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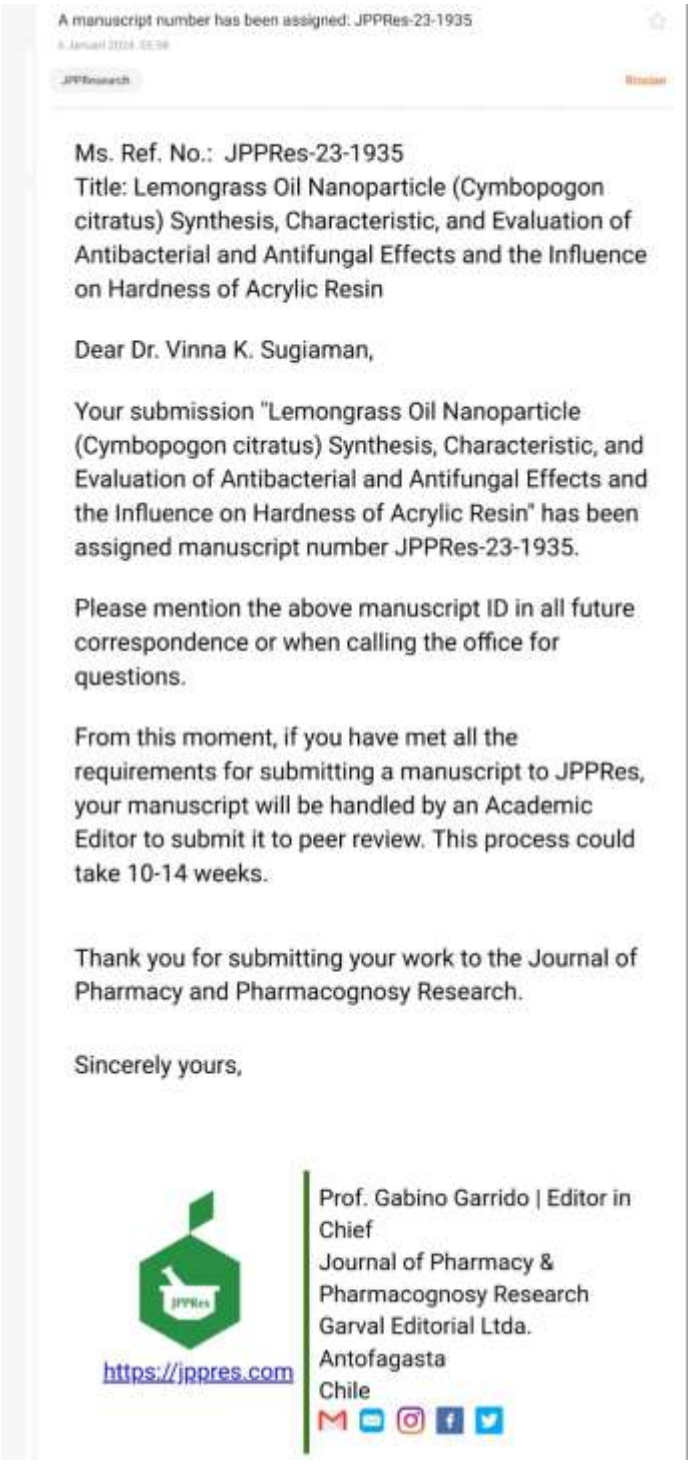

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Lemongrass Oil Nanoparticle (*Cymbopogon citratus*) Synthesis, Characteristic, and Evaluation of Antibacterial and Antifungal Effects and the Influence on Hardness of Acrylic Resin

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ABSTRACT

Introduction: Acrylic resin is used in dentistry as a removable denture base. It can cause various pathologies when not properly cleaned. One of the pathologies is denture stomatitis caused by *Candida albicans* and *Streptococcus mutans* accumulation on the acrylic resin surface. Therefore, microbial agents such as denture cleansers are needed. This study observed lemongrass (*Cymbopogon citratus)* as an herbal material in nanoparticles to provide better antimicrobial effects. **Methods:** Lemongrass oil nanoparticle (LON) was synthesized and analyzed by Transmission Electron Microscopy (TEM). Electrospray ionization tandem mass spectrometry (Esi-Ms) analysis was used to analyze the characteristics of LON bioactive components. Minimum Inhibitory Concentration (MIC) and Minimum Bacterial Concentration (MBC) against *Candida albicans* and *Streptococcus mutans* and mechanical hardness test of acrylic were performed. **Results:** The LON concentration of MIC and MBC against Candida albicans and Streptococcus mutans was 25% and 100%, respectively. One-way ANOVA showed no significant difference between groups of LON with different concentrations (p-value = 0.687). A paired t-test showed significant differences in acrylic resin hardness before and after treatment of LON with 100% (p-value = 0.022) and 50% (p-value = 0.021) concentration. There was no significant difference in hardness before and after treatment of other concentrations of LON and chlorhexidine as positive control. **Conclusion:** LON treatment on acrylic resin decreased the growth of *Candida albicans* and *Streptococcus mutans* without altering the mechanical properties (hardness).

Keywords: *Streptococcus mutans*, *Candida albicans*, *Cymbopogon citratus*, *Acrylic resin*, *Nanoemulsion*

INTRODUCTION

The acrylic resin base is part of the denture, attached to oral soft tissue, and can cause denture stomatitis if oral hygiene is poor.[1] Denture stomatitis is when the mucosal tissue covered by the denture becomes inflamed and erythematous. The prevalence of denture stomatitis is

approximately 20-67% and is experienced by 2/3 of complete denture wearers.[2,3] This condition is triggered by decreased salivary flow and oxygen in the tissue underneath the denture, which causes microorganisms to grow. Poor oral hygiene also influences denture stomatitis by facilitating the fungal cells to attach to the denture base and increasing the growth of bacterial colonies in the oral cavity.[2,4]

Candida albicans (*C. albicans*) and *Streptococcus mutans (S. mutans)* are the most common fungi and bacteria that cause denture stomatitis.[5–8] At the beginning of this disease, the patients will have no symptoms. Nevertheless, as the disease progresses, patients will complain about discomfort, dry mouth with burning sensation, pain, mucosal bleeding, taste disorders, erythematous and mild inflammation.[2,7,9–12]

Therefore, insufficient cleaning of the denture will retain the fungi and bacteria, which will then cause the formation of plaque and calculus, which will play a role as a reservoir of more complex pathogen microorganisms.[13–15] Antimicrobial agents are then used to control the microorganism's growth. However, the efficient dose of this agent usage should be ideally considered. Prolonged denture submersion in the antibacterial agent will influence its mechanical properties. This alteration will cause erosion of the denture surface, a decrease in hardness, and micro-scratches on the surface, increasing microorganisms.[15–19]

Using chemical antimicrobial agents has adverse effects on the environment and human health compared to natural agents. Thus, recently, there have been increasing initiatives to utilize natural ingredients in medications.[20,21] These herbal agents can be obtained from various sources, such as animals, sea organisms, and plants, which contain secondary metabolites with antimicrobial potential.[22,23] These secondary metabolites include tannin, flavonoid, saponin, phenol, steroid, alkaloid, and glycoside, which can provide preventive and therapeutic effects.[17,18,20,24]

Several studies have shown that lemongrass has distinct and promising characteristics.[20,25] Lemongrass has antimicrobial, antifungal, antiprotozoal antioxidant, antimutagenic, and antiinflammation activities.[26] Lemongrass could also prevent denture biofilm formation by inhibiting bacterial cell membranes, altering ergosterol biosynthesis, and inhibiting fungi proliferation and spore germination.[27,28]

The utilization of natural materials' advantages as antimicrobials has the potential to develop into nanoparticles with sizes between 1-100 nm. Nanoparticles not only have superior physical and chemical properties compared to particles with bigger sizes, but the synthesis of nanoparticles is also environment-friendly, cost-effective, non-toxic, and more biocompatible.[29–31] Nanoparticles have unique characteristics based on size, distribution, and particle morphology. For example, smaller particle size will increase particle ability to penetrate bacterial cell membranes, increasing the antimicrobial effect.[31,32] These active biological components, characteristics, and physical properties of nanoparticles will produce the therapeutic results of nanoparticles, which can be applied as broad-spectrum antibacterial and antifungal agents.[33–35]

This research synthesized and analyzed a lemongrass oil nanoparticle (LON), Cymbopogon citratus, with Transmission Electron Microscopy (TEM). The bioactive component was characterized by an electrospray ionization tandem mass spectrometry (Esi-Ms) analysis. The minimum Inhibitory Concentration (MIC) and minimum Bactericidal Concentration (MBC) were performed against *S. mutans* and *C. albicans.* Then, after applied to acrylic resin, the hardness was tested to observe the influence of LON on the mechanical properties of acrylic.

METHODS

Lemongrass Extraction

Lemongrass extraction was performed in PT Indesso Aroma Pharmaceutical Company, Baturaden, Purwokerto, Central Java. The extraction process was part of 'the development of standardized herbal medicine from natural ingredients' project, including lemongrass extract as biofilm in acrylic resin. The extraction result is in oil form, then processed for certification of analysis for safety production.

Lemongrass Oil Nanoemulsion (LON) Production

Five milliliters of lemongrass oil (PT Indesso) was mixed with 5 ml of propylene glycol (Carbowax - X29160), 5 ml of PEG 400 (Carbowax - X29160**),** and 10 ml of glycerin (Merck-104094). All mixtures were stirred until homogenous. After that, 5 ml of chromophore was added and mixed with the previous solution.

***Transmission Electron Microscopy* (TEM) Morphological Characteristics Testing**

Morphology analysis was performed with a *Transmission Electron Microscopy* (TEM) analyzer (JEOL JEM-1400) at FMIPA Laboratory, Gajah Mada University, Yogyakarta. Ten μL of the sample were dropped into a *grid* and left still for 1 minute, and then the remaining liquid was removed with a micropipette. Ten μL of uranyl acetate was dropped into a grid, and the remaining liquid was removed with a micropipette. The grid was dried for 30 minutes and then observed under the TEM. TEM is a preferred nanoparticle size, granule size, size distribution, and morphology measurement method.[36]

***Electrospray Ionization-Mass Spectra* (ESI-MS) Measurement**

A sample of 0.03 grams was weighed with an analytic balance (Mettler Toledo, Swiss) and dissolved in 90% acetonitrile before being sonicated (DAW, USA) for 30 minutes. After that, quenchers were added to separate the sample contaminant and centrifuged for 10 minutes with a velocity of 10000 rpm. Then, the sample was filtered by a 0.2-micron membrane and tested with ESI-MS (TSQ Quantum Access Max) with a velocity of 5 microliters per minute and added eluent methanol with 0.1% formic acid.

Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) Analysis of *Streptococcus mutans* dan *Candida albicans*

Culture Media Preparation

Mueller Hinton Agar (MHA) (Himedia, M096-500G) and *Mueller Hinton Broth* (MHB) (Himedia, M403-500G) were used as the culture media. Thirty-eight grams of MHA and 21 grams of MHB were dissolved in 1000 mL of ddH_2O with the aid of a microwave (Shivaki, SMW 103). Then, the media were sterilized with an autoclave (HiClave, H-50) with a temperature of 121^oC and pressure of 1.5 atm for 25 minutes.

LON Preparation

Thirty ml of 100% LON stock were diluted using the Serial Working Solution method with ddH_2O and 10% DMSO to prepare series concentrations of 100%, 50%, 25%, 12.5%, 6.25%,

3.125%. After that, the sample concentrations were filtered using a 0.22 µm pore *syringe filter* (Sartorius, 17845) to obtain sterile samples.

MIC and MBC Analysis

S. mutans and *C. albicans* inoculums preparation

Inoculums of *S. mutans* (ATCC-25175) and *C. albicans albicans* (ATCC-10231) were cultured using the *direct colony suspension* method from the primary culture after 24 hours on MHA and MHB media. The turbidity of the inoculums was adjusted with McFarland 0,5 Standard to get bacteria suspensions of 1-5×10^8 CFU/mL. Then, inoculum suspensions were dissolved in MHB with a ratio of 1:50 to get 2×10^6 - 1×10^7 CFU/mL of bacteria suspensions. After that, further dissolving with a ratio of 1:20 was done to get 1-5×10^5 CFU/mL of bacteria suspension.

Broth Microdilution Method

One hundred µl of inoculum were added to microplate wells and mixed with series concentrations of LON. One hundred µl working solution was added to each concentration until it reached the final concentration. After that, 100 µl of chlorhexidine 0.2% (0011221-C107) was added to wells as the positive control. One hundred µl MHB and 100 µl of inoculum were added to wells as a growth or negative control to antibacterial inhibition. One hundred µl of MHB and 100 µl of working solution of each LON concentration and Chlorhexidine 0.2% (0011221-C107) were added to wells as blank groups.

The microplates were incubated for 24 hours at 37^{o}C and then observed with *spectrophotometry* (Multiskan GO Thermo Scientific 51119300) with 450 nm wavelength for *S. mutans* and 520nm wavelength for *C. albicans. S. mutans* and *C. albicans* growth was defined by Optical Density (OD) comparison between each treatment group and its blank groups. MIC was defined from the lowest LON concentration, which provided inhibition for more than 50% against microorganism growth. MBC was defined from the lowest LON concentration, which provided inhibition against microorganism growth for 99%.

