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Synergistic antimicrobial properties of nanoencapsulated clove oil and thymol against oral bacteria

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Abstract This study aimed to improve the antimicrobial activity of natural extracts against oral bacteria by synergistic combination and nanoencapsulation. Among five natural antimicrobials: clove oil, thymol, naringin, naringenin, and licorice, clove oil and thymol were selected by comparing the antimicrobial activities against *Streptococcus mutans* and *Streptococcus sobrinus* before and after nanoencapsulation. The combination of clove oil and thymol (CLTY) was nanoencapsulated using chitosan and poly-γ-glutamic acid. While free CLTY showed additive and synergistic antimicrobial activity against *S. mutans* and *S. sobrinus*, respectively, CLTY nanoparticles (NPs) exhibited synergistic activity against both strains in a time-kill kinetic assay. CLTY NPs significantly decreased the growth of salivary *S. mutans* during testing, compared with free CLTY in the mouth rinse test. These results indicate that nanoencapsulation can significantly increase the synergistic antimicrobial activity of CLTY and maintain its antimicrobial activity in oral cavities for a longer time.

Keywords Clove oil · Thymol · Synergistic antimicrobial effect · Nanoencapsulation · Oral bacteria

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Introduction

Dental caries and periodontal disease are major oral health problems that are mainly initiated by dental plaque, known as bacterial communities, on the surface of teeth (Loesche, 2007). The major causative agents of these oral diseases are cariogenic bacteria, such as *Streptococcus mutans* and *Streptococcus sobrinus* (Ekstrand et al., 1997; Loesche, 2007). Antibiotics and antimicrobial agents such as hydrogen peroxide, fluorides, chlorhexidine, and penicillin are used to effectively inhibit the growth of oral pathogenic bacteria and are now included in oral care products (Bidault et al., 2007). However, concerns exist among consumers regarding their side effects, such as tooth staining, diarrhea, taste perception, tooth discoloration, and vomiting. These concerns have increased the demand for safe antimicrobials (Claydon et al., 2006; Van Strydonck et al., 2005). Moreover, due to the repeated use of antibiotics and increase in microbial resistance, natural antimicrobial agents against oral bacteria that are safe enough to be used in food are growing in popularity and have received much attention for their use against oral bacteria (Soares et al., 2011; Wang et al., 2007).

In dentistry, several herbal extracts such as garlic (*Allium sativum*), clove (*Eugenia caryophyllata*), aloe vera (*Aloe barbadensis Miller*), neem (*Azadiracta indica*), cinnamon (*Cinnamon zeylanicum*), and thyme (*Thymus vulgaris*) have been used for the treatment of oral infections (Fani and Kohanteb, 2017; Pandita et al., 2014). Licorice (*Glycyrrhiza glabra*) showed potent antimicrobial activity in disk diffusion tests and favorable minimum inhibitory concentration (MIC) values against *S. mutans* and *Lactobacillus acidophilus* (Ajagannanavar et al., 2014). Furthermore, grape seed extract has been shown to possess significant antimicrobial properties against pathogenic oral

bacteria, with its antimicrobial activity being attributed to its main active ingredients, naringin and naringenin (Lee et al., 2009; Tsui et al., 2008; Yue et al., 2018). However, these natural antimicrobials have shown weaker activity and lower stability profiles compared to those of chemical antimicrobial agents. Moreover, natural ingredients, despite their obvious safety benefits, have unique flavors and tastes and therefore their application in the oral health care industry has been limited.

Natural antimicrobials have been shown to act together to more effectively inhibit the growth of pathogenic bacteria, compared to when single antimicrobials were used. This is thought to occur as a result of a synergistic effect when more than one natural antimicrobial is used (Pei et al., 2009). The synergistic combination of herbal extracts, including *Tribulus terrestris*, *Capsella bursa-pastoris*, and *Glycyrrhiza glabra* exhibited higher antibacterial activity against six oral pathogens compared with those of the individual extracts (Soleimanpour et al., 2015). The MIC in the combination of honey and propolis against oral bacteria was less than their individual MICs, indicating the synergistic antimicrobial effect (Eslami et al., 2016). In addition, various natural antimicrobials such as clove oil, silibinin, apigenin, and *Ficus carica* extract exhibited synergistic antimicrobial activity in association with antibiotics against oral bacteria (Cha et al., 2016; Jeong et al., 2009; Lee et al., 2011; Moon et al., 2011).

