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The Use of Lemongrass/Lemon Blend Essential Oil Nanoemulsions in Chewy Candy Formulations and Its Evaluation Against *Streptococcus Mutans* and *Porphyromonas Gingivalis*

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Abstract

Lemongrass (Lg) and lemon (Lm) essential oils (EOs) mainly contain citral and limonene, respectively, which are not limited to flavor for food but can also provide antibacterial activity. The information on the enrichment of these combined EOs in chewy candy products has never been documented yet. This study aimed to evaluate the physical-chemical properties and volatile compound of nanoemulsion formulations of Lg/Lm blend EOs as well as the physical-chemical properties, total plate count (TPC), and sensory acceptability of chewy candy formulated with nanoemulsions. Antibacterial evaluations against *Streptococcus mutans* and *Porphyromonas gingivalis* were performed for both nanoemulsion and chewy candy. Blending Lg and Lm EOs at ratios of Lg1.85 : Lm2.25 and Lg3.65 : Lm0.45 showed a broadly different constituent of the volatile compound than the ratio with the least amount of Lg (Lg0.05 : Lm4.05) but all the formulations possessed eugenol, β-Bisabolene, and caryophyllene oxide. The combination of Lg1.85 : Lm2.25 produced the nanoemulsion with the lowest particle size but lower zeta potential and emulsion stability. At this ratio, the significant inhibitory activity of the nanoemulsion was found to be 92.40% and 84.14% for *S. mutans* and *P. gingivalis*, respectively ($p < 0.05$). Its application in chewy candy formulation also resulted in the highest inhibitory activity against *S. mutans* (85%) and *P. gingivalis* (77.20%). Chewy candy formulated with Lg1.85 : Lm2.25 nanoemulsion was also found to have the lowest TPC of 3.72 log CFU/g. The overall acceptability of the chewy candy was around neutral (3.77) by panelists for its higher color score (4.73) and aroma (4.37)

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in average to the other samples. This study discovered the potential of Lg/Lm blend EOs nanoemulsion for enhancing the antibacterial effect and improving the texture and color of chewy candy.

Introduction

Almost all people, particularly children and older people living in developing countries, have experienced oral health problems. It was reported by WHO that oral disease was suffered by nearly 50% of the world's population.[1] It was dental caries and periodontal disease as the most common oral disorders. In Indonesia, dental caries has been recognized as the most prevalent oral disease with a prevalence of 88.8%, while the prevalence of periodontal disease accounted for 75%.[2]

It can be due to limited access to oral health services which are relatively costly, thus contributing to the lack of knowledge of how important toothbrushing is and the correct technique of doing it, and resulting in poor efficiency of personal oral care. Such conditions trigger an excessive growth of pathogenic bacteria in the oral cavity which is known to be the major cause of oral diseases. This can be one of the reasons that the current effort, such as the addition of fluoride in toothpaste, becomes ineffective. Therefore, one of the alternatives such as the use of natural antibacterial agents may be beneficial to effectively inhibit the growth of *Streptococcus mutans* and *Porphyromonas gingivalis.*

It is well acknowledged that essential oils (EOs) contain a wide range of volatile compounds with antimicrobial properties. For this reason, they have been massively used in the food sector not only by providing potential protection against undesired microbial growth but also by giving an extra benefit to the flavor of the food, for example, gummy candy.[3] Lemongrass (Lg) or *Cymbopogon flexuosus* is one of the most used spices in Indonesia due to its remarkable antimicrobial activity respective to its important volatile compounds, predominantly citral, neral, camphene, cyclohexene, α-sinensal, linalool, geraniol, and geranial.[4] Lg EO has been proven to inhibit the growth of those main pathogenic bacteria in the oral cavity.[5] In order to enhance its antibacterial effect, another EO can be combined.

In this study, we used lemon (Lm) EO which has been studied in previous works to effectively inhibit the growth of *S. mutans* and *P. gingivalis*.[6,7] Those two EOs have been reported to have synergistic effects with other EOs such as the combination of Lg with clove EOs and Lm with thyme EOs.[8,9] However, the combination of Lg and Lm EOs to give a synergistic antibacterial effect against *S. mutans* and *P. gingivalis* has not been investigated yet. Another underlying reason for such a combination is that they have a lemony scent and their volatile constituents will complement each other to create a pleasant aroma.

One of the promising food products as the carrier to deliver these EOs to combat pathogenic bacteria in the oral cavity is chewy candy. Chewing activity can be beneficially effective in stimulating the production of saliva to help the self-cleansing activity of the oral cavity, and importantly distributing the EOs evenly. Instead of using sucrose and glucose, we used sugar alcohols in this study to eliminate the possibility of the pathogenic bacteria producing acid waste from the utilization of the carbon of conventional sugar for their energy sources. However, the direct incorporation of EOs into chewy candy formulation gives a major challenge to their solubility, stability, partial inactivation, and intense aroma. Nano-emulsification process is one of the promising approaches to tackle the drawbacks as it entraps these EOs within compatible biopolymers to prevent the adverse effects from the external factors. Based on the points outlined above, this study was undertaken to develop and evaluate nanoemulsion formulations of Lg/Lm blend EOs to strengthen their antibacterial activity against *S. mutans* and *P. gingivalis* and results in an enhanced chewy candy quality.

Materials and Methods

Materials

Food-grade Lg and Lm EOs were purchased from PT Young Living Indonesia (Indonesia). Soy protein

isolate (SPI) and soy lecithin were supplied by Para Agribusiness (Indonesia) and Lansida (Indonesia), respectively. Sorbitol was donated by PT Cargill Indonesia (Pasuruan branch, Indonesia), isomalt was supplied by BENEO-Palatinit GmbH (Germany), and the other chewy candy ingredients such as sucralose, canola oil, maltodextrin, gum arabic, and glycerol monostearate were obtained from local markets. Microbiological media used were Mueller Hinton Agar (MHA) (Himedia, India), Mueller-Hinton Broth (MHB) (Himedia, India), and Plate Count Agar (PCA) (Merck, Germany). *Porphyromonas gingivalis* ATCC 33277 and *Streptococcus mutans* ATCC 25175 were supplied by Faculty of Dentistry, Jember University (Indonesia). All the other chemicals used were of analytical grade and were obtained from commercial sources.

