activity against *S. mutans* cells within biofilm. In a previous study, *S. mutans* biofilms were treated with xanthorrhizol at different maturation stages, namely the early stage of biofilm growth after 4 h, the active stage after 12 h, the early maturity stage after 20 h, and the mature stage after 24 h. The results showed that it had antimicrobial effects at all stages (Rukayadi and Hwang 2006b). In this study, the periodic exposure of *S. mutans* biofilm to CXO nanoemulsion and emulsion inhibited its growth and formation. This result indicates that both the nanoemulsion and emulsion were successfully solubilized and that

CXO concentration had a greater influence on their antibacterial effects than particle size. However, the emulsion had a higher IQR of the log value of the average particle size than the nanoemulsion, which indicates that the emulsion would not have as consistent of an antimicrobial effect as the nanoemulsion, making the nanoemulsion more widely applicable.

CLSM analysis showed that the live-to-dead cell ratio of the nanoemulsion group was approximately half that of the DW group, which indicates that the nanoemulsion damaged the bacterial cells within the biofilm more than DW. The thickness of the biofilm treated with the nanoemulsion was 22% lower than that of the biofilm treated with the unsonicated emulsion, indicating that biofilm formation was inhibited by the nanoemulsion. A previous study reported that CHX nanoemulsions damage the cell membranes of *S. mutans* and inhibit biofilm formation more effectively than CHX itself (Li et al. 2015). The effect of eucalyptus globulus oil (EO) nanoemulsion on biofilms of *Candida* spp. as evaluated by atomic force microscopy showed that EO nanoemulsions showed a greater inhibitory effect on biofilm growth than EO itself (Quatrin et al. 2017). The antimicrobial droplets in EO were protected and delivered by nanoencapsulation, which also increased their contact area. Similarly, this study's results suggest that nanoparticles effectively inhibit biomass formation by penetrating biofilm and killing the bacteria within it, thereby inhibiting the production of biofilm matrices, such as extracellular polysaccharides and bacteria metabolites. Biofilm matrices can hinder drug application and increase drug resistance, which in turn increases biofilm resistance to antimicrobial agents above that of planktonic

bacteria (Koo et al. 2017; Takahashi and Nyvad 2011). The results of this study confirmed that the nanoemulsification of CXO facilitates the penetration of antimicrobial agents into biofilm. Thus, CXO nanoemulsions can be utilized as effective antibacterial agents.

In the third study, the antibacterial and anti-biofilm effects of CXO nanoemulsion on polymicrobial biofilm were evaluated through various methods and it was shown to have significant effects. The QLF technique, which is commonly used in microcosm biofilm models, was also used in this study. Plaque, which is a dental biofilm, can be observed through red fluorescence using a QLF camera (Van der Veen et al. 2006). Red fluorescence increases significantly in biofilms that have matured for more than 3 days (Kim et al. 2014). Similarly, in this study, red fluorescence was observed in all groups through QLF imaging on the third day of maturation. There was no difference in red fluorescence between the groups until day 2 and after day 3, the difference between the groups increased significantly. The reason for this result is the fact that the first treatment in this model was performed after QLF imaging on day 2. The red fluorescence of the biofilm treated with the CXO nanoemulsion was lower than that of the negative control group beginning on the third day of maturation and was significantly lower on the fourth day. Red fluorescence intensity increases as the maturity and cariogenicity of the microcosm biofilm increased (Kim et al. 2014; Lee et al. 2013). These results indicate that the CXO nanoemulsion significantly inhibited biofilm maturation and cariogenicity more than DW. A previous study showed that red fluorescence was negatively correlated with CHX concentration (Lee et al. 2018). Similarly, this study showed that CHX had the strongest antibacterial effect, followed by

the CXO nanoemulsion and Listerine.