Nanoencapsulation of antimicrobials has been investigated to ensure that the antimicrobials fully exert their inherent biological properties, in addition to increasing their stability profiles (Prakash et al., 2018; Rai et al., 2017). Nano-sized particles have the potential to increase the water solubility (or dispersibility) and the contact with pathogenic bacteria, resulting in improved antimicrobial activity. Additionally, through nanoencapsulation, sensitive antimicrobials could be protected against oxidative environments such as oxygen, heat, and light during food processing and storage. Furthermore, their shelf life could also be extended (Bahrami et al., 2020; Prakash et al., 2018). Thus, nanoencapsulation technology may be used to alleviate the problems of natural antimicrobials. Moreover, as the synergistic combination was encapsulated together at the desired concentration in the same particles, the synergistic biological activity could be increased by simultaneous release, while maintaining the desired concentration (Windbergs et al., 2013). However, relatively little progress has been made on the natural preparations for oral health by combination and nanoencapsulation of synergistic antimicrobials.

Chitosan (CS) has been used as a wall material for many bioactive substance due to its nontoxic, biodegradable, and biocompatible properties. Poly-γ-glutamic acid (PGA) is a naturally-occurring anionic polymer composed of D- and L-glutamic acid linked by amide bonds (Lin et al., 2005). Particularly, CS/PGA nanoparticles (NPs) are spontaneously obtained through ionic gelation interaction between the positively charged amino groups of CS and the negatively charged carboxyl group of PGA in a mild conditions without using toxic solvent and heating process (Buescher and Margaritis, 2007). CS/PGA nanoencapsulation was reported to sustain release of catechin and increase its paracellular transport (Tang et al., 2013). In a previous study, we found that CS/PGA nanoencapsulation improved the solubility, stability, and cellular uptake of resveratrol (Jeon et al., 2016) and the antimicrobial activity of rosemary extract in vitro and food model (Lee et al., 2019). Thus, nanoencapsulation using CS and PGA may be suitable for nanodelivery system for oral health care.

The objective of this study was to improve natural antimicrobial properties through synergistic combination and nanoencapsulation methodology. Five natural antimicrobials, clove oil, thymol, naringin, naringenin, and licorice, were nanoencapsulated using CS and poly-γ-glutamic acid (PGA). By comparing the antimicrobial activities of nanoencapsulated and non-nanoencapsulated free form, clove oil and thymol were selected. The synergistic antimicrobial activity of the nanoencapsulated clove oil and thymol were investigated in time-kill studies and mouth rinse tests against oral bacteria.

Materials and methods

Materials

Clove oil and thymol were purchased from Newdia Co. (Seoul, Korea) and Neumond Co. (Raisting, Germany), respectively. Naringin and naringenin were obtained from Sigma-Aldrich Co. (St Louis, MO, USA). Licorice was obtained from CS chemical Co. Ltd. (Seoul, Korea). CS (water soluble, 24 cps, 95% deacetylated) and PGA were purchased from Kittolife Co. (Seoul, Korea) and BioLeaders Corp. (Daejeon, Korea), respectively. Ethanol (99% fermented ethanol), used in the mouth rinse test, was purchased from Korea ethanol supplies Co. (Seoul, Korea). All other reagents were of analytical grade.