Nanoemulsion Preparation

The nanoemulsion was prepared with SPI and soy lecithin as the carriers.[10] The determination of the mixing ratio (v/v) was based on the minimum inhibitory concentration of Lg and Lm EOs against *P. gingivalis* and *S. mutans*, respectively.[5,7] The ratios of the EOs mixture were F1 (Lg0.00 : Lm4.10), F2 (Lg0.05 : Lm4.05), F3 (Lg1.85 : Lm2.25), F4 (Lg3.65 : Lm0.45), and F5 (Lg4.10 : Lm0.00). Briefly, SPI was previously mixed with lecithin at 10:1 (w/w) under stirring using a magnetic stirrer at 400 rpm for 10 min. The mixture was then dissolved in phosphate buffer solution 0.1 M at pH 7 (1.1% w/v) and further stirred for 2 h to obtain SPI-lecithin solution. The Lg/Lm blend EOs were added into the SPI-lecithin solution at a ratio of 1:3 (v/v). The mixture was homogenized at 21,500 rpm for 1 min using an Ultra-Turrax homogenizer to obtain coarse emulsion, and it was then sonicated at 150 W for 24 min to produce nano-sized emulsion. The nanoemulsion was stored and sealed at room temperature (28 ± 2°C) before analysis.

Determination of Physical-chemical Properties of Nanoemulsion

The particle size, polydispersity index, and zeta potential of nanoemulsion samples were measured using Particle Size Analyzer (Malvern Zetasizer, UK). An aliquot of nanoemulsion (1 ml) was dissolved in distilled water to a volume of 30 mL. The condition of analysis was set at a refractive index of 1.33, viscosity of 0.797-1.002, and temperature of 20-30°C. pH value was measured using a portable pH meter (HI98107 pHep® Hanna Instrument, USA) at 25°C.

Morphological Observation

The emulsion was frozen overnight and was then freeze-dried at -30°C for 48 h. Freeze-dried samples were spread onto adhesive carbon tape mounted on aluminum stubs. Samples were briefly coated with gold metal using a sputter coater (IB2, Japan). Images were obtained by using a Scanning Electron Microscopy (Hitachi TM-3000, Japan) at 5000× magnification.

Determination of Nanoemulsion Stability

Nanoemulsion stability was evaluated by measuring the weight of the separated aqueous phase after centrifugation.[11] About 8 g of nanoemulsion was weighed in a centrifuge tube and then centrifuged at 3,500 rpm for 30 min (Sigma 2-16 KL refrigerated benchtop centrifuge, Germany). The separated aqueous phase at the bottom of the tube was taken out using a pipette and weighed. The nanoemulsion stability was calculated based on the following formula:

Creaming index (%) = separated aqueous phase (g) /emulsion total (g) ×100%

Analysis of Volatile Compound

Three nanoemulsion formulations (F2, F3, and F4) were analyzed for their volatile constituents using Gas Chromatography-Mass Spectrometry (GC-MS Shimadzu-QP2010 Plus, Japan). Separation of the oil phase of the nanoemulsion sample was performed by dissolving the sample in n-hexane (1:10, v/v) and was then centrifuged at 1,000 rpm for 3 min.[11] The separated oil was taken from the upper part and the remaining solvent in the oil fraction was evaporated using a vacuum rotary evaporator at 35°C. This analysis employed GC-MS equipped with a capillary column consisting of 5% phenyl and 95% methylpolysiloxane with a length of 60 m, a diameter of 0.25 mm, and a film thickness of 0.25 µm. Helium was used as a carrier gas at a pressure of 60 kPa and at a flow rate of 0.9 mL/min. The split ratio was set at 1:100 with an injector temperature of 250°C and detector temperature of 300°C. The column temperature was initially programmed at 90°C for 5 min, then increased to 250°C at 5°C/min, and held at this temperature for 25 min. The mass range was set from 40 m/z to 600 m/z. An aliquot of oil fraction

(1 ml) was injected using a split injector. Identification of volatile compounds was made on the basis of the linear retention index and the mass spectra of each compound detected in the spectrometer which was compared with the electronic mass spectral database of the National Institute of Standards and Technology.

Chewy Candy Preparation

Sorbitol (47.75%), isomalt (32.8%), maltodextrin (2.25%), gum arabic (1.45%), sucralose (0.01%), and water (5.04%) were mixed and heated on a hot plate until the temperature reaches 136°C. The mixture was then cooled down to 110°C, and canola oil (5.9%), glycerol monostearate (0.3%), and lecithin (0.4%) were subsequently added. Further stirring was done at 800 rpm for 20 min to obtain a homogeneous mixture and cooled down again to 40°C. Nanoemulsion (4.1%, v/v) was added to the mixture before pulling and stretching.[12] The resulting candies were flattened, cut, and wrapped in baking paper.

Moisture Content Analysis

The moisture content of chewy candy was assayed according to the protocol of AOAC 925.10.[13] Ceramic bottles with lids were cleaned and dried at 130±3°C for 30 min prior to analysis. About 5 g of sample was weighed into a ceramic bottle with a lid, and dried in an oven at 130±3°C until constant weight. Moisture content was measured by the mass loss of 5 g of the sample after the drying process, and the result was expressed as a percentage.

Reducing Sugar Analysis

The reducing sugar of chewy candy was measured using the method of Nelson-Somogyi.[14] The candy was dissolved in distilled water at a ratio of 1:1, then 1 ml of the mixture was added into a test tube. Afterwards, 1 ml of Somogyi reagent was added and the mixture was homogenized using a vortex for 1 min. The mixture was subsequently placed in a water bath at 100°C for 20 min. After cooling down to 25°C, the Nelson's reagent was added. Homogenization was done again and the absorbance of the mixture was determined at 520 nm using a spectrophotometer UV-Vis (Thermo Scientific Genesys 10s, USA). Reducing sugar was estimated by a calibration curve (R^2 = 0.9998) obtained from the measurement of the absorbance of known concentrations (0.010, 0.025, 0.050, 0.075, 0.100, 0.125, and 0.150 ppm) of glucose standard.