Pour Plate Method

After the spectrophotometry measurement from each treatment in the microplate, the pour plate method confirmed that the MBC measurement observed could inhibit microorganism growth in agar media. Twenty µl from each well were cultured in MHA media with the pour plate method and then incubated (Thermo IH3543) for 24 hours at 37^{o}C. Visual observation was done to count the colony number with the colony counter (Funke Gerber 8500). The MBC was defined from the concentration that showed almost no agar media colony growth. The inhibition percentage was counted with the following formula:

$$\%\ Inhibition = \frac{Negative\ Control\ Colony - \ Total\ Treatment\ Colony}{Negative\ Control\ Colony} \times 100\%$$

Acrylic Specimen Fabrication

The wax model was sculpted in a flat cylinder with 5 mm diameter and 2 mm thickness with baseplate wax (Cavex) and placed into the cuvette. Then, a cold mold seal (CMS) was smeared

into the cuvette, and the wax was boiled out. Heat-cured acrylic (ADM, ISO 1567) was stirred and packed into the cuvette, then cured for 60 minutes at 100^{o}C. After that, the acrylic was de-flasked, finished, and polished with grit number 400.

Vickers Hardness Test

Acrylic resin hardness was measured with a Micro Vickers Hardness Tester (Shimadzu, HMV-G21ST Series). Specimens were pressed with a pyramid-shaped diamond indenter with a rectangle base and face-to-face surface angled 136^{o}. Test load ranges were 5, 10, 30, and 50 kgf with 10-15 seconds dwell time. The minimal distance between the central point trace and the specimen edge is 2.5 times the (ASTM) distance of the diagonal of the trace, and the minimal distance between neighboring traces was 2.5 times the distance of the diagonal of the trace. The applied pressure would result in traces such as indentation on the specimen surface. The Vickers hardness value was obtained by dividing the test load size by the traces' surface area.

RESULTS

Lemongrass Oil Nanoemulsion (LON) Certificate of Analysis (CoA)

CoA was done by observing the fabricated nanoparticle's color and odor. This analysis was one of the important steps in determining product quality and grade. The result obtained from the current study was that LON had a yellow color without a specific odor.

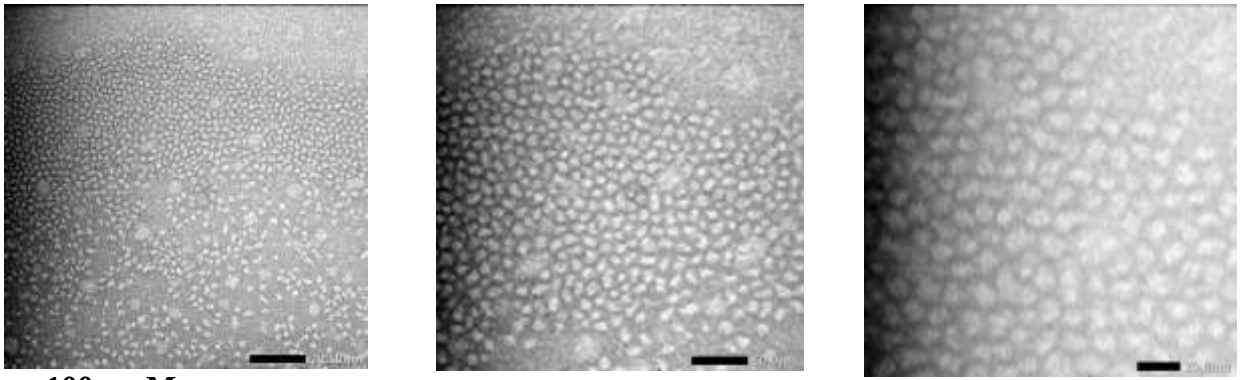
Table 1 Lemongrass Oil Nanoemulsion (LON) CoA

Characteristic	**Specification**	**Result**
Appearance	Liquid	Liquid
Color (Visual)	Pale Yellow-Yellow Brown	Yellow
Organoleptic (odor)	Conform to standard	Conform to standard
Acid value (titration)	Max 8.0	2.4
Optical rotation (25^{O}C)	(-)6-0	-2
Refractive index (20^{O}C)	1.480-1.493	1.486
Specific gravity (D25/25)	0.872-0.897	0.892

Physical properties of LON (Table 1) showed that the observation result was within the standard range. Therefore, LON could be used to develop herbal medicine from lemongrass as an antimicrobial.

Morphological Characteristics Analysis with *Transmission Electron Microscopy* (TEM)

TEM is the gold standard and most efficient method for obtaining the nanoparticle morphological characteristics and size. The method provides accurate characterization, such as shape, structure, and size information. This method will present a two-dimensional nanoparticle illustration, which can be observed for number base shape distribution.[37,38]



100nm Measurement **50nm Measurement** **20nm Measurement**

Figure 1 Transmission Electron Microscopy Result from Lemongrass Oil Nanoemulsion (LON)

Figure 1 showed that LON sample size measurements were uniformly between 10-100 nm with irregular individual particle shapes. In TEM measurement, the sample observed should be between 60-90 nm thick to penetrate by electron.[37] However, TEM is favorable for characterizing nanoparticles because the resolution reaches approximately 0.07 nm.[39]

Mass Spectrophotometry (ESI-MS) Result of LON

Determination of the substance's component of LON could be characterized with ESI-MS (TSQ Quantum Access Max). ESI-MS was also used to analyze herbal compounds' nanoparticles, substance composition, and molecule mass.[40] The chromatogram and substance of LON compounds are presented in Figure 2 and Table 2.

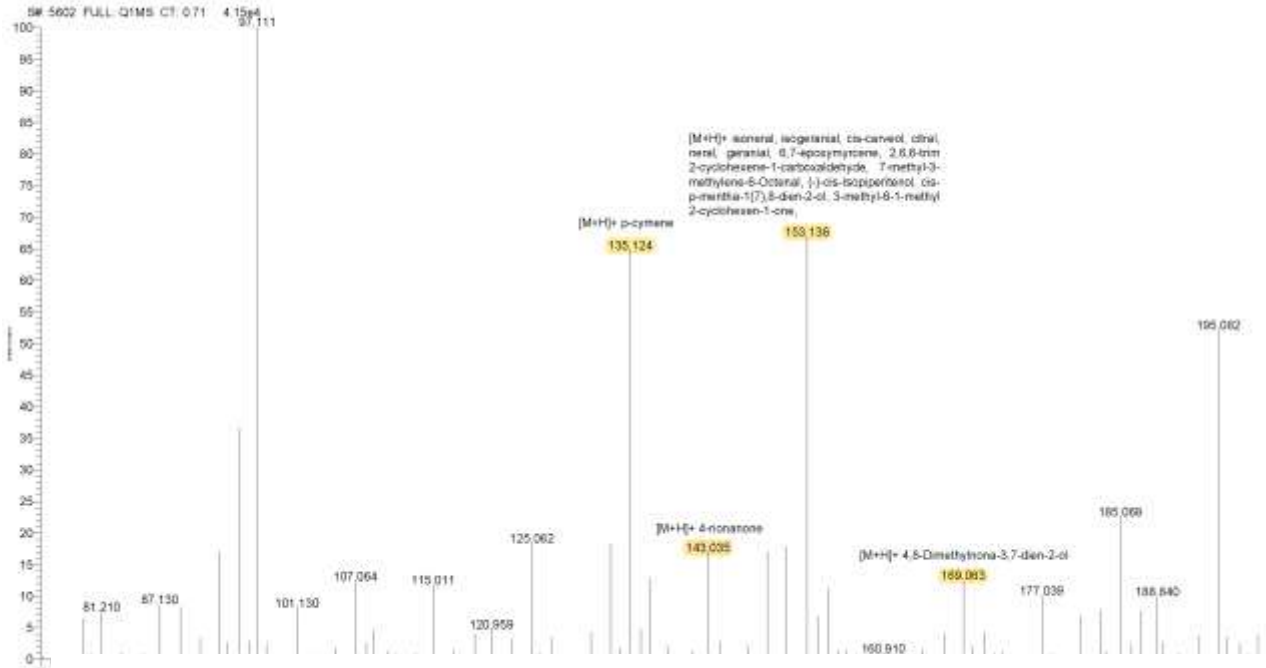


Figure 2A Mass spectra 75-200 m/z of lemongrass oil nanoemulsion (LON) with positive

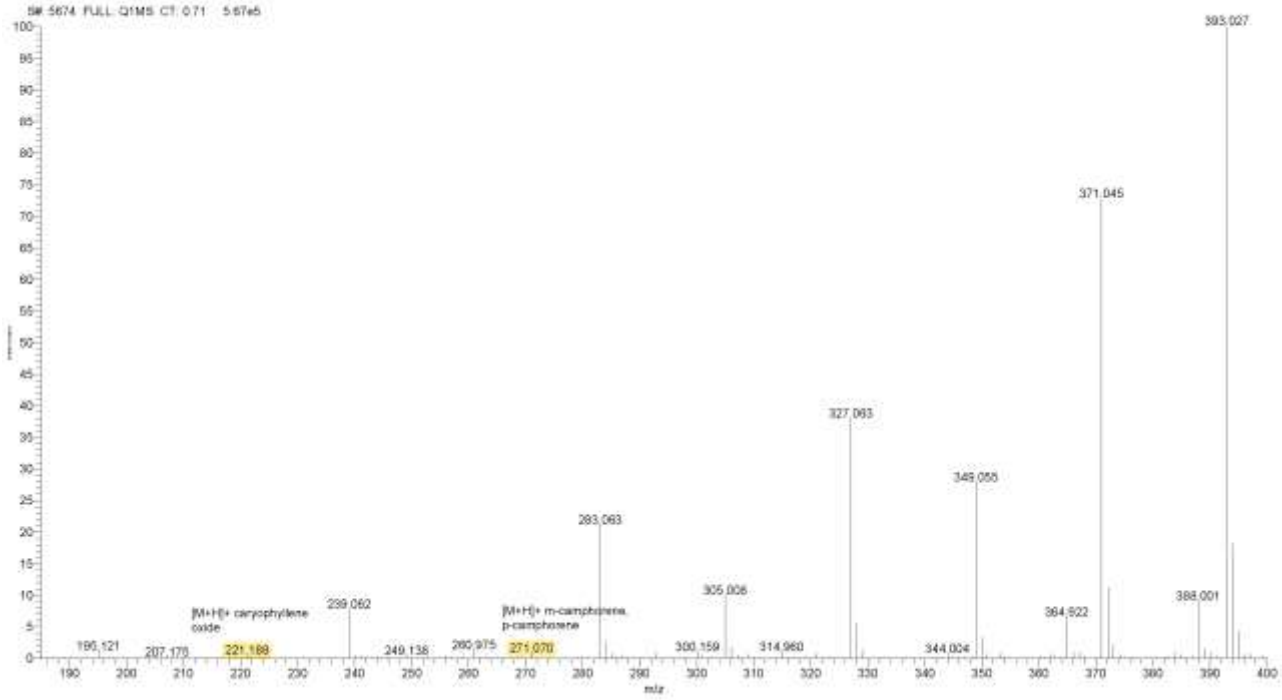


Figure 2B Mass spectra 200-400 m/z of lemongrass oil nanoemulsion (LON) with positive ionization.

Figures 2A and 2B illustrated that LON contained several compounds, as presented in Table 2.

Table 2 Target compounds identification of lemongrass oil nanoemulsion (LON) with ESI-MS

Lemongrass Oil Nanoemulsion	**Molecular Weight (MW) (g/mol)**	**Mass Spectra (MS) $[M-H]^+$**
p-cymene	134.220	135.124
4-nonanone	142.240	143.035
Isoneral	152.233	153.136
Isogeranial	152.233	153.136
Cis-carveol	152.233	153.136
Citral	152.233	153.136
Neral	152.233	153.136
Geranial	152.233	153.136
6,7-epoxy myrcene	152.233	153.136
2,6,6-trim 2-cyclohexene-1-carboxaldehyde	152.233	153.136
7-methyl-3-methylene-6-Octenal	152.233	153.136
(-)-cis-Isopiperitenol	152.233	153.136
Cis-p-mentha-1(7),8-dien-2-ol	152.233	153.136
3-methyl-6-1-methyl 2-cyclohexen-1-one	152.233	153.136
4,8-Dimethylnona-3,7-dien-2-ol	168.280	169.063
Caryophyllene oxide	220.350	221.188
m-camphorene	272.468	271.070
p-camphorene	272.468	271.070

Minimal Inhibitory Concentration (MIC) and Minimal Bactericidal Concentration (MBC) Results Against *Streptococcus mutans* and *Candida albicans*

The antimicrobial activity of LON against *S. mutans* and *C. albicans* was determined by the MIC and MBC.