Preparation of CS/PGA NPs

NPs entrapping each of the five natural antimicrobials (clove oil, thymol, naringin, naringenin, and licorice) were prepared by ionic gelation using CS and PGA (Liu et al., 2013). The stock solutions of clove oil, thymol, naringin, and naringenin were prepared using absolute ethanol and diluted with distilled water at a concentration of 5.0 mg/

mL in 25% ethanol, respectively. The stock solutions of licorice was dissolved in distilled water and mixed with ethanol at a concentration of 5.0 mg/mL in 25% ethanol. CS and PGA were dissolved in distilled water at 4.0 mg/mL, respectively. Antimicrobial solution (2.0 mL) was premixed with 7 mL of CS solution. The NPs were prepared by adding 1.0 mL of PGA solution dropwise into the premixed solution using a pipette tip under magnetic stirring at 1000 rpm for 10 min. The clove oil and thymol-loaded NPs were prepared by the same method; however, the concentration and volume of each solution was different; 2.0 mL of clove oil solution (5.0 mg/mL), 2.0 mL of thymol solution (5.0 mg/mL), 4.0 mL of CS solution (7.0 mg/mL), and 2.0 mL of PGA solution (2.0 mg/mL). Blank NPs were prepared using the same procedure without adding any antimicrobials. The final concentration of ethanol in all NP dispersions was 5%.

Physicochemical properties of NPs

The mean particle size and polydispersity index (PDI) of antimicrobial NPs was measured by dynamic light scattering using Zetasizer Nano ZS (Malvern Instruments Ltd., Malvern, Worcestershire, UK). The measurements were performed at multiple narrow modes at 25 ± 1 °C. Samples were analyzed in triplicate, at the least, for each treatment.

The EE of NPs were indirectly determined by measuring the amount of unencapsulated free total phenolic content within NPs using a 3-k-Da filter device (Amicon Ultra-4 Centrifugal Filter, Millipore, Billerica, MA, USA). The total phenolic content in filtered solution was analyzed after centrifuging the NP dispersion at 7000×*g* (VS-24SMTi, Vision Scientific, Bucheon, Korea) for 30 min (Lee et al., 2019).

The total phenolic content was determined using the method of Anesini et al. (2008) with some modification. Approximately, 0.1 mL of the sample was reacted with 0.5 mL of 0.2 N Folin-Ciocalteu's reagent for 3 min and then 0.4 mL of sodium carbonate (7.5%, w/v) was added and incubated at room temperature for 30 min. Absorbance was read at 765 nm on a spectrophotometer (DU 650, Beckman Coulter Inc., Fullerton, CA, USA) and the total phenolic content was calculated using gallic acid as a standard. The EEs of the NPs were calculated by the following equation: EE (%) = ((total phenolic content − total phenolic content in filtered solution)/total phenolic content) × 100 (Lee et al., 2019).

Determination of antimicrobial activity

Bacterial strain and growth conditions

The antimicrobial assays were carried out with *S. mutans* (Korean Culture Center of Microorganisms, KCCM 40105) and *S. sobrinus* (KCCM 11898). All cultures were stored at − 80 °C in brain heart infusion broth (BHIB, Difco, Detroit, MI, USA) with 87% glycerol. Working cultures were maintained in BHIB for 24 h at 37 °C and adjusted to the required concentration of 10^6 colony forming units (CFU)/mL.

Minimum inhibitory concentration (MIC)

The effect of combination and nanoencapsulation on the MIC values of antimicrobials was determined using 96-well microtiter plates (Fu et al., 2007; Koo et al., 2000). The wells containing stock solution in the first row were serially diluted two-fold with BHIB along each column. Next, 100 μL of BHIB containing 10^6 CFU/mL of the bacterial strain was added to each well. The positive controls contained BHIB inoculated with the bacterial strain and 5% ethanol without any antimicrobial agent in the place of sample. The negative controls contained the antimicrobial agent and BHIB only. The well plate was incubated at 37 °C for 24 h and the MIC was defined as the lowest concentration of antimicrobial agent that inhibited visible growth after incubation.