Sensory Evaluation

A hedonic test was performed according to the protocol of Indonesian National Standard (SNI) 01-2346 to indicate the sensory properties of chewy candy as perceived by the attributes of color, aroma, taste, texture, and overall.[15] Thirty untrained panelists were recruited to assess the candy quality on a scale of 7 points, starting from strongly disliked=1, moderately disliked=2, slightly disliked=3, neither liked nor disliked=4, slightly liked=5, moderately liked=6, to extremely liked=7.

Total Plate Count Analysis

The total plate count (TPC) of chewy candy was conducted according to the protocol of FDA Chapter 3.[16] A weight (25 g) of the sample was dissolved in 225 6mL of Butterfield's phosphate-buffered solution and homogenized for 2 min (10^{-1} dilution). A set of serial dilutions (10^{-2}, 10^{-3}, 10^{-4}, and 10^{-5}) was made, and 1 ml of each dilution was placed into a petri dish. A volume (12 mL) of PCA was then poured into each petri dish and gently shaken to mix them homogeneously. Incubation lasted for 72 h at 37°C. TPC values were expressed as log CFU/g.

Determination of Inhibitory Activity Against *S. mutans* and *P. gingivalis*

Inhibitory activities of nanoemulsion and chewy candy against *S. mutans* and *P. gingivalis* were evaluated by measuring optical density after incubation.[12,17] *S. mutans* and *P. gingivalis* suspensions were prepared in MHB at 37°C for 24 h under anaerobic conditions. The initial concentration of the bacteria was 10^7 CFU/mL (estimated using the TPC method). Sterile MHB medium was added simultaneously to 1 ml of suspensions (*S. mutans* or *P. gingivalis*) and then to 1 ml of nanoemulsion samples. The incubation period lasted for 24 h at 37°C under anaerobic conditions. Afterward, the mixtures were homogenized using a vortex, and the optical density (OD) was measured using a spectrophotometer UV-Vis (Thermo Scientific Genesys 10s, USA) at 600 nm to obtain OD of samples. Media solution with bacterial suspension was prepared as a control and the OD (OD of control) was measured after the incubation period. Whereas, OD of nanoemulsion was recorded from the mixture of media solution

with nanoemulsion without conducting an incubation period. Inhibitory activity (%) was calculated using the following formula:

Inhibitory activity (%) = OD of control - (OD of sample - OD of nanoemulsion) / OD of control ×100%

Table 1: Volatile compound of Lg/Lm blend EOs nanoemulsion

Compound	Retention time (min) (chromatogram area in %)		
	F2	F3	F4
Monoterpene			
Neral	-	-	29.34 (0.69)
trans-Geranic acid	-	-	32.51 (11.04)
cis-Geranic acid	-	-	38.07 (0.92)
4,8-dimethyl-3,7-nonadien-2-ol	-	33.46 (9.17)	34.02 (4.10)
Verbenone	-	16.97 (0.37)	16.94 (3.18)
Oxygenated Monoterpene			
Eugenol	18.27 (2.92)	18.28 (1.71)	18.26 (12.58)
cis-limonene oxide	-	-	20.56 (1.62)
Linalool oxide	-	21.44 (0.60)	16.04 (10.96)
Epoxy-Linalool oxide	-	16.06 (0.87)	17.15 (1.02)
Monoterpene alcohol			
Santolina alcohol	-	32.54 (6.65)	-
Sesquiterpene			
trans-Caryophyllene	-	16.71 (0.29)	16.70 (1.29)
β-Bisabolene	15.89 (48.61)	15.85 (3.05)	15.83 (4.47)
Bicyclogermacrene	16.93 (2.08)	-	-
Humuladienone	-	20.59 (0.19)	-
Valeranone	-	-	52.70 (0.13)
Longifolene	33.00 (2.95)	-	-
Oxygenated sesquiterpene			
Caryophyllene oxide	19.72 (3.64)	19.75 (2.19)	19.72 (12.08)
Sesquiterpene alcohol			
α-Bisabolol	21.66 (3.37)	-	-
α-cedrol	-	-	36.93 (0.31)
Ledol	-	34.79 (3.83)	-
Widdrol	-	47.43 (1.75)	-
Hydrogenated diterpene			
Dihydrotorulosol	-	42.87 (2.28)	-
Diterpene alcohol			
Geranyl linalool	-	37.00 (11.20)	-
Triterpenoid			
Squalene	47.60 (1.25)	-	-
Coumarin			
Scoparone	33.62 (3.33)	33.63 (4.02)	-

F2 (Lg0.05 : Lm4.05); F3 (Lg1.85 : Lm2.25); F4 (Lg3.65 : Lm0.45).

Statistical Analysis

Statistical analysis was done using the software Minitab 17 for Windows. A one-way analysis of variance (ANOVA) was used to analyze the data (physical-chemical properties, TPC, and antibacterial activity) and a Tukey test was then used to analyze

the differences in the related parameters at a level of significance of $p < 0.05$.