The CXO nanoemulsion-treated biofilm had significantly lower total and aciduric bacteria viability than DW-treated biofilm and Listerine-treated biofilm. These results were similar to those of study II, which evaluated the antimicrobial effect of the solutions on *S. mutans* biofilm. However, the difference in the log values of the viability values between DW- and the CXO nanoemulsion-treated biofilms was smaller in study III than in study II. This result was likely a product of the fact that polymicrobial biofilms are more resistant to antibacterial agents than single-bacteria biofilms. When *Staphylococcus aureus* form biofilm with *C. albicans,* the biofilm's resistance to vanomycin increases (Harriott and Noverr 2009). Biofilm with both *S. mutans* and *Veillonella parvula* is less sensitive to CHX than when it only contains one bacteria or the other (Kara, Luppens, and ten Cate 2006). Consistent with the results of previous studies, the CXO nanoemulsion did not strongly inhibit CFU growth in study III because the microcosm biofilm had greater antibacterial resistance because it was a polymicrobial biofilm model. However, only the CXO nanoemulsion group had an aciduric bacteria viability value that was significantly different from that of the DW group and the Listerine group, indicating that the CXO nanoemulsion has a stronger antimicrobial effect than commercial mouthwash.

In this study, dry weight and CLSM analyses were performed to evaluate the solutions' anti-biofilm effects quantitatively and qualitatively, respectively. Both analyses' results showed similar trends. Dry weight analysis showed that only the CXO nanoemulsion and CHX groups were statistically significantly different from the DW group. The CLSM

images of the two groups showed that the CXO nanoemulsion and CHX groups had significantly less fluorescence for bacterial cells and EPS than the DW group. Thus, the CXO nanoemulsion exhibited not only a strong antimicrobial effect but also an antibiofilm effect, inhibiting biomass formation. A previous study showed that the combination of ferumoxtol nanoparticles and hydrogen peroxide had a strong antibacterial effect on *S. mutans* biofilm and inhibited EPS formation, while H_2O_2 alone reduced cell viability but did not inhibit EPS formation (Liu et al. 2018). On the other hand, when only H_2O_2 was applied, cell viability was reduced, but EPS formation was not inhibited. Thus, there can be no antibiofilm effect without a sufficiently strong antibacterial effect. In addition, the thickness of the biofilm treated with the CXO nanoemulsion was the second-lowest behind the biofilm treated with CHX, indicating that the CXO nanoemulsion inhibited biofilm formation and had an antimicrobial effect on the microcosm biofilm.

In this study, CHX had the strongest antibacterial effect. Cationic CHX molecules interact with the anionic extracellular matrix to produce antibacterial and anti-biofilm effects, resulting in cell membrane damage and enzyme activity inhibition (Wang, Shen, and Haapasalo 2014). However, frequent use of CHX may cause side effects such as discoloration, burning, and irritation (Gürgan et al. 2006; Zanatta, Antoniazzi, and Rösing 2010). Therefore, a CXO nanoemulsion can be used as an alternative anti-agent and in combination with CHX. A previous study found that a 5% cinnamon essential oil nanoemulsion caused biofilm to have a red fluorescence similar to that of 0.1% CHX (Jeong et al. 2021). The antibacterial effect of CXO is proportional to its xanthorrhizol

concentration (Hwang et al. 2000), so CXO nanoemulsion can have a similar effect as CHX if it has a sufficiently high CXO concentration.

CXO nanoemulsion safety is important to its use in commercialized oral care products. Nano-sized substances are potentially toxic and can be absorbed, distributed, metabolized, and excreted from the body (Hu, Mao, and Gao 2009). However, these substances' toxicities depend on their composition and physicochemical properties, such as their concentration and size distribution (McClements and Rao 2011). In this study, a nanoemulsion was prepared using CXO. *C. xanthorrhiza* is edible and has been used as a traditional folk medicine in Southeast Asia (Devaraj et al. 2010; Ruslay et al. 2007). Its effect is a result of the hydroxyl group in xanthorrhizol, which is the main active ingredient in *C. xanthorrhiza* extract. The hydroxyl radical, generated in the hydroxyl group, is an active oxygen that affects both pathogens and the human body (Lobo et al. 2010). However, radicals are unstable and have a short lifespan because of their non-covalently active electrons (Gligorovski et al. 2015; Prinn et al. 2001). For this reason, CXO's antimicrobial effects are likely not long-lasting. A study tested the safety of *C. xanthorrhiza* extract by administering 5,000 mg/kg of it to mice for 14 days and found no effect on toxicity, mortality, or behavior (Devaraj et al. 2010). Clinical trials have tested the gingivitis-inhibiting and antimicrobial effects of toothpastes that contain *C. xanthorrhiza* extract (Hwang and Rukayadi 2006; Hwang et al. 2005; Kim et al. 2005). They showed that it is safe enough that toothpaste products containing CXO are now sold in South Korea. CXO nanoemulsion also contains Tween 80 as a surfactant. The toxicity and side effects of

several surfactants, including Tween 80, have been studied (Arechabala et al. 1999). However, toxicity is the product of chemical concentration and application duration. Low concentrations of Tween 80 are safe enough to be used in food and medicine. Its concentration in this study was 1%, which is its same concentration as in commercial eye drops (Kim, Choi, and Joo 2009). CXO nanoemulsions are removed after 1 minute in the oral cavity when used as a mouthwash, so their Tween 80 concentrations would not likely be harmful. Therefore, it is believed that the use of CXO nanoemulsion would likely exhibit a safe antimicrobial effect while alleviating the side effects of CHX.