Fractional inhibitory concentration (FIC) index

The synergistic antimicrobial effect in combination with clove oil and thymol was determined by the checkerboard method, using 96-well microtiter plates to obtain the FIC index (Schelz et al., 2006). Clove oil and thymol were diluted two-fold with BHIB along the x- and y-axes, respectively. All wells had a total volume of 100 μL, comprising 50 μL of each dilution. Subsequently, 100 μL of BHIB containing 10^6 CFU/mL of the bacterial strain was added to each well. After 24 h incubation at 37 °C, the MIC of each solution alone, and in combination, was determined, as described above. The FIC indices were determined using the following Eq. (1):

$$\text{FIC index} = \text{FIC}_\text{A}\left(\frac{\text{MIC}_\text{A}\text{combination}}{\text{MIC}_\text{A}\text{alone}}\right) + \text{FIC}_\text{B}\left(\frac{\text{MIC}_\text{B}\text{combination}}{\text{MIC}_\text{B}\text{alone}}\right) \quad (1)$$

This method defines synergism, additivity, indifference, and antagonism by the FIC index of ≤ 0.5, 0.5–1.0, 1.0–4.0, and > 4.0, respectively.

In order to determine the FIC index for nanoencapsulated clove oil and thymol combination (CLTY), clove oil NPs, thymol NPs, and CLTY NPs were prepared with various concentration of clove oil, thymol and CLTY, respectively and used as samples in the checkerboard method described above.

Time-kill assay

A time-kill assay was performed according to a modified method from Koo et al. (Koo et al., 2002) The final concentration of clove oil and thymol was 1/8 × MIC. BHIB containing 10^7 CFU/mL of *S. mutans* ATCC 25175, or *S. sobrinus* ATCC 27351, was incubated at 37 °C for 48 h. Samples were removed at 0, 3, 6, 24, and 48 h. Counts were performed by serially diluting samples 10-fold in PBS and spreading 50 µL volumes onto BHI agar. Plates were incubated at 37 °C for 48 h and the number of colonies were counted.

Mouth rinse test

Approval for the study was obtained from the Hanyang University Institutional Review Board, and all subjects provided informed consent prior to participation. The effect of nanoencapsulation of CLTY was evaluated by a mouth rinse test according to a modified method from Arunakul et al. (2011). A total of 18 healthy individuals, 1 male and 17 females, between 24 and 29 years in age (mean age: 26.2 years), were selected. All subjects were non-smokers with no current caries activity, periodontal disease, or other oral pathologies.

For the mouth rinse test, the subjects were randomly divided into three groups: (1) control (5% ethanol solution), (2) non-nanoencapsulated free CLTY, and (3) nanoencapsulated CLTY. Regardless of nanoencapsulation, both concentrations of CLTY were 0.125 mg/mL. The ethanol concentration of all three samples was fixed at 5% to eliminate the interference from ethanol.

The subjects brushed their teeth immediately after lunch and pre-experimental saliva was collected 2 h later. The subjects rinsed their mouths for 1 min with 10 mL of the test mouth rinse solution, or the control solution. Saliva samples were collected at 30, 60, and 90 min after mouth rinsing from each subject. All saliva samples were serially diluted ten-fold in PBS, and inoculated on Mitis Salivarius Sucrose Bacitracin Agar (MSBA, MBcell, Los Angeles, CA, USA). The plates were then incubated at 37 °C for 48 h and the number of colonies were counted.

Statistical analysis

All experiments were performed in triplicate and data are presented as mean ± standard deviation (SD). Statistical analysis was conducted using the Statistical Package for Social Science software (SPSS, Version 21.0, SPSS Inc., Chicago, IL, USA). The statistical analysis to evaluate the differences among multiple groups was carried out by one-way analysis of variance (ANOVA), followed by Duncan's multiple range test ($p < 0.05$).

Results and discussion

Effect of five natural antimicrobials against *S. mutans* and *S. sobrinus*

Based on several antimicrobial studies that have investigated the buccal cavity (Ajagannanavar et al., 2014; Botelho et al., 2007; Gafner et al., 2011; Lee et al., 2009; Moon et al., 2011; Tsui et al., 2008; Yue et al., 2018), clove oil, thymol, naringin, naringenin, and licorice were selected as antimicrobials against oral bacteria. Their antimicrobial activities were determined by an MIC assay (Table 1). The MIC value of clove oil, thymol, and licorice against *S. mutans* and *S. sobrinus* was 0.5 mg/mL, and that of naringin and naringenin was 1.0 mg/mL. These results indicate that the antimicrobial activity of clove oil, thymol, and licorice against *S. mutans* and *S. sobrinus* is higher than that of naringin and naringenin.