Results and Discussion

Volatile Compound of Lg/Lm Blend EOs Nanoemulsion

From Table 1, all three formulations showed their own characteristics which were indicated by their major and minor constituents. F3 and F4 formulations showed more volatile compounds than F2. Monoterpene and sesquiterpene groups are mostly found in EOs but none of the volatile compounds from the monoterpene group was detected in F2. In our study, neral, *trans*-geranic acid, and *cis*-geranic acid were only observed in F4 (formulation with a higher ratio of Lg EO). It is clear because neral is the major component of Lg EO with other oxidized derivatives, those are *cis*- and *trans*- geranic acid. But squalene, α-Bisabolol, longifolene, and bicyclogermacrene were only found in F2. It was also reported β-bisabolol in Lm EO at low concentration (0.21%).[20]

In addition, eugenol, β-Bisabolene, and caryophyllene oxide were recorded in all nanoemulsion formulations, but at different levels. Eugenol and caryophyllene oxide were found to be the major constituent in F4 comprising just over 12% of both compounds. Also, a previous study has reported eugenol and caryophyllene oxide in *Cymbopogon* species at low concentrations.[4] Neral, *cis*-Geranic acid, valeranone, and α-cedrol were minor volatile compounds in F4 accounting for 0.69, 0.92, 0.13, and 0.31%, respectively. In contrast, F2 and F3 recorded both compounds as the minor constituent, 1.71-2.92% for eugenol and 2.19-3.64% for caryophyllene oxide. Neral and α-cedrol were minor volatile compounds in this study but they have been previously reported to exhibit antimicrobial and anticancer activities.[21,22] In F2, β-Bisabolene was observed as the major constituent comprising a little less than 50% while F3 and F4 contained that compound at a minimal amount. It is not surprising since β-bisabolene is one of the main sesquiterpenes in Lm peel.[23] Furthermore, not surprisingly, the alteration of chemical components during emulsification can be possible. It was reported that some compounds were decreased quantitatively in the form of nanoemulsion.[11] The use of a high-energy process (homogenization) probably decomposes some chemical components in EOs.

Table 2: Physical-chemical properties of Lg/Lm blend EOs nanoemulsion

Nanoemulsion sample	Particle size (nm)	Polydyspersity index	Zeta potential (mV)	pH	Creaming index (%)
F1	363.04 ± 9.90^{c}	0.64 ± 0.06^{c}	-26.83 ± 2.91^{b}	7.01 ± 0.06	56.85 ± 0.80^{b}
F2	366.62 ± 18.51^{c}	0.45 ± 0.00^{b}	-30.51 ± 3.98^{b}	7.00 ± 0.07	57.57 ± 1.01c^{b}
F3	155.74 ± 41.18^{a}	0.44 ± 0.08^{b}	-27.22 ± 0.35^{b}	7.01 ± 0.06	60.43 ± 1.53^{c}
F4	420.02 ± 14.83^{c}	0.21 ± 0.02^{a}	-36.08 ± 5.91^{b}	6.96 ± 0.04	55.65 ± 1.70^{ab}
F5	265.46 ± 30.58^{b}	0.11 ± 0.03^{a}	-53.85 ± 2.93^{a}	6.91 ± 0.04	53.49 ± 1.08^{a}

F1 (Lg0.00 : Lm4.10); F2 (Lg0.05 : Lm4.05); F3 (Lg1.85 : Lm2.25); F4 (Lg3.65 : Lm0.45); F5 (Lg4.10 : Lm0.00). Mean ± standard deviation (n=3). Means with different superscripts in the same column for each sample are significantly different ($p < 0.05$)

Physical-chemical Properties of Nanoemulsion

Results of particle size, polydyspersity index (PDI), zeta-potential, pH, and stability of nanoemulsions prepared with different ratios of Lg and Lm EOs were listed in Table 2. The combination of Lg/Lm EOs significantly influenced the particle size, PDI, zeta-potential, and creaming index of nanoemulsions ($p < 0.05$). Particle sizes of nanoemulsions were successfully made in the nano-size range (155.70–420 nm). Interestingly, blending Lg and Lm EOs at a close ratio (F3, Lg1.85 : Lm2.25) significantly decreased the particle size of the nanoemulsion by 57.10% and 41.33% as compared to their single uses F1 and F5, respectively. Also, compared to the nanoemulsions prepared with single Lg and Lm EOs, the particle size of F3 was still lower than those previous works.[24,25] This indicates that the emulsifier and emulsification process were suitable for the

blend of these EOs. The formation and stabilization of the Lg/Lm EOs blend nanoemulsion can be achieved with regards to the ability of lecithin that effectively lowers the surface tension and viscosity of the EOs/SPI system to form the emulsion, while SPI stabilizes the emulsion by coating oil droplets with viscoelastic films.[26]

Further, it was noted that a slight change in the ratio of the combination could increase the particle size of the nanoemulsions. They were exemplified by the change of particle size from F1 to F2 and F5 to F4 which became bigger than their single EOs. This could be a result of the instability when two types of EOs (Lg and Lm) with different compounds and sizes combined at big differences in ratios (Lg0.05 : Lm4.05 and Lg3.65 : Lm0.45). Spaces between larger particles will be occupied by smaller particles and they will form bigger particles progressively due to flocculation during homogenization and ultrasonication (high-energy processes).

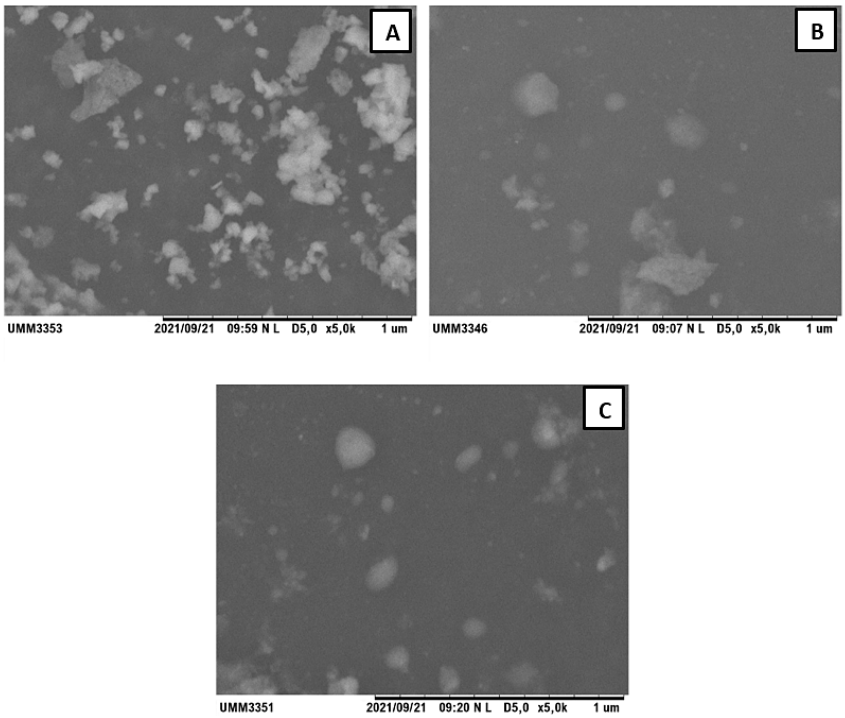


Fig. 1: Morphology of freeze-dried F2 (A), F3 (B), and F4 (C) nanoemulsion samples