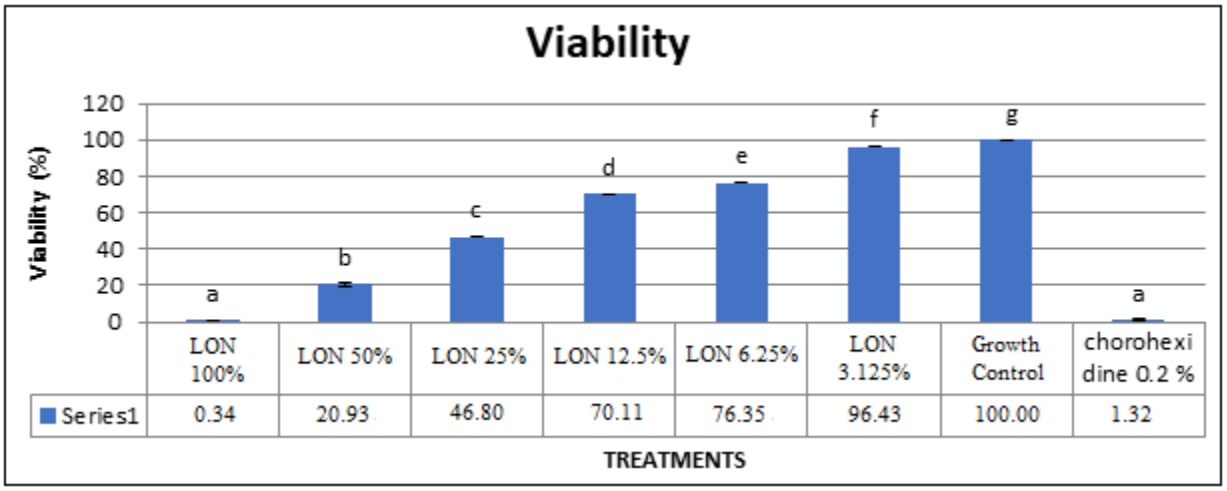
MIC and MBC against *S. mutans*

Table 3 Viability and Inhibition of Lemongrass Oil Nanoemulsion (LON) against *S. mutans*

Sample	**Viability (%)**	**Inhibition (%)**	
LON1	**0.34 ± 0.19^{a}**	**99.66 ± 0.19^{g}**	**MBC**
LON2	20.93 ± 1.10^{b}	79.07 ± 1.10^{f}	
LON3	**46.80 ± 0.19^{c}**	**53.20 ± 0.19^{e}**	**MIC**
LON4	70.11 ± 0.19^{d}	29.89 ± 0.19^{d}	
LON5	76.35 ± 0.28^{e}	23.65 ± 0.28^{c}	
LON6	96.43 ± 0.22^{f}	3.57 ± 0.22^{b}	
Negative Control	100.00 ± 0.11^{g}	0.00 ± 0.11^{a}	

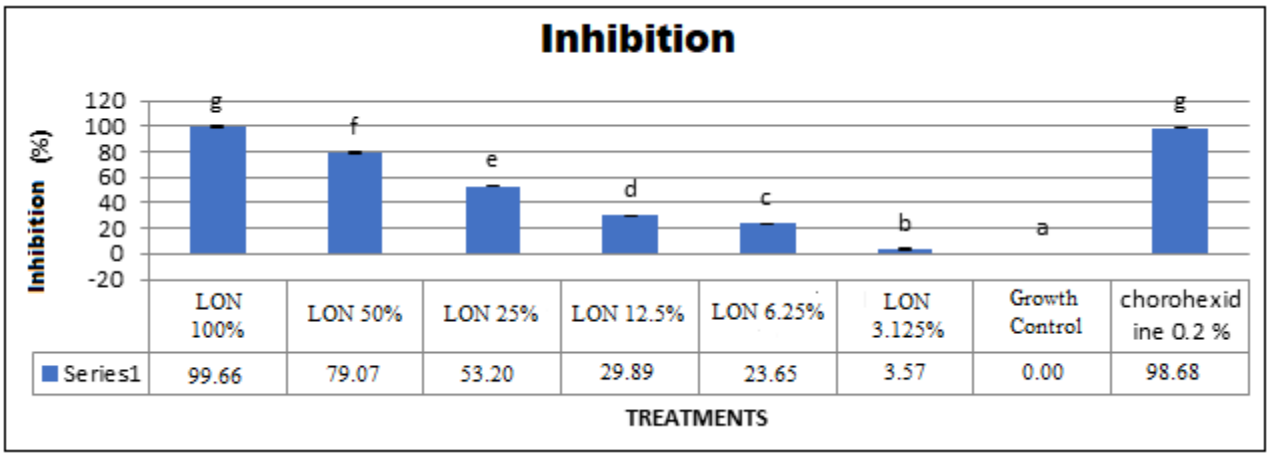
Positive Control (Chlorhexidine 0.2%)	1.32 ± 0.45^{a}	98.68 ± 0.45^{g}

* Data were presented in average ± deviation standard. a b, c, d, e, f, g symbols showed significant differences in the Tukey HSD test ($p<0.05$).



* Data were presented in average ± deviation standard. a, b, c, d, e, f, g symbols showed significant differences in the Tukey HSD test ($p<0.05$).

Figure 3. Viability percentage of *S. mutans* after Lemongrass Oil Nanoemulsion (LON) treatment.



* Data were presented in average ± deviation standard. a, b, c, d, e, f, g symbols showed significant differences in the Tukey HSD test ($p<0.05$).

Figure 4. Inhibition percentage of Lemongrass Oil Nanoemulsion (LON) against *S. mutans*.

Table 4 Colony Forming Unit of *S. mutans* (CFU/ml)

Sample	Dilution	Colony Count	CFU/ml	Average	Average

	Factor	1	2	3	4	1	2	3	4		
Positive Control	10000	0	0	0	0	0	0	0	0	0	0
Negative Control	10000	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC
LON 100%	10000	0	0	0	0	0	0	0	0	0.00	0
LON 50%	10000	31	33	35	33	31×10^3	33×10^3	35×10^3	33×10^3	33.00	33×10^3
LON 25%	10000	72	71	75	77	72×10^3	71×10^3	75×10^3	77×10^3	73.75	73.75×10^3
LON 12.5%	10000	120	126	123	128	120×10^3	126×10^3	123×10^3	128×10^3	124.25	124.25×10^3
LON 6.125%	10000	181	185	175	168	181×10^3	185×10^3	175×10^3	168×10^3	177.25	177.25×10^3
LON 3.125%	10000	210	215	220	218	210×10^3	215×10^3	220×10^3	218×10^3	215.75	215.75×10^3

*TNTC: too numerous to count

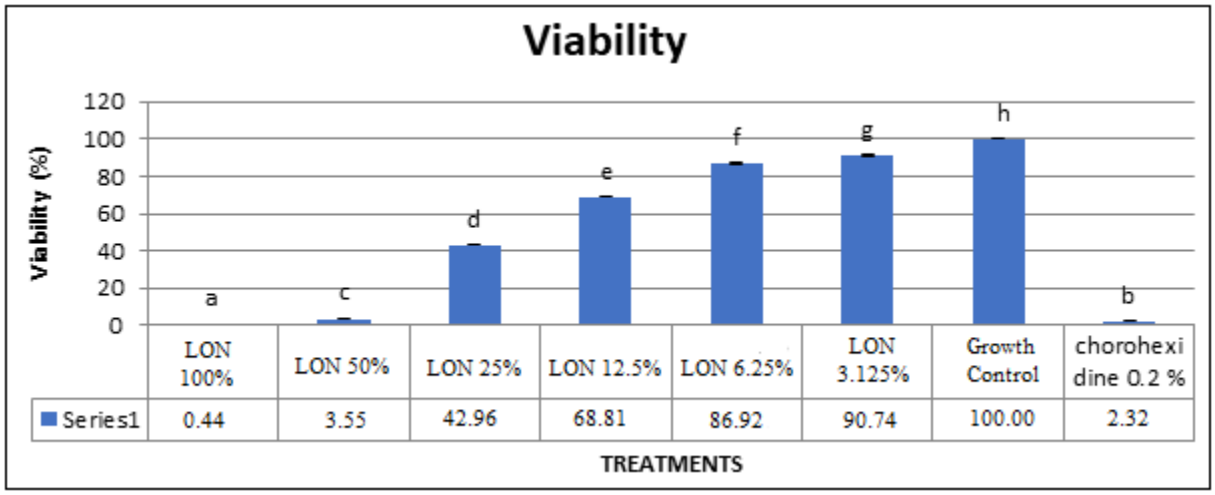
The MIC value of LON against *S. mutans* was at 25% concentration, and the MBC was at 100%. The inhibition level of LON against *S. mutans* growth was considered high because it showed results similar to the positive control. The inhibition level was proportional to the concentration; higher concentrations showed more potent *S. mutans* inhibition.

MIC and MBC against *C. albicans*

Table 5 Viability and Inhibition of Lemongrass Oil Nanoemulsion (LON) against *C. albicans*

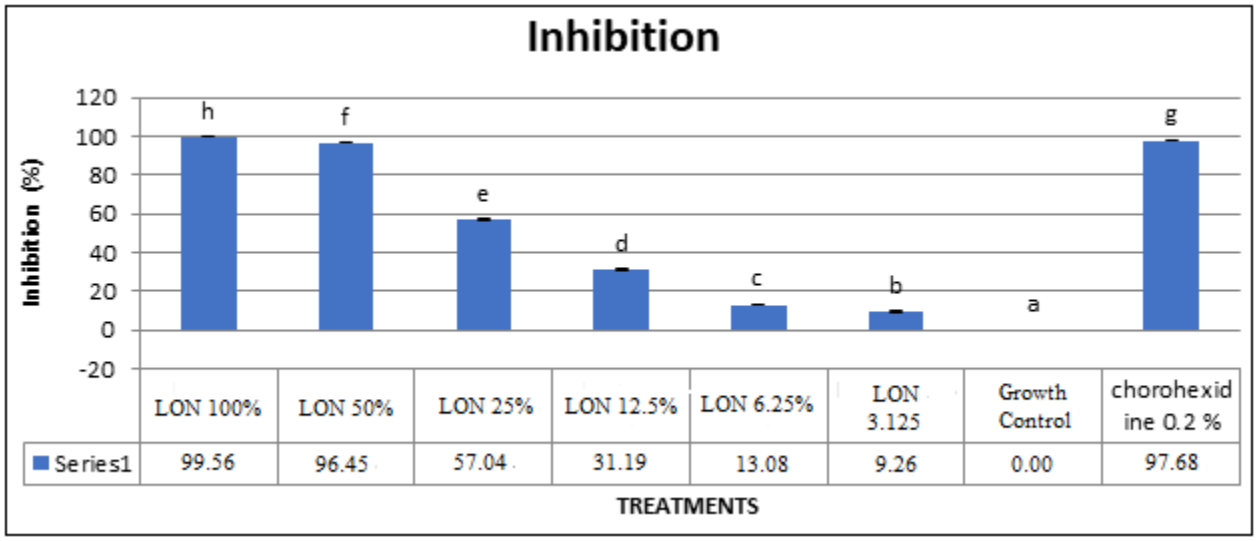
Sample	Viability (%)	Inhibition (%)	
LON1	**0.44 ± 0.04^a**	**99.56 ± 0.04^h**	**MBC**
LON2	3.55 ± 0.08^c	96.45 ± 0.08^f	
LON3	**42.96 ± 0.03^d**	**57.04 ± 0.03^e**	**MIC**
LON4	68.81 ± 0.12^e	31.19 ± 0.12^d	
LON5	86.92 ± 0.11^f	13.08 ± 0.11^c	
LON6	90.74 ± 0.60^g	9.26 ± 0.60^b	
Negative Control	100.00 ± 0.05^h	0.00 ± 0.05^a	
Positive Control (Chlorhexidine 0.2%)	2.32 ± 0.04^b	97.68 ± 0.04^g	

* Data were presented in average ± deviation standard. a, b, c, d, e, f, g symbols showed significant differences in the Tukey HSD test ($p<0.05$).



* Data were presented in average ± deviation standard. a, b, c, d, e, f, g symbols showed significant differences in the Tukey HSD test ($p<0.05$).

Figure 5. Viability percentage of *C. albicans* after Lemongrass Oil Nanoemulsion (LON) treatment.



* Data were presented in average ± deviation standard. a, b, c, d, e, f, g symbols showed significant differences in the Tukey HSD test ($p<0.05$).

Figure 6. Inhibition percentage of Lemongrass Oil Nanoemulsion (LON) against *C. albicans*.

Table 6 Colony Forming Unit of *C. albicans* (CFU/ml)

Sample	Dilution Factor	Colony Count				CFU/ml				Average	Average
		1	2	3	4	1	2	3	4		
Positive Control	1000	0	0	0	0	0	0	0	0	0	0
Negative	1000	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC

Control											
LON 100	1000	0	0	0	0	0	0	0	0	0	0
LON 50	1000	5	2	2	4	5×10^3	2×10^3	2×10^3	4×10^3	3.25	3.25×10^3
LON 25	1000	29	31	33	33	29×10^3	31×10^3	33×10^3	33×10^3	31.50	31.50×10^3
LON 12.5	1000	43	45	45	48	43×10^3	45×10^3	45×10^3	48×10^3	45.25	45.25×10^3
LON 6.125	1000	58	56	51	55	58×10^3	56×10^3	51×10^3	55×10^3	55.00	55.00×10^3
LON 3.125	1000	62	61	63	61	62×10^3	61×10^3	63×10^3	61×10^3	61.75	61.75×10^3

*TNTC: too numerous to count

The MIC value of LON against *C. albicans* was at 25% concentration, while the MBC was at 100% concentration. The inhibition rate of LON against *C. albicans* was proportional to the concentration. Higher concentrations showed a more potent inhibition rate.

Hardness Test of Acrylic Specimen

A hardness test was performed to evaluate acrylic resin hardness differences before and after LON treatment, as shown in Table 7.