Particle properties and MIC values of natural antimicrobial-loaded NPs

Five NPs that entrapped clove oil, thymol, naringin, naringenin, and licorice were prepared by ionic gelation of soluble CS and PGA. CS and PGA nanoencapsulation was reported to produce NPs with high solubility and uniform particle size (Jeon et al., 2016; Kim et al., 2016; Lee et al., 2019). Therefore, CS/PGA NPs are proposed here as appropriate nanocarriers for antimicrobials. All the NPs formulated in this study ranged from 237 to 375 nm in diameter (Table 2). Their PDIs were lower than 0.3, indicating a homogeneous and monodisperse system. Their EEs ranged from 52.1 to 56.7%, with no significant difference between samples (data not shown).

To investigate the effect of nanoencapsulation on the antimicrobial activities of clove oil, thymol, naringin, naringenin, and licorice, their MIC values against *S. mutans* and *S. sobrinus* were determined after nanoencapsulation (Table 1). Blank NPs without any antimicrobial agent were used for MIC assays to evaluate the influence of the wall materials on the antimicrobial activities of NPs.

Table 1 Minimum inhibitory concentration (MIC, mg/mL) values and fractional inhibitory concentration (FIC) index of natural antimicrobials against *S. mutans* and *S. sobrinus,* before and after nanoencapsulation

	Antimicrobials	*S. mutans* ATCC 25175		*S. sobrinus* ATCC 27351	
		Free	NPs	Free	NPs
MIC	Clove oil	0.5	0.5	0.5	0.5
	Thymol	0.5	0.5	0.5	0.5
	Naringin	1.0	1.0	1.0	1.0
	Naringenin	1.0	1.0	1.0	1.0
	Licorice	0.5	> 1.0	0.5	> 1.0
FIC	Clove oil + Thymol	0.75 (A)[a]	0.5 (S)	0.5 (S)	0.5 (S)

[a]FIC index are interpreted as synergy (S, FIC ≤ 0.5), additive (A, 0.5 < FIC ≤ 1), indifference (I, 1 < FIC ≤ 4), or antagonism (AN, FIC > 4)

Table 2 Physical properties of natural antimicrobial-loaded nanoparticles

	Particle size (nm)	Polydispersity index
Clove oil NP	374.9 ± 29.6[b]	0.17 ± 0.05[b]
Thymol NP	371.2 ± 29.6[b]	0.16 ± 0.05[b]
Naringin NP	318.5 ± 24.7[c]	0.03 ± 0.02[c]
Naringenin NP	287.9 ± 10.3[d]	0.12 ± 0.05[b]
Licorice NP	236.6 ± 26.3[e]	0.38 ± 0.08[a]
Clove oil + Thymol NP	483.2 ± 21.7[a]	0.18 ± 0.03[b]

[a–f]Means with different letters are significantly different at $p < 0.05$

However, blank NPs did not showed antimicrobial activity in MIC assays (data not shown). The MIC values of clove oil, thymol, naringin, and naringenin against *S. mutans* and *S. sobrinus* were not affected by nanoencapsulation. However, MIC values of licorice showed an increase after nanoencapsulation. These results may be explained by the difference in solubility of nanoencapsulated antimicrobials. It has been reported that the antimicrobial activity of poorly soluble antimicrobials is improved by nanoencapsulation as their water solubility, or dispersibility, is increased by their larger surface areas, which arises from the fact they are nano-sized particles (Bhawana et al., 2011; Lee et al., 2017). However, as the nanoencapsulated water-soluble substance must be released from within the capsule, any activity could take time and this can result in a reduction of initial activity. Therefore, the MIC values of water soluble licorice were increased after nanoencapsulation, resulting in decreased antimicrobial activity. Nevertheless, as MIC values of the poorly soluble clove oil, thymol, naringin, and naringenin were not affected by nanoencapsulation, it was not confirmed that their antimicrobial activity was increased from the results of the MIC assay in this study. This result may be due to the fact that MIC determination was not sensitive enough to clearly indicate the effect of nanoencapsulation (Lee et al., 2019). Moreover, among the five natural antimicrobials, the antimicrobial activities of clove oil and thymol were the highest in MIC tests and were not reduced by nanoencapsulation. Thus, CLTY was selected for further studies.