In the present work, the PDI of F3 seemed to be highly dependent on the Lg and Lm EOs ratio. On one hand, an increase in the ratio of Lm EO caused PDI to increase significantly, as seen from F5 to F3. On the other hand, the addition of Lg EO significantly reduced the PDI, as demonstrated from F1 to F3. This result implies that the particle size distribution of F5 is narrower than F1, or in other words, F5 can be considered as a monodisperse particle (PDI ≤ 0.1) while the other formulations represent polydisperse particles (PDI ≥ 0.1).[27] Sample F4 was significantly lower in PDI and it demonstrated more individual particles and less agglomeration (Figure 1). This condition maintained the higher moisture content of chewy candy (Table 3) and darker appearance (Figure 4A). Interestingly, less agglomeration effectively amplified the inhibitory activity of nanoemulsion against *S. mutans* and *P. gingivalis* (Figure 3A). Formulation with a lower value of PDI can be more favorable as its uniformity of the particle size affects the stability of the emulsion, but it depends on the core purpose of emulsion making.

This finding is supported by a significantly decreased creaming index in the F5 formulation with the lowest

PDI and a lower particle size. This result is in line with the result that reported the effect of particle size of EOs nanoemulsion on phase separation.[28] Despite F3 having the smallest particle size, its stability was found to be the lowest as it also indicated a higher PDI. Taken together, such a condition can also be affected by the low value of absolute zeta potential (F3 = -27.20 mV) which indicates poor repulsion between each particle and leads to a higher chance of aggregation and flocculation. Even so, all the nanoemulsion formulations possessed a high negative zeta potential at pH values ranging from 6.91–7.01. F2, F4, and F5 might be more resistant to aggregation as their zeta potential values were less than -30 mV.[29] The small particle size may not proportionally be related to good zeta potential and PDI.[24] It seems reasonable to suggest that the particle size, particularly the smallest particle, should not be pointed out as a lone reference to indicate the good properties of an emulsion. Furthermore, our emulsification method can be a promising alternative as a delivery system for other flavoring agents in food products because the favorable physical properties of the nanoemulsion were obtained in this study, particularly for the particle size and zeta-potential.

Table 3: Physical-chemical properties of chewy candy formulated without and with nanoemulsions

Chewy candy samples	Moisture (%)	Reducing sugar (%)	L* value	b* value
F0	7.42 ± 0.43^{b}	4.20 ± 0.10	85.81 ± 0.70^{d}	5.80 ± 0.40^{a}
F1	5.93 ± 0.22^{a}	4.16 ± 0.15	81.28 ± 0.77^{c}	8.21 ± 0.53^{b}
F2	5.99 ± 0.24^{a}	3.90 ± 0.19	79.49 ± 0.48^{b}	9.28 ± 0.68^{b}
F3	6.23 ± 0.26^{a}	4.05 ± 0.08	79.27 ± 0.42^{b}	9.55 ± 0.68^{b}
F4	6.97 ± 0.13^{b}	4.13 ± 0.17	76.27 ± 0.82^{a}	11.91 ± 0.95^{c}
F5	7.29 ± 0.04^{b}	4.13 ± 0.13	75.11 ± 0.36^{a}	13.88 ± 0.88^{d}

F0 (candy without nanoemulsion); F1 (Lg0.00 : Lm4.10); F2 (Lg0.05 : Lm4.05); F3 (Lg1.85 : Lm2.25); F4 (Lg3.65 : Lm0.45); F5 (Lg4.10 : Lm0.00). L* represents lightness and b* represents yellowness. Mean ± standard deviation (n=3). Means with different superscripts in the same column for each sample are significantly different ($p < 0.05$)

Physical-chemical Properties of Chewy Candy

The addition of Lg/Lm blend EOs nanoemulsion in chewy candy formulations significantly affected the moisture content, lightness, and yellowness of the candy ($p < 0.05$). The moisture contents of chewy candies formulated with nanoemulsions at different levels of Lg/Lm blend EOs varied significantly and ranged from 5.93% to 7.42% (Table 3). Our result was lesser than the commonly used standard moisture content of chewy candy which was 8%.[30] It was shown that the addition of Lg/Lm blend EOs nanoemulsion reduced the moisture content of the chewy candy. The moisture content of F5 (candy formulated with Lg EO nanoemulsion) was higher than F1 (candy formulated with Lm EO nanoemulsion). This is because several major volatile compounds in Lg EO such as neral, geranial, and linalool are less non-polar so they can still be soluble in water at a very slight solubility (0.06%). Whereas, limonene, pinene, and squalene of Lm EO are completely water-insoluble.[31] Clearly, such compounds in Lg EO will lead to the water binding more strongly and reduce water loss due to drying. This is the reason why the moisture content of candy formulated with F4 nanoemulsion was also higher than those made with F2 and F3 nanoemulsion formulations.

In the present study, the addition of Lg/Lm blend EOs nanoemulsion at different ratios in chewy candy formulation did not affect the content of reducing sugar, ranging from 3.90% to 4.16% (Table 3). The use of sugar alcohols, sorbitol (47.75%) and isomalt (32.8%), as an alternative to conventional sugar, is certainly a major reason since they are not a group of reducing sugar. The reducing sugar that was observed in our candy could be from the glucose and arabinose of maltodextrin and gum

arabic, respectively. Because these ingredients were added at a similar proportion for all chewy candy formulations, it was obvious that the reducing sugars were also comparable among the samples.