Table 7 Mean Hardness Value and Standard Deviation

Group	Before	After
LON 100%	22.88±0.56	19.36±2.06
LON 50%	22.38±0.88	19.24±1.14
LON 25%	22.38±1.36	21.04±2.01
LON 12.5%	21.44±0.75	19.62±1.09
LON 6.25%	21.12±0.73	19.50±2.15
LON 3.125%	21.60±0.38	19.90±1.78
Chlorhexidine 0,2%	21.00±1.69	20.06±1.18

A one-way ANOVA test was performed on hardness value, and the p-value was 0.687 (>0.05), which can be concluded that there were no significant differences between treatment groups. A paired t-test was performed to evaluate the hardness before and after LON treatment. Only the 100% and 50% LON groups showed significant differences between treatments with p-values of 0.022 and 0.021, respectively. However, other groups, including the control group with chlorhexidine 0.2%, showed no significant differences statistically with p-value >0.05.

DISCUSSION

Lemongrass oil was extracted according to standard and safety production of Certificate of Analysis (CoA). The CoA result showed the specification of compounds, such as appearance, color, odor, titration, purity, solubility, and water content.[41] The physical properties of LON in current research met the chosen standard. Therefore, lemongrass was suitable as an antimicrobial agent from the development of standardized herbal medicine. The lemongrass oil was fabricated into nanoparticles that proved superior characteristics and properties.

The characterization of LON in current research was analyzed with TEM. The results showed that the particles' size and shape characterized the physicochemical properties of LON, which significantly contributed to better particle performance. Other physicochemical characteristics

that influence the particle performance are texture and surface structure.[42,43] Other than affecting the performance, the characteristics also influence nanomaterials' physical and chemical properties, such as electronic, optic, and catalytic aspects.[44] By understanding its characteristics, a nanoparticle's stability can be identified by forming particle aggregation because of inter-particle force. This force will induce interaction between particles and create a more extensive cluster.[45]

Herbal materials have important antimicrobial roles against pathogens such as bacteria and fungi. The antimicrobial activity depends on the biochemical content and morphology of the materials.[20,46,47] The biochemical content of lemongrass is usually affected by several factors, such as plants' characteristics, climate, geographical conditions, part of the plant used, ecological conditions, and harvest timing.[20,46,48]

Lemongrass essential oil has proved to have the ability to inhibit the growth of bacteria and fungi.[47] Essential oil is a mixture of herbal compounds that is easy to evaporate, has low molecular weight, and is hydrophobic. These properties allow the oil to split the lipid component in bacteria cells' membranes and mitochondria, disrupting cell structure and rendering it more permeable. After that, the cell will experience molecular and essential ion leakage and induce cell death.[48,49]

The microbial activities of LON were associated with a high oxygen content of the essential oil, which contains monoterpene and hydrocarbon sesquiterpene, mostly aldehyde and alcohol such as neral/geranial and nerol/geraniol. These contents determined the gram-negative and gram-positive antibacterial properties.[50,51] Higher oxygen content, such as in geranial, has more effective antimicrobial activity.[52] The antibacterial activities of essential oil are also affected by a mixture of several complexes, such as monoterpene, sesquiterpene, and the oxygenated derived.[53]

According to several studies, there are several components of essential oil which have antimicrobial activities, such as monoterpene (C10H16), sesquiterpene (C15H24), diterpene (C20H32), triterpene (C30H40), and other components such as 1,8-cineole, p-cymene, α-terpineol acetate, eugenol, limonene, estragole, menthol, anethole, borneol, thymol, geraniol, cinnamyl alcohol, α-thujone, β-thujone, α-pinene, sabinene, caryophyllene oxide, dan terpinene.[48,53]

LON's ability to inhibit S. mutans was similar to chlorhexidine 0.2%, as shown by the number of *S. mutans* formed in LON with 100% concentration, which is 0 CFU/mL. Therefore, current research proved that the essential oil of lemongrass has antibacterial effects. Smaller particle sizes also gave more substantial antibacterial effects.[31,54]

The current study proved that LON had antibacterial and antifungal effects, as shown by the MIC and MBC results, which align with a previous study by Koseki et al.[55] LON's ability to inhibit the growth of *S. mutans* and C. albicans was similar to chlorhexidine by 0.2%.[31,54] Essential oil in the nanoparticle form of LON has more effective and efficient roles as an antifungal by inhibiting the metabolism process of fungi and then inhibiting its growth.[31]

Older people have a limitation in performing denture mechanical cleaning because of physical deterioration. Therefore, a combination of mechanical and chemical cleaning is suggested. Although this combination sometimes failed to kill C. albicans completely.[56]

This failure can be caused by the extracellular matrix polymer material of the denture, which limits cleaning agents' access to microorganisms located far inside the biofilm.[57] Therefore, denture biofilm should be adequately cleaned daily because biofilm accumulation can be the source of local and systemic disease.[58] Several studies found that there is an accumulation of

Candida after biofilm formation.[57] The porosity of acrylic resin increases the difficulty of mechanical cleaning and infection control in dentures. Thus, denture submersion in disinfectant liquid has become a routine procedure.[59] Submersion of dental prosthesis in chemical cleanser aims to deactivate bacteria, viruses, and fungi activity.[60] However, the commonly used disinfectant agent has disadvantages such as being toxic (glutaraldehyde), corrosive to metal, and inducing skin irritation and mucosal staining. Therefore, an alternative agent that does not influence the denture's properties is needed.[59] Other studies showed that various cleaning agents affect the physical properties of denture bases, such as hardness, transverse strength, roughness, and color.[61] The denture cleaning method should be effective without affecting denture material properties.[62]

Hardness is one of the material properties that influences the surface characteristics of acrylic resin. Hardness is also used to evaluate the alteration because of denture cleaning.[63] Hardness value measurement is an indication of the possibility of polymer matrix degradation. This degradation decreases the hardness value, thereby increasing the possibility of fracture and reducing the length of use of the denture.[64] In the current study, the hardness value of resin acrylic samples after LON treatment declined compared to before treatment. These results were possible because of the water and chemical absorption, which caused decreasing mechanical properties of acrylic resin.[58] In contrast, previous studies showed that hardness values increased after applying coated material and before applying a denture cleaner solution.[57] Another study showed that the hardness value of conventional acrylic resin increased after submersion in sodium hypochlorite cleaning solution and chlorhexidine, mainly after 120 days, which means the longer the submersion time, the greater the hardness value.[65] However, another study showed decreased hardness value after one year of submersion in chlorhexidine disinfectant gels.[66] The variety of results can be affected by diverse materials, procedures, and analysis time for each study.

The hardness value of the current research was observed after treatment, which is in line with other studies that observed that chlorhexidine did not significantly decrease the acrylic resin hardness.[60] Another study comparing vinegar as a denture cleaning agent and chemical agents showed insignificant differences in hardness values between the two groups.[59] This result aligned with current research, which showed insignificant differences in hardness values after control groups and LON treatment groups. The negligible differences in hardness values were also observed from another study, which compared four disinfectant agents, including chlorhexidine.[61] Acrylic resin is hydrophilic, easy to absorb water, and acts as a plasticizer; therefore, the decline of hardness value was also possible because of the formation of cracking zones due to the water absorption process and cycle.[67]

CONCLUSION

The submersion of acrylic resin in lemongrass nanoemulsion effectively inhibited the colony growth of *Streptococcus mutans* and *Candida albicans* without affecting the acrylic resin's mechanical properties.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

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Bukti melakukan review yang pertama (14 Mei 2024)

Interim Decision

JPPRes-23-1935.R1

Title: Lemongrass (Cymbopogon citratus) Oil Nanoparticle Synthesis, Characteristic, and Evaluation of Antibacterial and Antifungal Effects and the Influence on Hardness of Acrylic Resin

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Lemongrass (*Cymbopogon citratus*) Oil Nanoparticle ~~(*Cymbopogon citratus*)~~ Synthesis, Characteristic, and Evaluation of Antibacterial and Antifungal Effects and the Influence on Hardness of Acrylic Resin

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Contribution Details (to be ticked marked (X) as applicable and add columns according to the number of authors):

Contribution	Sugiaman VK	Saputri RI	Naliani S	Amalia J	Jeffrey
Concepts or Ideas					
Design					
Definition of intellectual content					
Literature search					
Experimental studies					
Data acquisition					
Data analysis					
Statistical analysis					
Manuscript preparation					
Manuscript editing					
Manuscript review					

It is mandatory that all authors have reviewed the manuscript before submitting it to the journal.

ABSTRACT

Context: Acrylic resin is used in dentistry as a removable denture base. It can cause various pathologies when not properly cleaned. One of the pathologies is denture stomatitis caused by *Candida albicans* and *Streptococcus mutans* accumulation on the acrylic resin surface. Therefore, microbial agents such as denture cleansers are needed.

Objectives: To evaluate lemongrass (*Cymbopogon citratus)* as an herbal material in nanoparticles to provide better antimicrobial effects.

Methods: Lemongrass oil nanoparticles (LON) were synthesized and analyzed by Transmission Electron Microscopy (TEM). Electrospray ionization tandem mass spectrometry (Esi-Ms) analysis was used to analyze the characteristics of LON bioactive components. Minimum Inhibitory Concentration (MIC) and Minimum Bacterial Concentration (MBC) against *Candida albicans* and *Streptococcus mutans* and mechanical hardness test of acrylic were performed.

Results: The LON concentration of MIC and MBC against *Candida albicans* and *Streptococcus mutans* was 25% and 100%, respectively. One-way ANOVA showed no significant difference between groups of LON with different concentrations (p-value = 0.687). A paired t-test showed significant differences in acrylic resin hardness before and after treatment of LON with 100% (p-value = 0.022) and 50% (p-value = 0.021) concentration. There was no significant difference in hardness before and after treatment of other concentrations of LON and chlorhexidine as positive control.

Conclusions: LON treatment on acrylic resin decreased the growth of *Candida albicans* and *Streptococcus mutans* without altering the mechanical properties (hardness).

Keywords: acrylic resin; *Candida albicans*; *Cymbopogon citratus*; nanoparticle; *Streptococcus mutans.*

The style of citations and references do not comply with JPPRes requirements throughout the manuscript. We appreciate your correction. Also, citations and References must be written free of codes from any bibliography processor.

INTRODUCTION

The acrylic resin base is part of the denture, attached to oral soft tissue, and can cause denture stomatitis if oral hygiene is poor (Alqutaibi AY et al., 2023). Denture stomatitis is when the mucosal tissue covered by the denture becomes inflamed and erythematous. The prevalence of denture stomatitis is approximately 20-67% and is experienced by 2/3 of complete denture wearers (Sartawi SY et al., 2021; Bukhari MA, 2022). This condition is triggered by decreased salivary flow and oxygen in the tissue underneath the denture, which causes microorganisms to grow. Poor oral hygiene also influences denture stomatitis by facilitating the fungal cells to attach to the denture base and increasing the growth of bacterial colonies in the oral cavity (Sartawi ~~SY~~ et al., 2021; Inayat ~~A~~ et al., 2019). *Candida albicans* (*C. albicans*) and *Streptococcus mutans (S. mutans)* are the most common fungi and bacteria that cause denture stomatitis(Volchkova ~~IR~~ et al., 2020; Fujiwara ~~N~~ et al., 2020; Günther ~~E~~ et al., 2020; Archilla ~~AR~~ and Galan ~~CG~~., 2020). At the beginning of this disease, the patients will have no symptoms. Nevertheless, as the disease progresses, patients will complain about discomfort, dry mouth with burning sensation, pain, mucosal bleeding, taste disorders, erythematous and mild inflammation (Sartawi ~~SY~~ et al., 2021; Günther ~~E~~ et al., 2020; Ayavoo ~~T~~ et al, 2021; Bajunaid ~~SO.~~, 2022; O'Donnell ~~LE~~ et al., 2015; Bansal ~~P~~ et al., 2013).

Therefore, insufficient cleaning of the denture will retain the fungi and bacteria, which will then cause the formation of plaque and calculus, which will play a role as a reservoir of more complex pathogen microorganisms (Sahin C et al., 2013; Abdurahman SNS et al., 2020; Kaypetch R et al., 2023). Antimicrobial agents are then used to control the microorganism's growth. However, the efficient dose of this agent usage should be ideally considered. Prolonged denture submersion in the antibacterial agent will influence its mechanical properties. This alteration will cause erosion of the denture surface, a decrease in hardness, and micro-scratches on the surface, increasing microorganisms (Kaypetch R et al., 2023; Song X et al., 2019; Barua DR et al., 2017; Sugio., 2020; An S et al., 2018)

Using chemical antimicrobial agents has adverse effects on the environment and human health compared to natural agents. Thus, recently, there have been increasing initiatives to utilize natural ingredients in medications(Basera P et al., 2019; Jeffrey J et al., 2020). These herbal agents can be obtained from various sources, such as animals, sea organisms, and plants, which contain secondary metabolites with antimicrobial potential (Azghar A et al., 2023; Jeffrey et al., 2023). These secondary metabolites include tannin, flavonoid, saponin, phenol, steroid, alkaloid, and glycoside, which can provide preventive and therapeutic effects (Barua DR et al., 2017; Sugio., 2020; Basera P et al., 2019; Ribeiro SM et al., 2019).

Several studies have shown that lemongrass has distinct and promising characteristics (Basera P et al., 2019; Bossou AFAD et al., 2020). Lemongrass [*Cymbopogon citratus* (DC.) Stapf, family *Poaceae*] has antimicrobial, antifungal, antiprotozoal antioxidant, antimutagenic, and antiinflammation activities (Manvitha K and Bidya B., 2014). Lemongrass could also prevent denture biofilm formation by inhibiting bacterial cell membranes, altering ergosterol biosynthesis, and inhibiting fungi proliferation and spore germination (Sahal G et al., 2020; Shariati A et al., 2022).