Effect of combination and nanoencapsulation on the in vitro antimicrobial activity of CLTY

To evaluate the effect of combination and nanoencapsulation on the antimicrobial activity of CLTY, after CLTY-loaded NPs were prepared using CS and PGA, the particle properties and antimicrobial activities were evaluated. The average particle size of CLTY-loaded NP was 483 nm, which is approximately 30% larger than that of clove oil or thymol NPs (Table 2). The EE of CLTY-loaded NP (58.7%) was slightly higher than those of single antimicrobial-loaded NPs with no significant difference (data not shown, $p < 0.05$). Although the particle size is increased, their PDI still remained below 0.3, indicating that CLTY NPs had a relatively narrow size distribution.

To numerically determinate the synergistic antimicrobial effects of CLTY, their FIC index was determined against *S. mutans* and *S. sobrinus,* before and after nanoencapsulation (Table 1). The FIC scale revealed synergistic and additive effects against *S. sobrinus* and *S. mutans*, respectively, before nanoencapsulation. However, the nanoencapsulated CLTY displayed synergistic activity against both *S. sobrinus* and *S. mutans*. This showed that non-nanoencapsulated CLTY exhibits partial synergistic antimicrobial activity, whereas nanoencapsulated CLTY is found to have synergistic effects against both *S. sobrinus* and *S. mutans.* This result can be explained by inferring that synergistic combinations increase the probability of synergy when present together within the same NP (Windbergs et al., 2013).

With regards to the effect of combination and nanoencapsulation on the antimicrobial activity of CLTY over time against *S. mutans* and *S. sobrinus* by the time-kill assay (Fig. 1A, B), clove oil and thymol as single agents, non-nanoencapsulated free and nanoencapsulated CLTY,