Lg/Lm blend EOs nanoemulsion influenced the color of the chewy candy slightly. An increase in L* and a decrease in b* were observed in candy with a higher addition of Lm EO, indicating that Lm EO brightened the color of the candy. In contrast, the higher addition of Lg EO resulted in a darker candy. Lm EO tends to be clear and pale yellow, while Lg EO appears to be brown to yellowish.[32,33] It is obvious that the coloring agent influences the lightness of the candy, as presented by the previous study that the addition of darker coloring agents such as açai resulted in a darkening color of the chewy candy.[34] Our result showed a higher L* than their study, so this means that the nanoemulsion kept the candy color lighter (Figure 4A). The apparent advantage in the moisture content and color improvements of the chewy candy was gained by incorporating Lg/Lm blend EOs nanoemulsion; thus, this nanoemulsion can be potentially used for the development of other types of candies.

Total Plate Count of Chewy Candy

The TPC values of candy without nanoemulsion (F0) and with nanoemulsion formulations F1, F2, F3, F4, and F5 are shown in Figure 2. In our study, we found a significantly different increasing trend of TPC among chewy candy manufactured with different ratios of Lg/Lm blend EOs nanoemulsion. The addition of Lg/Lm blend EOs nanoemulsion at all ratios exhibited lower TPC of candy as compared to candy without nanoemulsion (control).

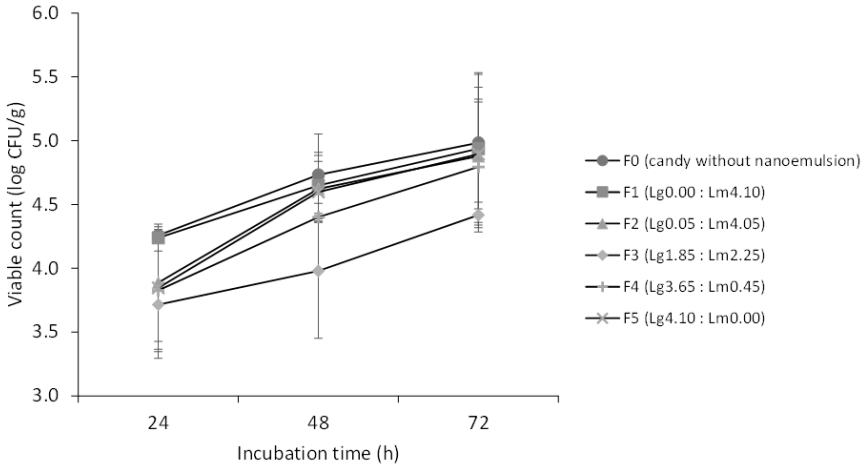


Fig. 2: Total plate count of chewy candy formulated without and with nanoemulsions

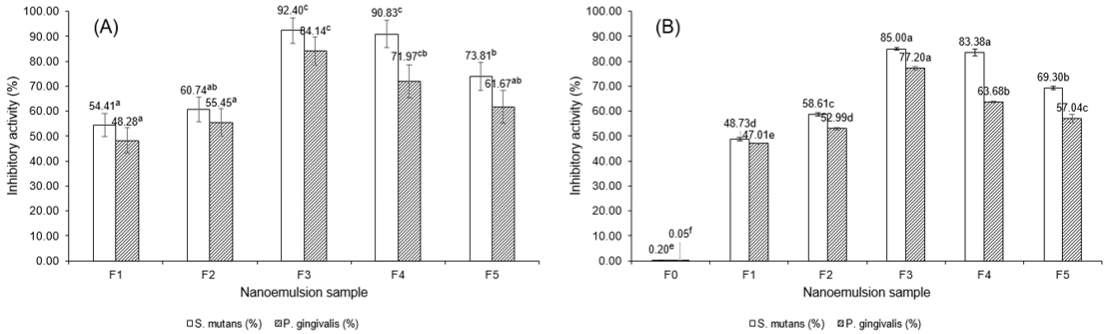
The lowest TPC (4.42 log CFU/g after 72 h of incubation) was found in candy made with F3 formulation ($p < 0.05$), at a close combination ratio of Lg and Lm EOs. It is undoubtedly that F3 nanoemulsion provides more antimicrobial activity since it has the most diverse volatile compounds (F3 in Table 1). Given that F3 also had the smallest particle size (Table 2), this further leads to the better efficiency of the nanoemulsion to slow down or even inhibit the microbial growth. In nano-sized forms, previous works documented excellent antimicrobial activities of Lg EO and Lm EOs.[8,25] A decrease in particle size increases the surface area to volume ratio and probably also allows the encapsulated EOs to be more efficiently transported into the inner part of microbial cells via the membrane system, thereby boosting the reactivity. However, the lowest TPC value of candy is not able to satisfy SNI 3547.2 that recommends the maximum TPC of candy is 2 log CFU/g.[35]

Furthermore, there were only small differences between the TPC values of candies formulated with F0 (control), F1 (with Lm EO nanoemulsion only), and F5 (with Lg EO nanoemulsion only) during 72 h of incubation. This indicates that single

use of Lg and Lm EOs nanoemulsions has poor antimicrobial activity. There are some determining factors that account for this result but it is believed that the presence of volatile compounds is the most influential factor. A previous study uncovered the superior efficiency of Lm EO in suppressing the microbial counts of peeled shrimp due to a wider variety of volatile compounds, particularly identified major compounds such as limonene, β-Pinene, γ-Terpinene, linalool, and β-Bisabolene.[20] Similarly, Lg EOs have been well-documented for their broader variety of volatile compounds, highlighting the major constituents detected in the previous report such as neral, geranial, and linalool, alongside their efficiency in reducing the microbial count in strawberry fruits.[18] Taken together, the smaller size of nanoemulsion that abundantly brings the volatile antimicrobial compounds, as exemplified in F3, might have led to a severe disruption of the microbial membrane system and leakage of microbial inner content.