As this is a journal specialized in medicinal plants, it is necessary that the species are named by their scientific names. Please replace hereafter "lemongrass" with "*C. citratu"s*

The utilization of natural materials' advantages as antimicrobials has the potential to develop into nanoparticles with sizes between 1-100 nm. Nanoparticles not only have superior physical and chemical properties compared to particles with bigger sizes, but the synthesis of nanoparticles is also environment-friendly, cost-effective, non-toxic, and more biocompatible (Ozdal M and Gurkok S., 2022; Ajayi E and Afolayan A., 2017; Riyanto et al., 2022). Nanoparticles have unique characteristics based on size, distribution, and particle morphology. For example, smaller particle size will increase particle ability to penetrate bacterial cell membranes, increasing the antimicrobial effect (Riyanto et al., 2022; Rakib-Uz-Zaman et al., 2022). These active biological components, characteristics, and physical properties of nanoparticles will produce the therapeutic results of nanoparticles, which can be applied as broad-spectrum antibacterial and antifungal agents (Sharmin S et al., 2021; Li X et al., 2014; Wang L et al., 2017).

Please, describe much better the research problem:

What is the importance of studying this species or essential oils with these properties and formulations?

What relationship exists between the ethnomedical uses of plants and the activity that it intends to demonstrate?

Why is it important to encapsulate this plant extract or essential oils in nanoparticles?

Justify here

Please be precise here in stating the research problem that this research will solve and how you intend to solve it.

This research synthesized and analyzed a lemongrass oil nanoparticle (LON), ~~*Cymbopogon citratus,*~~ with Transmission Electron Microscopy (TEM). The bioactive component was characterized by an electrospray ionization tandem mass spectrometry (Esi-Ms) analysis. The minimum Inhibitory Concentration (MIC) and minimum Bactericidal Concentration (MBC) were performed against *S. mutans* and *C. albicans.* Then, after applied to acrylic resin, the hardness was tested to observe the influence of LON on the mechanical properties of acrylic.

Please check the correspondence between the Title, the Hypothesis, the Objectives and the Conclusions. In our opinion, there is little correspondence between these parameters. This, and the wording, must be improved.

METHODS

To establish that the nanoemulsion has any advantage over the basic essential oil, the authors must test the same activity with the essential oil without nanoemulsification. We cannot assess whether the results reported here with the nanoemulsion are the most appropriate if we do not know what has happened with the essential oil without nanoemulsification.

Plant material

Non-indicated who identified the material. The manuscript must include references to voucher specimens of the plants (deposited in a major regional herbarium) or the material examined, including their registration number(s). It should be mentioned which plant parts have been used. The GPS coordinates of the place of collection of the species must also be given.

Lemongrass Extraction

Lemongrass extraction was performed in PT Indesso Aroma Pharmaceutical Company, Baturaden, Purwokerto, Central Java, Indonesia. The extraction process was part of 'the development of standardized herbal medicine from natural ingredients' project, including

lemongrass extract as biofilm in acrylic resin. The extraction result was in oil form and then processed for certification of analysis for safety production.

Lemongrass Oil Nanoemulsion (LON) Production

Five milliliters of lemongrass oil (PT Indesso) was mixed with 5 mL of propylene glycol (Carbowax - X29160), 5 mL of PEG 400 (Carbowax - X29160**),** and 10 mL of glycerin (Merck-104094). All mixtures were stirred (with which type of stirrer, mechanical, magnetic..?) until homogenous (temperature?). After that, 5 mL of chromophore (which was the chromophore?) was added and mixed with the previous solution.

Transmission Electron Microscopy (TEM) Morphological Characteristics Testing

Morphology analysis was performed with a ~~*Transmission Electron Microscopy* (~~TEM~~)~~ analyzer (JEOL JEM-1400) at FMIPA Laboratory, Gajah Mada University, Yogyakarta. Ten µL of the sample were dropped into a grid and left still for 1 minute, and then the remaining liquid was removed with a micropipette. Ten µL of uranyl acetate was dropped into a grid, and the remaining liquid was removed with a micropipette. The grid was dried for 30 minutes and then observed under the TEM. TEM is a preferred nanoparticle size, granule size, size distribution, and morphology measurement method (Smith DJ., 2015).

***Electrospray Ionization-Mass Spectra* (ESI-MS) Measurement**

A sample of 0.03 grams was weighed with an analytic balance (Mettler Toledo, Swiss) and dissolved in 90% acetonitrile before being sonicated (DAW, USA) for 30 minutes. After that, quenchers (??) were added to separate the sample contaminant and centrifuged for 10 minutes with a velocity of 10000 rpm. Then, the sample was filtered by a 0.2-micron membrane and tested with ESI-MS (TSQ Quantum Access Max) with a velocity of 5 microliters per minute and added eluent methanol with 0.1% formic acid. (bibliography?)

Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) Analysis of *Streptococcus mutans* dan *Candida albicans*

Culture Media Preparation

Mueller Hinton Agar (MHA) (Himedia, M096-500G) and Mueller Hinton Broth (MHB) (Himedia, M403-500G) were used as the culture media. Thirty-eight grams of MHA and 21 grams of MHB were dissolved in 1000 mL of ddH_2O with the aid of a microwave (Shivaki, SMW 103). Then, the media were sterilized with an autoclave (HiClave, H-50) with a temperature of 121^oC and pressure of 1.5 atm for 25 minutes. (bibliography?)

LON Preparation

Thirty mL of 100% LON stock were diluted using the Serial Working Solution method with ddH_2O and 10% DMSO to prepare series concentrations of 100%, 50%, 25%, 12.5%, 6.25%, 3.125%. After that, the sample concentrations were filtered using a 0.22 µm pore syringe filter (Sartorius, 17845) to obtain sterile samples. (bibliography?)

MIC and MBC Analysis

S. mutans and *C. albicans* inoculums preparation

Inoculums of *S. mutans* (ATCC-25175) and *C. albicans albicans* (ATCC-10231) were cultured using the direct colony suspension method from the primary culture after 24 hours on MHA and MHB media. The turbidity of the inoculums was adjusted with McFarland 0,5 Standard to get bacteria suspensions of 1-5×10^8 CFU/mL. Then, inoculum suspensions were dissolved in MHB with a ratio of 1:50 to get 2×10^6 - 1×10^7 CFU/mL of bacteria suspensions. After that, further dissolving with a ratio of 1:20 was done to get 1-5×10^5 CFU/mL of bacteria suspension. (bibliography?)

Broth Microdilution Method

One hundred µl of inoculum were added to microplate wells and mixed with series concentrations of LON. One hundred µL working solution was added to each concentration until it reached the final concentration. After that, 100 µL of chlorhexidine 0.2% (0011221-C107) was added to wells as the positive control. One hundred µL MHB and 100 µl of inoculum were added to wells as a growth or negative control to antibacterial inhibition. One hundred µL of MHB and 100 µL of working solution of each LON concentration and Chlorhexidine 0.2% (0011221-C107) were added to wells as blank groups. (bibliography?)

The microplates were incubated for 24 hours at 37ºC and then observed with spectrophotometry (Multiskan GO Thermo Scientific 51119300) with 450 nm wavelength for *S. mutans* and 520 nm wavelength for *C. albicans. S. mutans* and *C. albicans* growth was defined by Optical Density (OD) comparison between each treatment group and its blank groups. MIC was defined from the lowest LON concentration, which provided inhibition for more than 50% against microorganism growth. MBC was defined from the lowest LON concentration, which provided inhibition against microorganism growth for 99%.(bibliography?)

Pour Plate Method

After the spectrophotometry measurement from each treatment in the microplate, the pour plate method confirmed that the MBC measurement observed could inhibit microorganism growth in agar media (bibliography?). Twenty µL from each well were cultured in MHA media with the pour plate method and then incubated (Thermo IH3543) for 24 hours at 37ºC. Visual observation was done to count the colony number with the colony counter (Funke Gerber 8500). The MBC was defined from the concentration that showed almost no agar media colony growth. The inhibition percentage was counted with the following formula [1].

$$\%\ Inhibition = \frac{Negative\ Control\ Colony - Total\ Treatment\ Colony}{Negative\ Control\ Colony} \times 100\% \qquad [1]$$

Acrylic Specimen Fabrication

The wax model was sculpted in a flat cylinder with 5 mm diameter and 2 mm thickness with baseplate wax (Cavex) and placed into the cuvette. Then, a cold mold seal (CMS) was smeared into the cuvette, and the wax was boiled out. Heat-cured acrylic (ADM, ISO 1567) was stirred

and packed into the cuvette, then cured for 60 minutes at 100°C. After that, the acrylic was de-flasked, finished, and polished with grit number 400. (bibliography?)

Vickers Hardness Test

Acrylic resin hardness was measured with a Micro Vickers Hardness Tester (Shimadzu, HMV-G21ST Series). Specimens were pressed with a pyramid-shaped diamond indenter with a rectangle base and face-to-face surface angled 136°. Test load ranges were 5, 10, 30, and 50 kgf with 10-15 seconds dwell time. The minimal distance between the central point trace and the specimen edge is 2.5 times the (ASTM) distance of the diagonal of the trace, and the minimal distance between neighboring traces was 2.5 times the distance of the diagonal of the trace. The applied pressure would result in traces such as indentation on the specimen surface. The Vickers hardness value was obtained by dividing the test load size by the traces' surface area. (bibliography?)

Statistical analysis

The variation of data should be expressed in terms of the standard error of mean (S.E.M) or the standard deviation (S.D.), along with the number of observations (n). The details of the software and statistical tests used, and the significance level should be stated (p-value). If more than one test is used it is important to indicate which groups and parameters have been subjected to which test.

RESULTS

To establish that the nanoemulsion has any advantage over the basic essential oil, the authors must test the same activity with the essential oil without nanoemulsification. We cannot assess whether the results reported here with the nanoemulsion are the most appropriate if we do not know what has happened with the essential oil without nanoemulsification.

Lemongrass Oil Nanoemulsion (LON) Certificate of Analysis (CoA)

CoA was done by observing the fabricated nanoparticle's color and odor. This analysis was one of the important steps in determining product quality and grade. The result obtained from the current study was that LON had a yellow color without a specific odor.

Table 1 Lemongrass Oil Nanoemulsion (LON) CoA

Characteristic	**Specification**	**Result**
Appearance	Liquid	Liquid
Color (Visual)	Pale Yellow-Yellow Brown	Yellow
Organoleptic (odor)	Conform to standard	Conform to standard
Acid value (titration)	Max 8.0	2.4
Optical rotation (25°C)	(-)6-0	-2
Refractive index (20°C)	1.480-1.493	1.486

Specific gravity (D25/25)	0.872-0.897	0.892

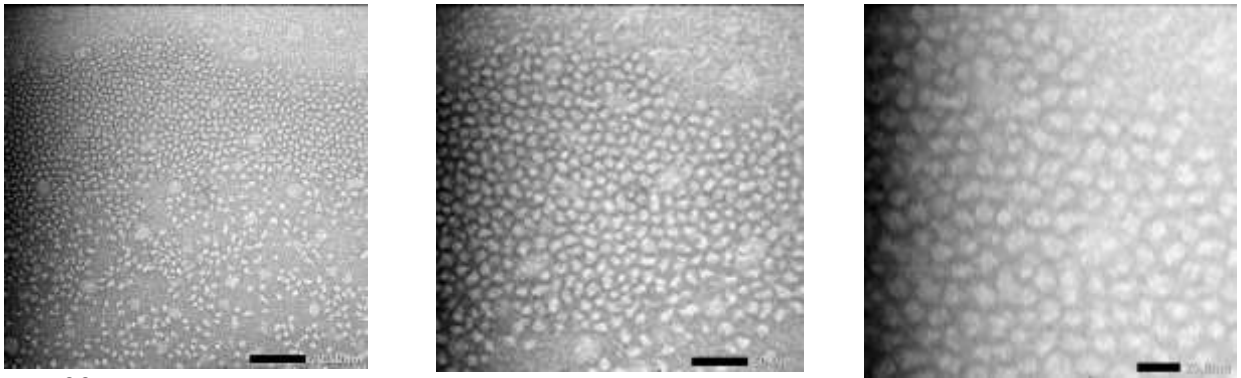
Physical properties of LON (Table 1) showed that the observation result was within the standard range. Therefore, LON could be used to develop herbal medicine from lemongrass as an antimicrobial.

Morphological Characteristics Analysis with Transmission Electron Microscopy (TEM)

TEM is the gold standard and most efficient method for obtaining the nanoparticle morphological characteristics and size. The method provides accurate characterization, such as shape, structure, and size information. This method will present a two-dimensional nanoparticle illustration, which can be observed for number base shape distribution (Tremi I et al., 2021; Al-Khafaji MA et al., 2020).

The results have not been written!

The results should be stated concisely without comments. Efforts should be made to avoid jargon, to spell out all non-standard abbreviations the first time they are mentioned and to present the contents of the study as clearly and concisely as possible. Results should be presented in logical sequence in the text with appropriate reference to tables and/or figures. The data given in tables or figures should not be repeated in the text. The same data should not be presented in both tabular and graphic forms. Simple data may be given in the text itself instead of figures or tables. Avoid discussions and conclusions in the results section. Results that do not have an adequate statistical analysis, including statistical significance, will be immediately rejected.