C. xanthorrhiza extract also has a photodynamic effect as a photosensitizer (Lee et al. 2017; Sears 2006). The antibacterial photodynamic effect is caused by exposure to light. Curcumin is a photosensitizer in *C. xanthorrhiza* extract and has a strong antibacterial photodynamic effect for visible light at a wavelength of 450 nm (Araujo et al. 2012). Therefore, CXO's photodynamic antibacterial properties could contribute to its effectiveness.

This study examined the antimicrobial and antibiofilm effects of CXO nanoemulsion through various analyzes, but further analysis is required to the mechanisms behind these effects. A previous study found that CHX nanoemulsion damages the membranes of *S. mutans* cells by through transmission electron microscopy analysis (Li et al. 2015). Another study used scanning electron microscopy to determine how EO nanoemulsion affected on *S. aureus* and found that there were intact cells in the negative control group but there were morphological changes in the EO nanoemulsion treatment group (Sugumar et al. 2014).

Therefore, transmission or scanning electron microscopy could be used to determine how CXO nanoemulsion affects bacteria and biofilm.

In order to clinically apply CXO nanoemulsion, its potential reactions in the oral cavity should be studied because it may affect not only oral bacteria but also saliva and soft tissues. In addition, the use of *C. xanthorrhiza* extract in the oral cavity is limited because it has a bitter taste, so in one study, it was mixed with xylitol as a sweetener and was still shown to have a strong antibacterial effect (Kim et al. 2006). Therefore, CXO can be mixed with alternative sweeteners without reducing its effectiveness, enhancing its attractiveness as an oral care product.

V. Conclusions

This study showed that 10 min of sonication produced a CXO nanoemulsion with the smallest average particle size, greatest uniformity of particle size, and lowest turbidity. This nanoemulsion was also more resistant to external stimuli and stable over time than CXO emulsion. Thus, CXO nanoemulsion was superior properties to CXO emulsion.

The antibacterial effects of the CXO nanoemulsion were evaluated through planktonic bacteria, single-species biofilm, and multi-species biofilm models. CXO nanoemulsion killed all planktonic *S. mutans* bacteria and reduced cell viability more than DW, the negative control. CLSM analysis showed that it inhibited biofilm formation. CXO nanoemulsion reduced red fluorescence intensity, cell viability, and biomass in microcosm biofilm more than DW. Taken together, these results show that CXO nanoemulsion had strong antimicrobial activity against oral bacteria and biofilms and can be used as an antimicrobial agent in oral care.

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ABSTRACT (IN KOREAN)

쿠르쿠마 잔토리자 오일 나노에멀젼의 항균효과

<지도교수 김백일>

연세대학교 대학원 응용생명과학과

조무열

Curcuma xantorrhiza oil (CXO)은 천연 에센셜 오일로서 구강 관련 균주에 뛰어난 항균 효과를 가졌다. 하지만, 소수성 특성을 가진 CXO는 가용화가 어렵기 때문에 안전하고 안정적인 가용화 방법이 필요하다. 따라서 CXO를 이용하여 새로운 가용화 방법인 나노에멀젼화를 수행하여 최적의 CXO 나노에멀젼 제작 조건을 탐색하고 그 항균 효과를 평가하고자 하였다.

첫 번째 연구의 목적은 CXO 나노에멀젼의 가장 작은 입자 크기를

나타내는 초음파 처치 시간을 탐색하는 것이었다. 두 번째 및 세 번째 연구에서는 각각 단일 균종 박테리아 및 다균종 바이오필름에 대한 항균 및 항바이오필름 효과를 평가하고자 하였다.