Antibacterial Activities of Nanoemulsion and Chewy Candy Against *S. mutans* and *P. gingivalis*

Inhibitory activities of Lg/Lm blend EOs nanoemulsion and chewy candy improved by nanoemulsion formulation were measured by the turbidity of broth media containing bacteria with known concentration after the incubation period. The growth inhibition of nanoemulsion formulations at different ratios ranged between 54.41–92.40% against *S. mutans* and 48.28–84.14% against *P. gingivalis* (Figure 3A). Regardless of the blending ratio of EOs, the nanoemulsion specifically showed greater inhibitory activity against *S. mutans* which could be associated with the structure of the bacterial cell wall.



F0 (chewy candy without nanoemulsions); F1 (Lg0.00 : Lm4.10); F2 (Lg0.05 : Lm4.05); F3 (Lg1.85 : Lm2.25); F4 (Lg3.65 : Lm0.45); F5 (Lg4.10 : Lm0.00)

Fig. 3: Inhibitory activities of Lg/Lm blend EOs nanoemulsion (A) and chewy candy (B) against *S. mutans* and *P. gingivalis*. Error bars represent the standard deviation of the mean (n=3). Means with different superscripts for each sample are significantly different ($p < 0.05$)

As Gram-positive bacteria, they are enveloped in a thicker (12.5–50 nm) peptidoglycan layer than that is found thinner (2–3 nm) in Gram-negative bacteria.[36] This polyaminosugar-peptide complex is hydrophilic, thus enabling our oil-in-water nanoemulsions to pass through the cell wall of *S. mutans* stronger than *P. gingivalis* (Gram-negative bacteria). In agreement with this result, it has been confirmed the efficacy of emulsified Lm and Lg EOs against *S. mutans*.[37,38] The thick layer of peptidoglycan in Gram-positive bacteria also has a teichoic acid, a molecule that is responsible for hydrophobicity and surface charge of the bacterial cell wall, but still possibly allowing the penetration of such nanoemulsions.

There was an increasing inhibitory effect of the nanoemulsion when Lg and Lm EOs were used in combination compared to their single use. It was particularly noted that blending these EOs at a close ratio (F3, Lg1.85 : Lm2.25) presented the highest activity of nanoemulsion against *S. mutans* (92.40%) and *P. gingivalis* (84.14%). This can be due to the nanoemulsion F3 being comprised of more volatile compounds (Table 1). When eugenol, one of the potential antibacterial compounds, is specifically highlighted, nanoemulsion F4 had the highest content but it was the second strongest antibacterial agent in this study. Moreover, with respect to the particle size of the nanoemulsion, F3

had the smallest particle size (155.7 nm) while F4 showed the biggest size (420 nm) (Table 2). These results imply that the variety of volatile compounds and the particle size of nanoemulsion are the key driving forces for improving antibacterial activity.

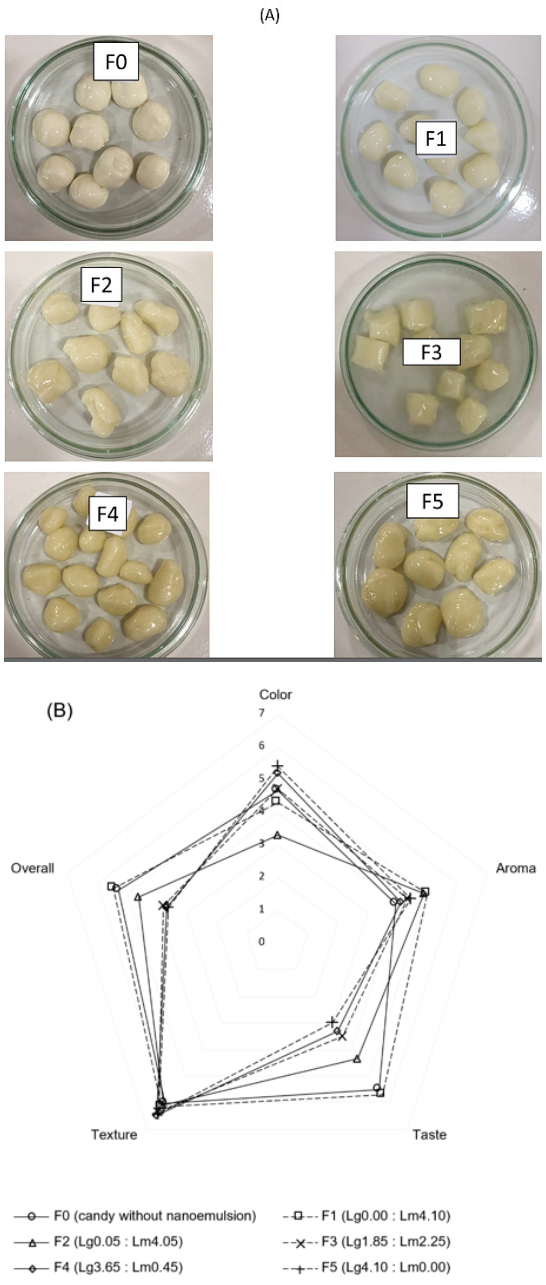


Fig. 4: Photograph (A) and sensory acceptability (B) of chewy candy

The results also showed that the lowest inhibitory activity was found in the nanoemulsion containing Lm EO only, while nanoemulsion with Lg EO at any ratio likely strengthened the antibacterial activity. A high antibacterial activity of Lg EO against pathogenic oral bacteria has also been proven.[5] Citral, geraniol, and myrcene are three major volatiles in Lg EO which are known to have high antibacterial activities. The result of the antibacterial activity of nanoemulsion also convinces the antibacterial activity of chewy candy enhanced by those nanoemulsion.