100nm Measurement **50nm Measurement** **20nm Measurement**

Figure 1 Transmission Electron Microscopy Result from Lemongrass Oil Nanoemulsion (LON)

Figure 1 showed that LON sample size measurements were uniformly between 10-100 nm with irregular individual particle shapes. In TEM measurement, the sample observed should be between 60-90 nm thick to penetrate by electron (Tremi I et al., 2021). However, TEM is

favorable for characterizing nanoparticles because the resolution reaches approximately 0.07 nm (Souza TGF et al., 2016).

Mass Spectrophotometry (ESI-MS) Result of LON

Determination of the substance's component of LON could be characterized with ESI-MS (TSQ Quantum Access Max). ESI-MS was also used to analyze herbal compounds' nanoparticles, substance composition, and molecule mass (Chen T et al., 2019). The chromatogram and substance of LON compounds are presented in Figure 2 and Table 2.

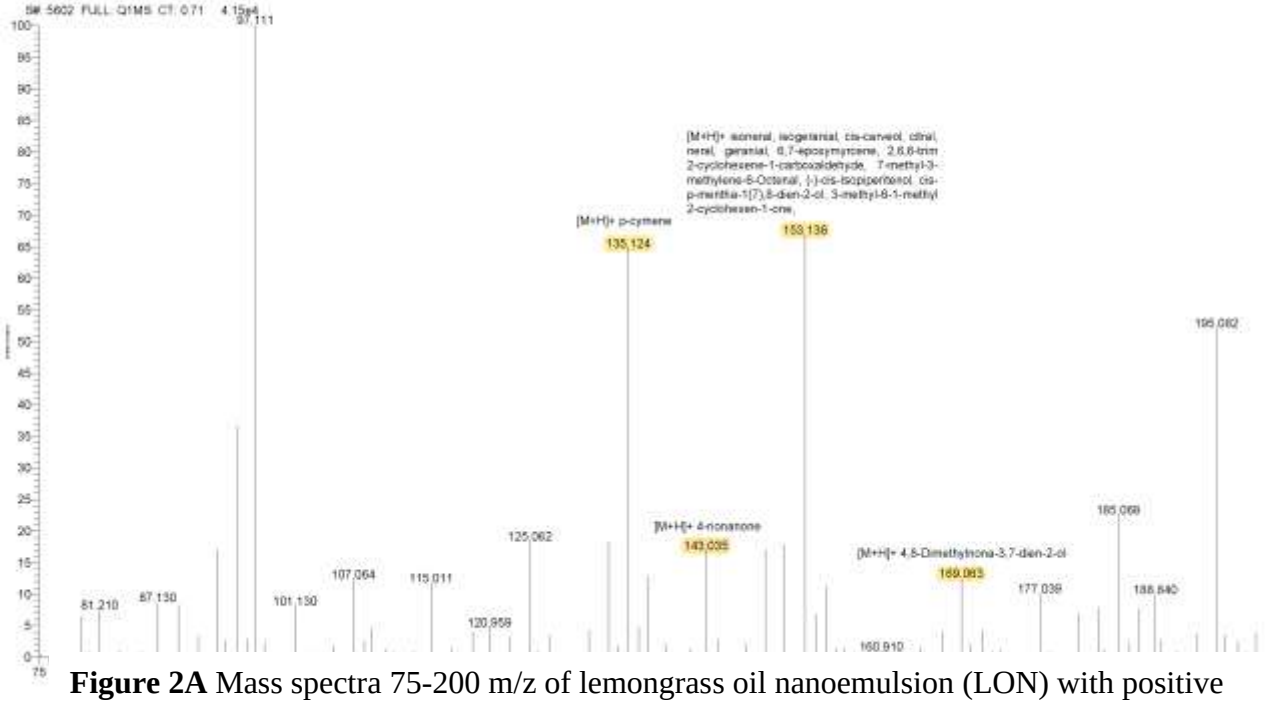


Figure 2A Mass spectra 75-200 m/z of lemongrass oil nanoemulsion (LON) with positive

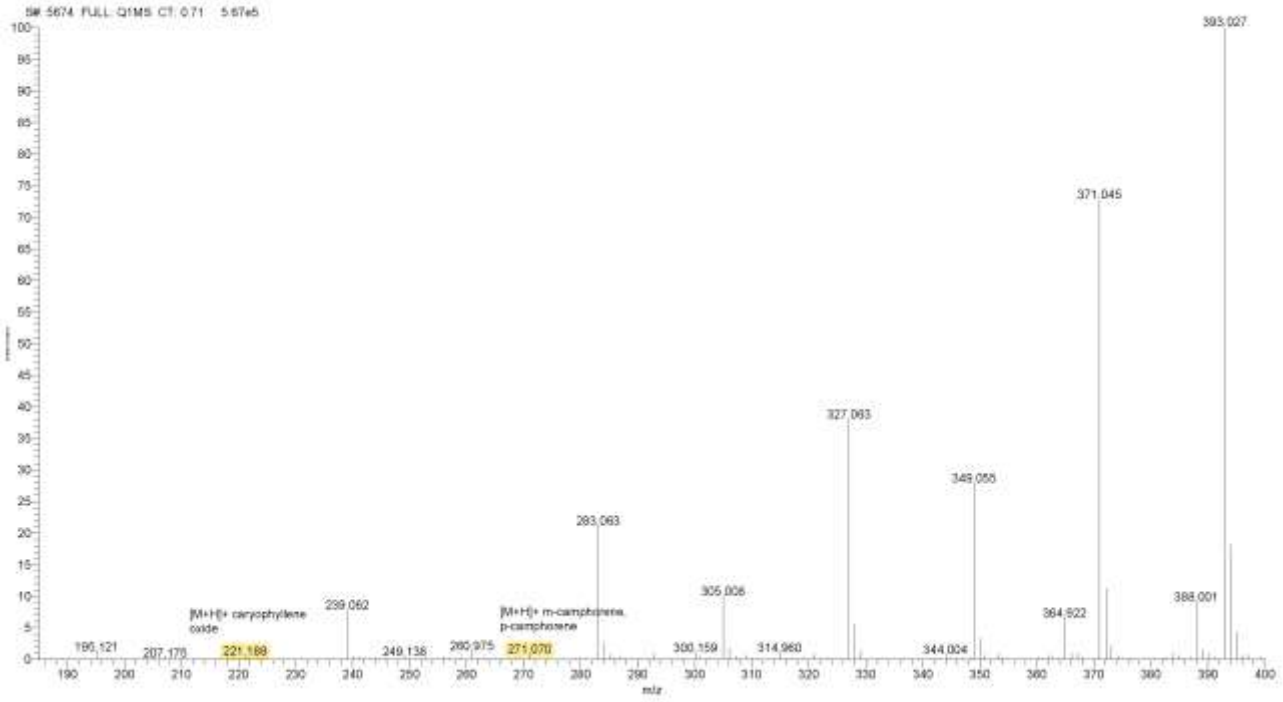


Figure 2B Mass spectra 200-400 m/z of lemongrass oil nanoemulsion (LON) with positive ionization.

Figures 2A and 2B illustrated that LON contained several compounds, as presented in Table 2.

Table 2 Target compounds identification of lemongrass oil nanoemulsion (LON) with ESI-MS

Lemongrass Oil Nanoemulsion	**Molecular Weight (MW) (g/mol)**	**Mass Spectra (MS) $[M-H]^+$**
p-cymene	134.220	135.124
4-nonanone	142.240	143.035
Isoneral	152.233	153.136
Isogeranial	152.233	153.136
Cis-carveol	152.233	153.136
Citral	152.233	153.136
Neral	152.233	153.136
Geranial	152.233	153.136
6,7-epoxy myrcene	152.233	153.136
2,6,6-trim 2-cyclohexene-1-carboxaldehyde	152.233	153.136
7-methyl-3-methylene-6-Octenal	152.233	153.136
(-)-cis-Isopiperitenol	152.233	153.136
Cis-p-mentha-1(7),8-dien-2-ol	152.233	153.136
3-methyl-6-1-methyl 2-cyclohexen-1-one	152.233	153.136
4,8-Dimethylnona-3,7-dien-2-ol	168.280	169.063

Caryophyllene oxide	220.350	221.188
m-camphorene	272.468	271.070
p-camphorene	272.468	271.070

Minimal Inhibitory Concentration (MIC) and Minimal Bactericidal Concentration (MBC) Results Against *Streptococcus mutans* and *Candida albicans*

The antimicrobial activity of LON against *S. mutans* and *C. albicans* was determined by the MIC and MBC.

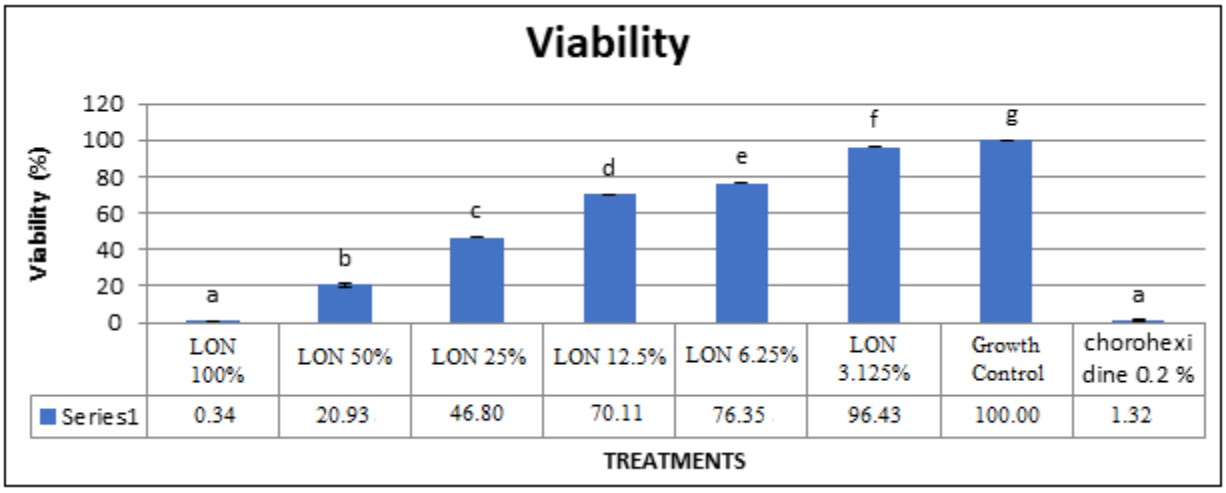
MIC and MBC against *S. mutans*

Table 3 Viability and Inhibition of Lemongrass Oil Nanoemulsion (LON) against *S. mutans*

Sample	Viability (%)	Inhibition (%)	
LON1	**0.34 ± 0.19^{a}**	**99.66 ± 0.19^{g}**	**MBC**
LON2	20.93 ± 1.10^{b}	79.07 ± 1.10^{f}	
LON3	**46.80 ± 0.19^{c}**	**53.20 ± 0.19^{e}**	**MIC**
LON4	70.11 ± 0.19^{d}	29.89 ± 0.19^{d}	
LON5	76.35 ± 0.28^{e}	23.65 ± 0.28^{c}	
LON6	96.43 ± 0.22^{f}	3.57 ± 0.22^{b}	
Negative Control	100.00 ± 0.11^{g}	0.00 ± 0.11^{a}	
Positive Control (Chlorhexidine 0.2%)	1.32 ± 0.45^{a}	98.69± 0.45^{g}	

* Data were presented in average ± deviation standard. a b, c, d, e, f, g symbols showed significant differences in the Tukey HSD test ($p<0.05$). LON1-6: ; MBC: ; MIC: please name these abbreviations.

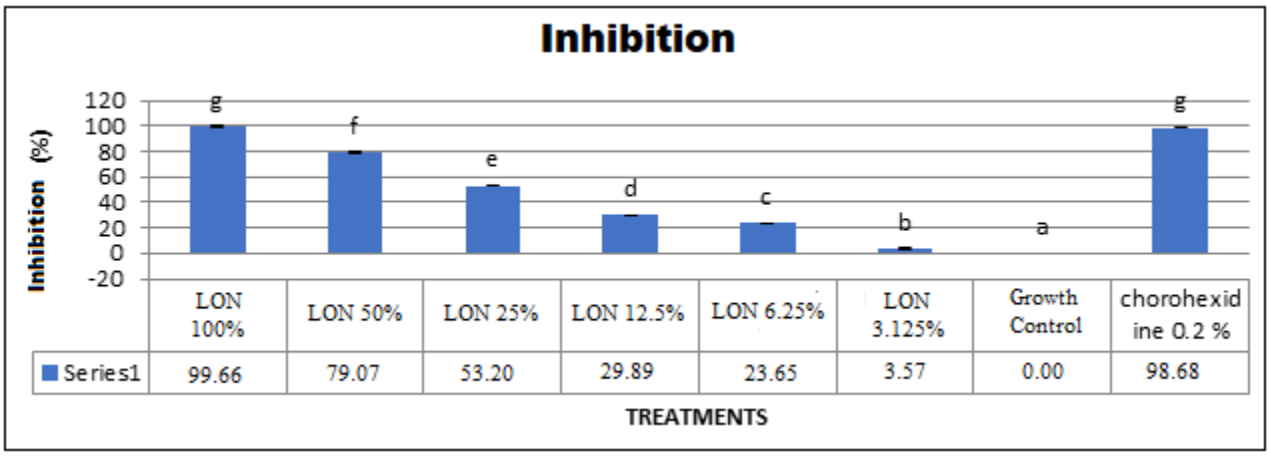
The authors must understand that the results should always be given by increasing, not decreasing, concentrations in all experiments (3.125 to 100% instead 100-3.125%).