첫 번째 연구에서 제작된 CXO 에멀젼을 5분 간격으로 15분 까지 초음파 처치를 수행하였다. 처치 후 각 용액에 대한 혼탁도와 용액 내 입자 크기와 그 크기의 균일성(Polydispersity index, PDI)을 평가하였다. 또한 원심 분리를 이용한 외부 자극 및 30일 동안의 보관 후 용액의 안정성을 평가하였다. 10분 동안 초음파 처치를 수행한 CXO 나노에멀젼은 가장 낮은 혼탁도를 보이며 가장 투명하였다. 또한 10분 동안 초음파 처치를 수행한 나노에멀젼은 평균 입자 크기, PDI에서 가장 낮은 값을 보였으며, 초음파 처치를 수행하지 않은 용액인 에멀젼과 유의한 차이를 보였다(각각, $p = 0.008$, $p = 0.008$). 나노에멀젼 용액은 원심 분리 및 보관 기간에 상관 없이 안정적인 상태를 보인 반면 에멀젼 용액에서는 원심 분리에 의한 침전물과 장기간 보관에 따른 층 분리가 관찰되었다.

두 번째 연구에서 부유성 *Streptococcus mutans* 및 *S. mutans* biofilm에 대한 CXO 나노에멀젼 용액의 항균효과를 평가하였다. 음성대조군으로 멸균 증류수(DW)와 양성대조군으로 Listerine을 포함하였고, 실험군으로 CXO 에멀젼과 10분 동안 초음파 처치한 CXO 나노에멀젼을 사용하였다. 항균

효과는 cell viability를 평가하는 방법인 colony forming units (CFUs)를 측정하는 방식을 통해 평가되었다. Biofilm 모델에서는 CLSM 을 이용한 live 및 dead cell 영상을 통해 바이오필름에 대한 평가를 추가적으로 수행하였다. 그 결과, CXO 나노에멀젼은 부유성 *S. mutans*에 대하여 완전 사멸 효과를 보였다. 또한 biofilm에 대한 평가에서 CXO 나노에멀젼은 DW 및 Listerine과 각각 통계적으로 유의한 차이를 보이며 낮은 CFUs 수치를 기록하였다(각각, $p < 0.001$, $p < 0.001$). CLSM 분석에서 CXO 나노에멀젼은 live 및 dead cell 형성 억제와 함께 가장 낮은 수치의 평균 biomass 및 biofilm의 두께를 기록하였다.

세 번째 연구에서는 다균종 biofilm 모델인 microcosm biofilm 모델을 이용하여 CXO 나노에멀젼의 항균 효과 및 항바이오필름 효과를 평가하였다. 정량 광유도 형광(QLF) 기술을 이용한 바이오필름의 붉은 형광 강도 분석 및 CFU 측정, dry weight을 이용한 biomass 분석, CLSM 을 이용한 bacterial cell 및 세포외 다당류 분석이 수행되었다. 본 연구에서는 양성대조군으로서 chlorhexidine (CHX)을 추가적으로 포함하였다. 평가 결과, CXO 나노에멀젼으로 처치한 바이오필름의 붉은 형광 강도는 DW 그룹에 비해 유의하게 낮았다($p < 0.001$). 또한, biofilm 내 total bacterial 및 aciduric bacterial CFU 측정에서도 CXO 나노에멀젼은 DW 보다 유의하게 낮은 값을 기록하였다(각각, $p < 0.001, p = 0.01$). CXO 나노에멀젼은 DW 및 Listerine과 비교했을 때 유의하게 낮은 biomass 수치를 보이며 강한 항바이오필름 효과를 나타냈다(각각, $p < 0.001$, $p < 0.001$).

CXO 나노에멀젼 그룹의 bacterial cell 및 EPS 형성 정도는 CHX 그룹 다음으로 적게 관찰되었다.

CXO 나노에멀젼은 나노에멀젼화를 통해 성공적으로 가용화되었으며 안정적인 유지력과 강한 항균 효과를 보였다. 특히, CXO 나노에멀젼은 구강 관련 균 종 및 실제 구강 바이오필름을 모사한 바이오필름 모델에 대해 강한 항균 및 항바이오필름 효과를 보였다. 따라서 CXO 나노에멀젼은 구강 관리를 위한 새로운 항균 제제로서 구강 질환 예방에 활용될 것으로 기대된다.

핵심되는 말: 쿠르쿠마 잔토리자 오일, 나노에멀젼, *S. mutans*, microcosm biofilm, antimicrobial effect, antibiofilm effect.