A similar trend of inhibitory activity against both *S. mutans* and *P. gingivalis* was also observed in enhanced chewy candies (Figure 3B). All nanoemulsion formulations significantly amplified the inhibitory activity of the chewy candy than that of without nanoemulsion (F0) which resulted in almost no inhibitory effect. However, it was shown that the incorporation of nanoemulsion into candy formulation significantly reduced the inhibitory activity against both bacteria, with an average decrease of 5.43% (*S. mutans*) and 4.72% (*P. gingivalis*). This can be due to the dilution of active compounds from the EOs, resulting in a complex matrix of ingredients. One of the well-studied mechanisms of EOs' antibacterial properties was attributed to the lipophilicity of the compounds to penetrate inside the cytoplasm of bacterial cells while collapsing the integrity of cell membranes.[39] Incorporating EOs in the food matrix results in less contact of the active compounds to reach the bacterial cells, thus decreasing their antibacterial activity.

Sensory Evaluation of Chewy Candy

The result of the sensory evaluation of chewy candy developed with Lg/Lm blend EOs nanoemulsion was presented in Figure 4B. The sensory scores varied for all of the sensory attributes, except for texture, in which all the formulated candies were very much liked (score 6) by the panelists. It was found that the color score decreased significantly as the ratio of Lm EO increased. This means that the yellowish-colored candies made with F4 and F5 nanoemulsion formulations are more favored since they had greater b* values, 11.91 and 13.88 (Table 3), respectively.

In contrast, for the aroma attribute, the use of high ratio of Lm EO (F1 and F2) was rated at level 5 (slightly liked) while the other candies were scored lower. Score 4 (neither liked nor disliked) was given to the rest of the candies, except for control with a lower score of 3 (slightly disliked). In comparison to the aroma of the candy without nanoemulsion, both the single and the blended EOs demonstrated their suitability to enhance the aroma of the candy. In the single use of the EO viewpoint, the previous study also reported the advantage of 20 µL/100 g of Lm EO over without Lm EO (control) in elevating the aroma of the hard candy remarkably.[40] Besides, It was suggested the use of Lg EO to enrich flavor was not limited to candy but could also be expanded to dairy-based frozen desserts, non-alcoholic beverages, puddings, and meat products.[41]

In the case of candies formulated with F2, F3, and F4 nanoemulsion formulations, Table 1 can be used to represent the volatile compounds of those candies. Some oxidation products such as *cis*-limonene oxide, linalool oxide, epoxy-linalool oxide, and caryophyllene oxide were more abundantly shown by F3 and F4 formulations, thus contributing to the undesirable off-flavors and eventually decreased the acceptability of candy aroma. Further study is needed to improve the ability of SPI/lecithin so that oxidative deterioration of major volatile compounds of both EOs can be halted.

Even though the aroma of the candy containing Lg EO nanoemulsion (F5) was more preferred than that of the candy without nanoemulsion, the taste was found to be slightly disliked (score 3). It is probably due to the pungent sensation of Lg EO.[41] Citral and geraniol were reported to cause moderate and slight pungencies, respectively, by activating and modulating transient receptor potential ion channels that are abundantly found in the nerve terminals of the mouth, nose, and tongue.[42,43] However, the score of the candy taste increased from 3 to 5 with an increased ratio of Lm EO which may be due to a minimum pungent sensation detected by the panelists. A quenching activity of limonene toward the intensity of citral sensitization has been studied.[44] It was also confirmed that candy prepared with Lm EO had a higher organoleptic score (3.5=neutral) than the control (without Lm EO) which scored 2.5 (do not like).[40] Consequently, it is likely that the Lm EO can be used as a taste enhancer.

The overall acceptability of formulated candy was rated at level 4 (neither liked nor disliked) for F3, F4, and F5; and 5 (slightly liked) for F2, F1, and F0. This result shows that the overall acceptability can be mainly associated with the taste due to a similar trend observed. This result also reveals that the use of Lm EO in combination with Lg EO can improve the overall acceptability of chewy candy, especially Lm EO, that strongly gives a pleasant lemony aroma. A previous study also found that gummy supplements enriched with blueberry anthocyanin vitamin D and mango-flavored phycocyanin were overall preferred because of their aroma and taste.[45] Therefore, this finding will help further studies and/or market-oriented practical applications to develop a chewy candy with a fruity flavor rather than an aromatic herb flavor.

Conclusion

The combination of Lg and Lm EOs improves the antibacterial activity of their nanoemulsion forms and when incorporated into chewy candy formulations against *S. mutans* and *P. gingivalis*. The smaller particle size of the nanoemulsion boosts the antibacterial activity while the lowest PDI and zeta potential stabilize the nanoemulsion. The diversity in volatile compounds of nanoemulsion affects the sensory acceptability of the chewy candy without altering the texture attribute. Due to the taste of the chewy candy being mainly compromised by the addition of Lg EO, further study using an appropriate natural antimicrobial agent rich in fruity flavor in combination with Lm EO needs to be done. The natural agents should have a wide-spectrum antimicrobial activity to tackle the unsatisfactory results of TPC in our study.

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Conflict of Interest

The authors declare no conflict of interest.

Authors' Contribution

Each author mentioned has significantly and directly contributed intellectually to the project and has given their approval for its publication. Jay Jayus: Conceptualization, Project administration, Supervision, Writing-review & editing. Yuli Witono: Conceptualization, Supervision, Writing-review & editing. Mohammad Rizky: Formal analysis, Investigation, Writing-review & editing. Maryam Tsaqifah Muwahhidah: Formal analysis, Investigation, Writing-review & editing. Jenny Marlissa: Formal analysis, Investigation, Writing-review & editing. Aji Sukoco: Conceptualization, Supervision, Methodology, Resources, Writing-original draft.

Data Availability

The manuscript incorporates all datasets produced or examined throughout this research study.

Ethics Approval

In the sensory evaluation, an ethical clearance to use panelists to assess the sensory attributes of the chewy candy was not enclosed as the ingredients used were food-grade and obtained from food-purposed companies/shops.

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