* Data were presented in average ± deviation standard. a, b, c, d, e, f, g symbols showed significant differences in the Tukey HSD test ($p<0.05$).

The same results repeated in tables and figures are not accepted. At JPPRes, we prefer tables.

Figure 3. Viability percentage of *S. mutans* after Lemongrass Oil Nanoemulsion (LON) treatment.



* Data were presented in average ± deviation standard. a, b, c, d, e, f, g symbols showed significant differences in the Tukey HSD test ($p<0.05$).

Figure 4. Inhibition percentage of Lemongrass Oil Nanoemulsion (LON) against *S. mutans*.

Table 4 Colony Forming Unit of *S. mutans* (CFU/ml)

Sample	Dilution Factor	Colony Count				CFU/ml				Average	Average
		1	2	3	4	1	2	3	4		
Positive Control	10000	0	0	0	0	0	0	0	0	0	0
Negative Control	10000	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC
LON 100%	10000	0	0	0	0	0	0	0	0	0.00	0
LON 50%	10000	31	33	35	33	31×10^3	33×10^3	35×10^3	33×10^3	33.00	33×10^3
LON 25%	10000	72	71	75	77	72×10^3	71×10^3	75×10^3	77×10^3	73.75	73.75×10^3
LON 12.5%	10000	120	126	123	128	120×10^3	126×10^3	123×10^3	128×10^3	124.25	124.25×10^3
LON 6.125%	10000	181	185	175	168	181×10^3	185×10^3	175×10^3	168×10^3	177.25	177.25×10^3
LON 3.125%	10000	210	215	220	218	210×10^3	215×10^3	220×10^3	218×10^3	215.75	215.75×10^3

*TNTC: too numerous to count. LON: ; CFU: please name these abbreviations.

The authors must understand that the results should always be given by increasing, not decreasing, concentrations in all experiments (3.125 to 100% instead 100-3.125%).

The MIC value of LON against *S. mutans* was at 25% concentration, and the MBC was at 100%. The inhibition level of LON against *S. mutans* growth was considered high because it showed results similar to the positive control. The inhibition level was proportional to the concentration; higher concentrations showed more potent *S. mutans* inhibition.

MIC and MBC against *C. albicans*

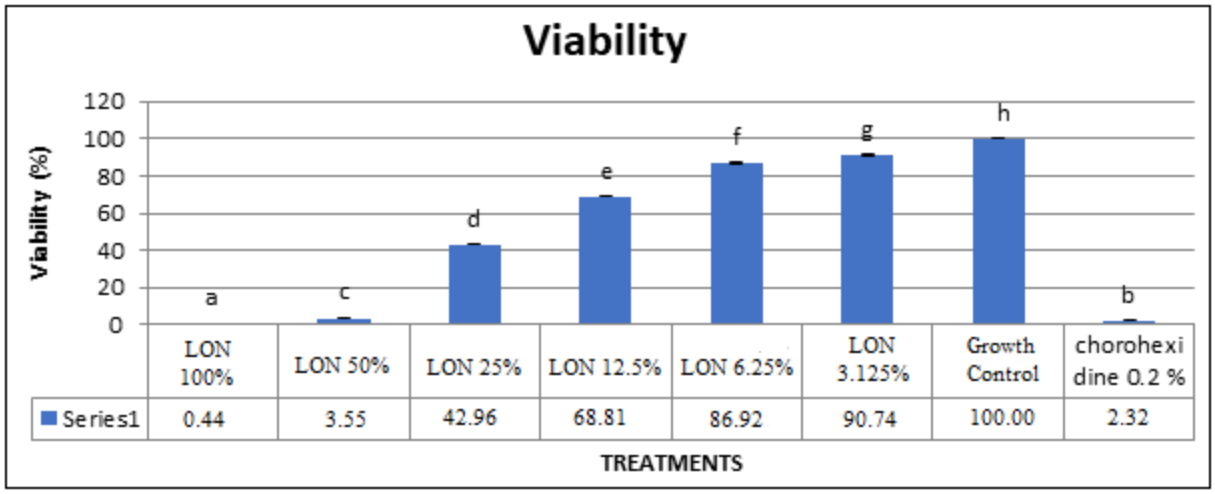
Table 5 Viability and Inhibition of Lemongrass Oil Nanoemulsion (LON) against *C. albicans*

Sample	Viability (%)	Inhibition (%)	
LON1	**0.44 ± 0.04^a**	**99.56 ± 0.04^h**	**MBC**
LON2	3.55 ± 0.08^c	96.45 ± 0.08^f	
LON3	**42.96 ± 0.03^d**	**57.04 ± 0.03^e**	**MIC**
LON4	68.81 ± 0.12^e	31.19 ± 0.12^d	
LON5	86.92 ± 0.11^f	13.08 ± 0.11^c	
LON6	90.74 ± 0.60^g	9.26 ± 0.60^b	
Negative Control	100.00 ± 0.05^h	0.00 ± 0.05^a	
Positive Control (Chlorhexidine 0.2%)	2.32 ± 0.04^b	97.68 ± 0.04^g	

* Data were presented in average ± deviation standard. a, b, c, d, e, f, g symbols showed significant differences in the Tukey HSD test ($p<0.05$). LON1-6: ; MBC: ; MIC: please name these abbreviations.

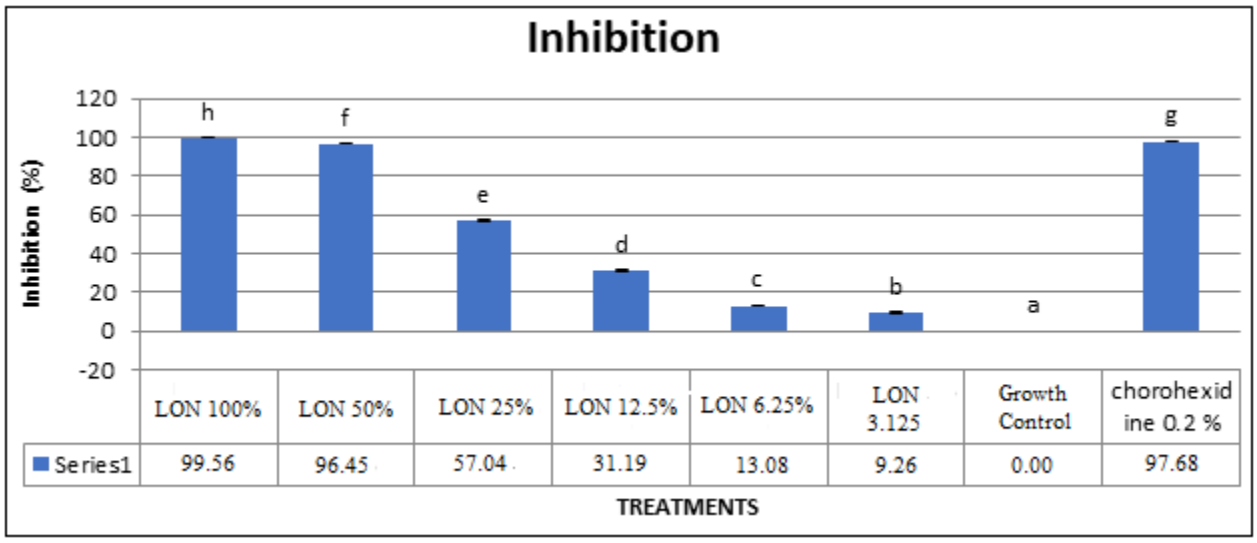
The authors must understand that the results should always be given by increasing, not decreasing, concentrations in all experiments (3.125 to 100% instead 100-3.125%).

The same results repeated in tables and figures are not accepted. At JPPRes, we prefer tables.



* Data were presented in average ± deviation standard. a, b, c, d, e, f, g symbols showed significant differences in the Tukey HSD test ($p<0.05$).

Figure 5. Viability percentage of *C. albicans* after Lemongrass Oil Nanoemulsion (LON) treatment.



* Data were presented in average ± deviation standard. a, b, c, d, e, f, g symbols showed significant differences in the Tukey HSD test ($p<0.05$).

Figure 6. Inhibition percentage of Lemongrass Oil Nanoemulsion (LON) against *C. albicans*.

Table 6 Colony Forming Unit of *C. albicans* (CFU/ml)

Sample	Dilution Factor	Colony Count	CFU/ml	Average	Average

		1	2	3	4	1	2	3	4		
Positive Control	1000	0	0	0	0	0	0	0	0	0	0
Negative Control	1000	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC
LON 100	1000	0	0	0	0	0	0	0	0	0	0
LON 50	1000	5	2	2	4	5×10^3	2×10^3	2×10^3	4×10^3	3.25	3.25×10^3
LON 25	1000	29	31	33	33	29×10^3	31×10^3	33×10^3	33×10^3	31.50	31.50×10^3
LON 12.5	1000	43	45	45	48	43×10^3	45×10^3	45×10^3	48×10^3	45.25	45.25×10^3
LON 6.125	1000	58	56	51	55	58×10^3	56×10^3	51×10^3	55×10^3	55.00	55.00×10^3
LON 3.125	1000	62	61	63	61	62×10^3	61×10^3	63×10^3	61×10^3	61.75	61.75×10^3

*TNTC: too numerous to count

The MIC value of LON against *C. albicans* was at 25% concentration, while the MBC was at 100% concentration. The inhibition rate of LON against *C. albicans* was proportional to the concentration. Higher concentrations showed a more potent inhibition rate.

Hardness Test of Acrylic Specimen

A hardness test was performed to evaluate acrylic resin hardness differences before and after LON treatment, as shown in Table 7.

Table 7 Mean Hardness Value and Standard Deviation

Group	Before	After
LON 100%	22.88±0.56	19.36±2.06
LON 50%	22.38±0.88	19.24±1.14
LON 25%	22.38±1.36	21.04±2.01
LON 12.5%	21.44±0.75	19.62±1.09
LON 6.25%	21.12±0.73	19.50±2.15
LON 3.125%	21.60±0.38	19.90±1.78
Chlorhexidine 0,2%	21.00±1.69	20.06±1.18

A one-way ANOVA test was performed on hardness value, and the p-value was 0.687 (>0.05), which can be concluded that there were no significant differences between treatment groups. A paired t-test was performed to evaluate the hardness before and after LON treatment. Only the 100% and 50% LON groups showed significant differences between treatments with p-values of 0.022 and 0.021, respectively. However, other groups, including the control group with chlorhexidine 0.2%, showed no significant differences statistically with p-value >0.05.

DISCUSSION

Lemongrass oil was extracted according to standard and safety production of Certificate of Analysis (CoA). The CoA result showed the specification of compounds, such as appearance, color, odor, titration, purity, solubility, and water content (Creff-Froger C et al., 2017). The physical properties of LON in current research met the chosen standard. Therefore, lemongrass was suitable as an antimicrobial agent from the development of standardized herbal medicine. The lemongrass oil was fabricated into nanoparticles that proved superior characteristics and

properties.

The characterization of LON in current research was analyzed with TEM. The results showed that the particles' size and shape characterized the physicochemical properties of LON, which significantly contributed to better particle performance. Other physicochemical characteristics that influence the particle performance are texture and surface structure (Rice SB et al., 2013; Wen H et al., 2021). Other than affecting the performance, the characteristics also influence nanomaterials' physical and chemical properties, such as electronic, optic, and catalytic aspects (Lee B et al., 2020). By understanding its characteristics, a nanoparticle's stability can be identified by forming particle aggregation because of inter-particle force. This force will induce interaction between particles and create a more extensive cluster (Oktavia IN and Sutoyo S., 2021).

Herbal materials have important antimicrobial roles against pathogens such as bacteria and fungi. The antimicrobial activity depends on the biochemical content and morphology of the materials (Basera P et al., 2019; Gunasena MT et al., 2022; Nitu S and Patidar KC., 2017). The biochemical content of lemongrass is usually affected by several factors, such as plants' characteristics, climate, geographical conditions, part of the plant used, ecological conditions, and harvest timing (Basera P et al., 2019; Gunasena MT et al., 2022; Chouhan S et al., 2017).

Lemongrass essential oil has proved to have the ability to inhibit the growth of bacteria and fungi (Nitu S and Patidar KC., 2017). Essential oil is a mixture of herbal compounds that is easy to evaporate, has low molecular weight, and is hydrophobic. These properties allow the oil to split the lipid component in bacteria cells' membranes and mitochondria, disrupting cell structure and rendering it more permeable. After that, the cell will experience molecular and essential ion leakage and induce cell death (Chouhan S et al., 2017; Sharma N et al., 2023).

The microbial activities of LON were associated with a high oxygen content of the essential oil, which contains monoterpene and hydrocarbon sesquiterpene, mostly aldehyde and alcohol such as neral/geranial and nerol/geraniol. These contents determined the gram-negative and gram-positive antibacterial properties (Chlif N et al., 2021; Islam M et al., 2018). Higher oxygen content, such as in geranial, has more effective antimicrobial activity (Hussein KA and Joo JH., 2018). The antibacterial activities of essential oil are also affected by a mixture of several complexes, such as monoterpene, sesquiterpene, and the oxygenated derived (Tanhaeian A et al., 2020).