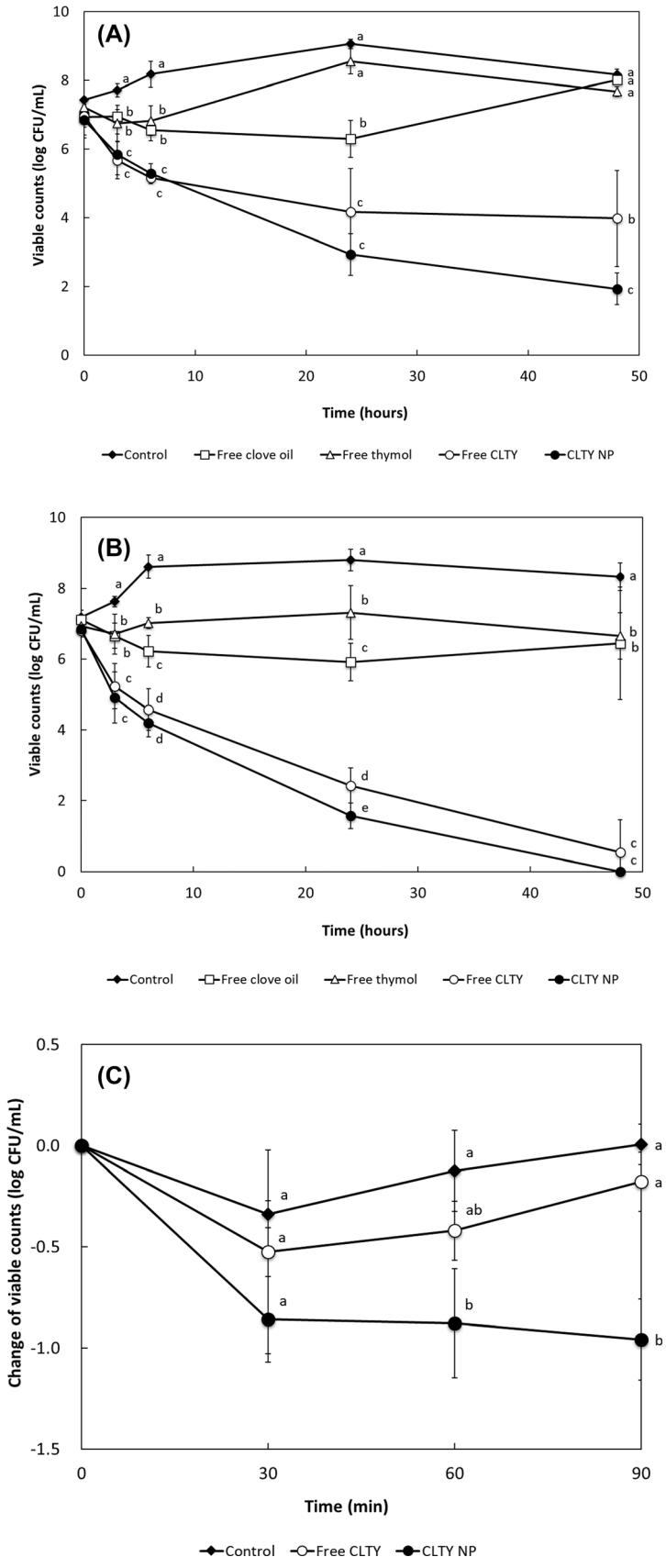

Fig. 1 The effect of combination and nanoencapsulation on the antimicrobial activity of clove oil and thymol by the time-kill assay against *S. mutans* (**A**) and *S. sobrinus* (**B**) and the mouth rinse test (**C**). [a−e]Means with different letters indicate significant difference between groups ($p < 0.05$)

were evaluated at concentrations of 1/8 × MIC for each strain. Blank NPs without any antimicrobial agent did not showed antimicrobial activity in time kill assays (data not shown). With the combination compared with colony count at 24 h incubation of the most active agent alone, synergy and antagonism in the time-kill assay were defined as greater than 2 $\log_{10}$ decrease and 2 $\log_{10}$ increase in colony count, respectively (Eliopoulos and Eliopoulos, 1988). Addition (or indifference) was defined as a less than 2 $\log_{10}$ change in colony count at 24 h with the combination, compared to the most active agent alone (Eliopoulos and Eliopoulos, 1988). Free CLTY and CLTY NPs showed additive and synergistic antimicrobial activity against *S. mutans*, respectively. In addition, both free CLTY and CLTY NPs showed significantly higher antimicrobial activity after 3 h than that of free clove oil or thymol. However, the significant difference between the antimicrobial activity of free CLTY and CLTY NPs was not observed at 24 h, but only at 48 h incubation. Regardless of nanoencapsulation, after 24 h, both free CLTY and CLTY NPs resulted in a greater than 2 log reduction in colony count compared to that by free clove oil or thymol, indicating a synergistic effect against *S. sobrinus*. In addition, CLTY NPs showed significantly higher antimicrobial activity against *S. sobrinus* at 24 h incubation than that of free CLTY. These results are similar to the results of the FIC index and indicate that the synergistic antimicrobial activity of CLTY is significantly improved by nanoencapsulation.

Effect of nanoencapsulation on the in vivo antimicrobial activity of CLTY

The effect of nanoencapsulation on the in vivo antimicrobial activity of CLTY against *S. mutans* was evaluated by a mouth rinse test (Fig. 1C). *S. mutans* has been reported to be more prevalent than *S. sobrinus* in dental plaque samples (de Carvalho et al., 2006). The number of *S. mutans* in the control and free CLTY slightly decreased at 30 min and then re-increased. Although the number of *S. mutans* in free CLTY group was lower than that in the control group, there was no significant difference. However, CLTY NPs significantly decreased the growth of salivary *S. mutans* at all three sampling times, compared to the control and free CLTY groups. These results indicate that the CLTY NPs can maintain their antimicrobial activity in oral cavities for a longer time than the free combination.

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Compliance with ethical standards

Conflict of interest None of the authors of this study has any financial interest or conflict with industries or parties.

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