According to several studies, there are several components of essential oil which have antimicrobial activities, such as monoterpene (C10H16), sesquiterpene (C15H24), diterpene (C20H32), triterpene (C30H40), and other components such as 1,8-cineole, p-cymene, α-terpineol acetate, eugenol, limonene, estragole, menthol, anethole, borneol, thymol, geraniol, cinnamyl alcohol, α-thujone, β-thujone, α-pinene, sabinene, caryophyllene oxide, dan terpinene (Chouhan S et al., 2017; Tanhaeian A et al., 2020).

LON's ability to inhibit S. mutans was similar to chlorhexidine 0.2%, as shown by the number of *S. mutans* formed in LON with 100% concentration, which is 0 CFU/mL. Therefore, current research proved that the essential oil of lemongrass has antibacterial effects. Smaller particle sizes also gave more substantial antibacterial effects (Riyanto et al., 2022; Choonharuangdej S et al., 2020).

The current study proved that LON had antibacterial and antifungal effects, as shown by the MIC and MBC results, which align with a previous study by Koseki et al (Koseki Y et al., 2018). LON's ability to inhibit the growth of *S. mutans* and C. albicans was similar to chlorhexidine by 0.2% (Riyanto et al., 2022; Choonharuangdej S et al., 2020). Essential oil in the nanoparticle

form of LON has more effective and efficient roles as an antifungal by inhibiting the metabolism process of fungi and then inhibiting its growth (Riyanto et al., 2022).

Older people have a limitation in performing denture mechanical cleaning because of physical deterioration. Therefore, a combination of mechanical and chemical cleaning is suggested. Although this combination sometimes failed to kill C. albicans completely (de Lucena-Ferreira SC et al., 2013).

This failure can be caused by the extracellular matrix polymer material of the denture, which limits cleaning agents' access to microorganisms located far inside the biofilm (Yodmongkol S et al., 2014). Therefore, denture biofilm should be adequately cleaned daily because biofilm accumulation can be the source of local and systemic disease (Rocha MM et al., 2021). Several studies found that there is an accumulation of Candida after biofilm formation (Yodmongkol S et al., 2014). The porosity of acrylic resin increases the difficulty of mechanical cleaning and infection control in dentures. Thus, denture submersion in disinfectant liquid has become a routine procedure (Pereira CJ et al., 2019). Submersion of dental prosthesis in chemical cleanser aims to deactivate bacteria, viruses, and fungi activity (Kati FA., 2021). However, the commonly used disinfectant agent has disadvantages such as being toxic (glutaraldehyde), corrosive to metal, and inducing skin irritation and mucosal staining. Therefore, an alternative agent that does not influence the denture's properties is needed (Pereira CJ et al., 2019). Other studies showed that various cleaning agents affect the physical properties of denture bases, such as hardness, transverse strength, roughness, and color (Carvalho CF et al., 2012). The denture cleaning method should be effective without affecting denture material properties (Porwal A et al., 2017).

Hardness is one of the material properties that influences the surface characteristics of acrylic resin. Hardness is also used to evaluate the alteration because of denture cleaning (Lira A et al., 2014). Hardness value measurement is an indication of the possibility of polymer matrix degradation. This degradation decreases the hardness value, thereby increasing the possibility of fracture and reducing the length of use of the denture (Ayaz EA et al., 2014). In the current study, the hardness value of resin acrylic samples after LON treatment declined compared to before treatment. These results were possible because of the water and chemical absorption, which caused decreasing mechanical properties of acrylic resin (Rocha MM et al., 2021). In contrast, previous studies showed that hardness values increased after applying coated material and before applying a denture cleaner solution (Yodmongkol S et al., 2014). Another study showed that the hardness value of conventional acrylic resin increased after submersion in sodium hypochlorite cleaning solution and chlorhexidine, mainly after 120 days, which means the longer the submersion time, the greater the hardness value (Talikoti A et al., 2012). However, another study showed decreased hardness value after one year of submersion in chlorhexidine disinfectant gels (Raszewski Z et al., 2021). The variety of results can be affected by diverse materials, procedures, and analysis time for each study.

The hardness value of the current research was observed after treatment, which is in line with other studies that observed that chlorhexidine did not significantly decrease the acrylic resin hardness (Kati FA., 2021). Another study comparing vinegar as a denture cleaning agent and chemical agents showed insignificant differences in hardness values between the two groups (Pereira CJ et al., 2019). This result aligned with current research, which showed insignificant differences in hardness values after control groups and LON treatment groups. The negligible differences in hardness values were also observed from another study, which compared four disinfectant agents, including chlorhexidine (Carvalho CF et al., 2012). Acrylic resin is hydrophilic, easy to absorb water, and acts as a plasticizer; therefore, the decline of hardness

value was also possible because of the formation of cracking zones due to the water absorption process and cycle (Taqa AA et al., 2017).

CONCLUSION

The submersion of acrylic resin in lemongrass nanoemulsion effectively inhibited the colony growth of *Streptococcus mutans* and *Candida albicans* without affecting the acrylic resin's mechanical properties.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

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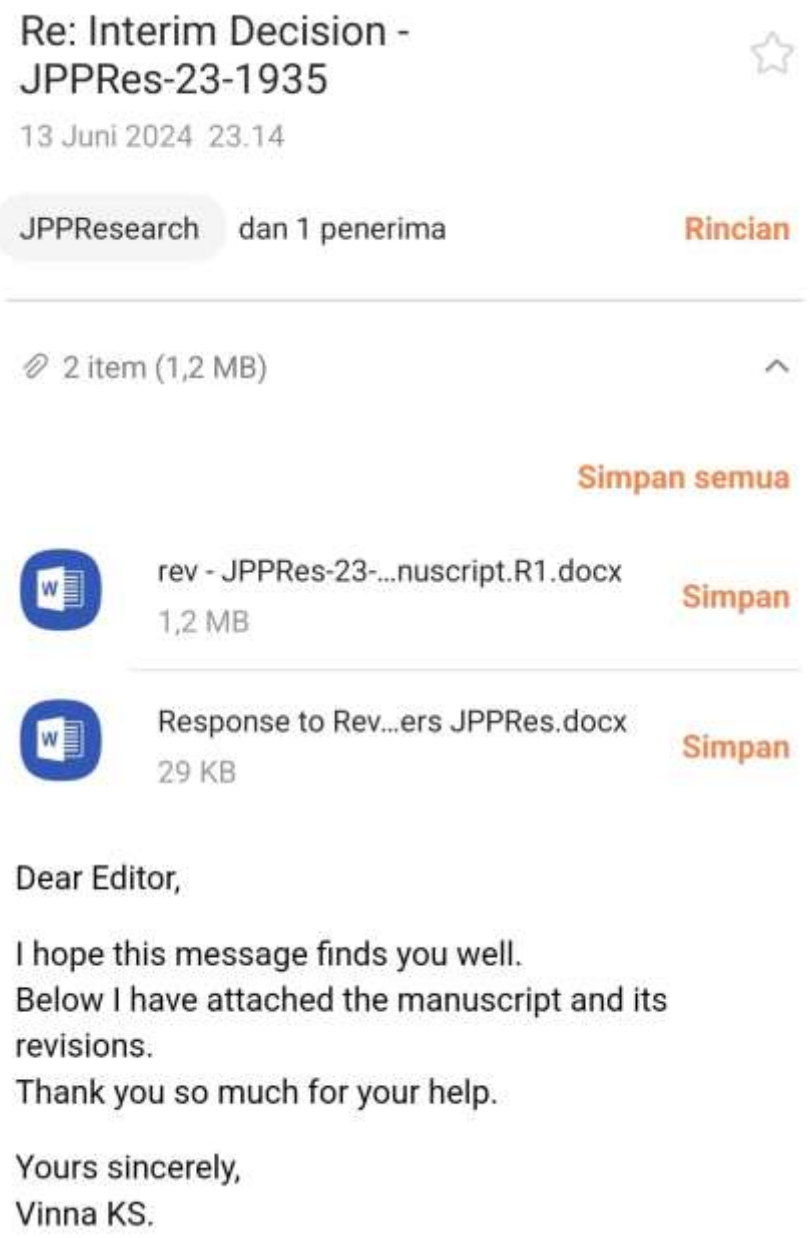
Bukti konfirmasi submit revisi pertama yang telah direvisi (13 Juni 2024)



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Dear Editor,

I hope this message finds you well.
Below I have attached the manuscript and its revisions.
Thank you so much for your help.

Yours sincerely,
Vinna KS.

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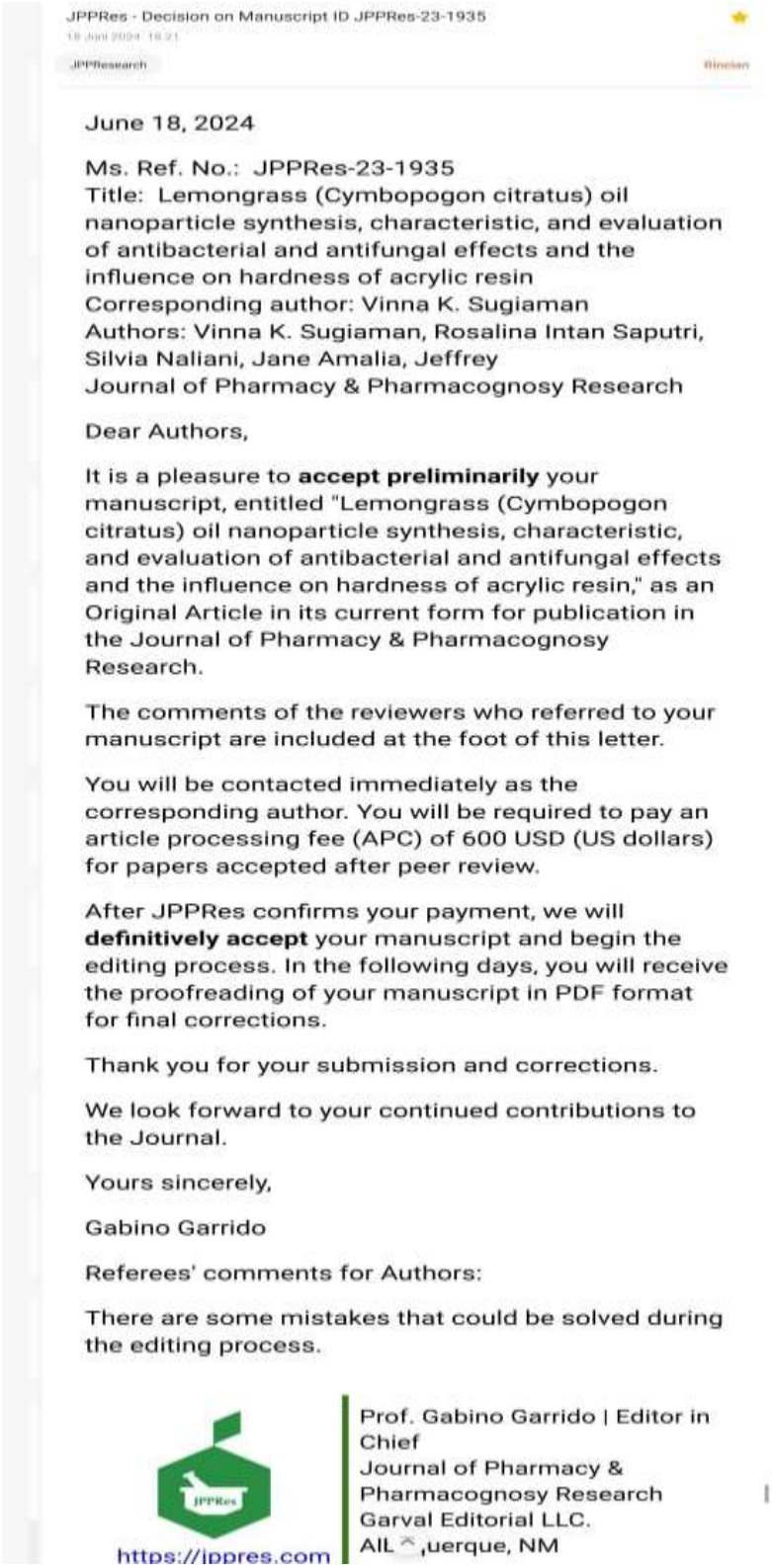

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Ms. Ref. No.: JPPRes-23-1935
Title: Lemongrass (Cymbopogon citratus) oil nanoparticle synthesis, characteristic, and evaluation of antibacterial and antifungal effects and the influence on hardness of acrylic resin
Corresponding author: Vinna K. Sugiaman
Authors: Vinna K. Sugiaman, Rosalina Intan Saputri, Silvia Naliani, Jane Amalia, Jeffrey
Journal of Pharmacy & Pharmacognosy Research

Dear Authors,

It is a pleasure to **accept preliminarily** your manuscript, entitled "Lemongrass (Cymbopogon citratus) oil nanoparticle synthesis, characteristic, and evaluation of antibacterial and antifungal effects and the influence on hardness of acrylic resin," as an Original Article in its current form for publication in the Journal of Pharmacy & Pharmacognosy Research.

The comments of the reviewers who referred to your manuscript are included at the foot of this letter.

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Thank you for your submission and corrections.

We look forward to your continued contributions to the Journal.

Yours sincerely,

Gabino Garrido

Referees' comments for Authors:

There are some mistakes that could be solved during the editing process.

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