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Antibacterial Test of The Essential Oil Fractions of Citronella (*Cymbopogon nardus* L.) Against *Escherichia coli* and Application as Hand Sanitizer Formulation

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ABSTRACT. Hand sanitizer is an essential item during the COVID-19 pandemic, posing potential side effects when containing synthetic ingredients. Therefore, this study aimed to replace hand sanitizer formulation with Citronella essential oil (*Cymbopogon nardus* L.). Essential oil was fractioned into 3 components, namely F1 (67.07% of limonene), F2 (92.39% of citronellal), and F3 (62.41% of geraniol) while evaluation was conducted on antibacterial properties against *E. coli*. Antibacterial test was performed using the well diffusion method, showing inhibition zone diameters of 7.40, 10.76, and 8.30 mm for F1 to F3 fractions, respectively. The results showed that F2, selected as the reference for hand sanitizer formulation, had the most potential antibacterial activity and a MIC of 3.125%. Comparative test with commercial alternatives, including hedonic, characteristic, and antibacterial activity test, were conducted to assess the formulated hand sanitizer. F2 was discovered to have an inhibition zone diameter of 29.56 mm as opposed to 9.06 mm of commercial hand sanitizer. Based on hendonic test, hand sanitizer formulated with citronella oil had a distinct smell, which was less preferred than lime fragrance.

Keywords: Antibacterial, *Escherichia coli*, and sanitizer, citronella essential oil.

INTRODUCTION

Pathogenic bacteria present in human hand pose significant health risk. *E. coli*, for instance, is notorious for causing infection in the digestive system, kidney failure, and death through contaminated food (Gomes et al., 2016). Infection from this bacterial is preventable by regular hand washing, as microbes can get into the body via hand. However, in the present days society, the development of antiseptic hand sanitizer has provided a practical solution for hand hygiene without the need for rinsing. A common product of hand sanitizer often contains alcohol or triclosan, which function as antibacterial agent by inhibiting and killing microorganisms without requiring water for rinsing (Muleba et al., 2022). Conversely, the excessive use of alcohol can lead to skin irritation and a burning sensation (Asngad & Nopitasari, 2018). Furthermore, regulatory bodies such as the Food Safety Agency and the United States Food and Drug Administration (FDA) have raised concerns about the potential health risks associated with triclosan, including bacterial resistance and adverse effects on immunity, reproduction, and endocrine function (FDA, 2017).

The use of alcohol and triclosan in hand sanitizer formulation has been reduced by incoporating natural ingredients. According to Acharya et al. (2018), in the era predating modern medicine, plants served as the primary solution for addressing diverse illnesses. As antibiotics surfaced, microbes began developing resistance to these substances. This phenomenon raised interest towards the exploration of plants antimicrobial properties. The aim is to harness the distinctive capabilities of various secondary metabolites, showing enduring and sustained efficacy against a wide range of microbes. Citronella plant (*Cymbopogon nardus* L.), is one of the natural components with a variety of advanteges and can be used to produce the essential oils (Anggreini & Asngad, 2018). Rastuti et al. (2020) reported the main components of citronella oils to be limonene, citronellal, citronellol, and geraniol. Sari et al. (2022) had explained that citronellal, geraniol, and citronellol, can inhibit bacterial activity. This study aims to determine antibacterial activity of citronella oil obtained from fractionation of *Cymbopogon nardus* L against *E. coli*. The results from this investigation could potentially offer an alternative to alcohol or triclosan as antibacterial agent in hand sanitizer formulation.

EXPERIMENTAL SECTION

Tools and Materials

The tools used in this study were incubator, gas stove, autoclave, aseptic cabinet, stirring rod, loop needle, bunsen burner, magnetic stirrer, test tube, Erlenmeyer, drugal sky, crock drill, calipers, pH meter, petri dish, hot plate, gauze, cotton wool, wrapping plastic, and aluminum foil.

The materials used included fractionated citronella oils (fraction F1, F2, F3) obtained from the previous report (Rastuti et al., 2019). *E. coli* bacteria were collected from the stock cultures at the Laboratory of Microbiology, Faculty of Biology, Jenderal Soedirman University. Antibacterial assay and formulation of hand sanitizer required Nutrient Agar (NA) media (merck millipore), Nutrient Broth (NB) (merck millipore), carbomer, ethanol, triethanolamine (TEA), glycerin, methylparaben, lime fragrance additive, and distilled water purchased at local store.

Antibacterial Activity Assay of Fractionated Citronella Oils

E. coli bacteria were cultured from the stock cultures before usage. A single loopful of the stock cultures was streaked on solid agar in a test tube, then incubated for 1×24 at 37°C.

Antibacterial assay of fractionated citronella oils was performed on the cultured *E. coli* bacteria. An ose of the regenerated bacteria was cultured for 18 to 24 hours at 37°C in 10 mL of Nutrient Broth liquid medium. Using a spreading plate technique, 50 μL of bacterial culture was inoculated onto the solidified Nutrient Agar media (15 mL) in a petri dish. Subsequently, the agar medium was perforated (diameter of ±8 mm) with a crock drill, and 50 μL of each fraction from fractionated citronella oils (fraction F1, F2, F3) were put into each well and incubated for 1×24 hours at 37°C. Ethanol was used as the solvent as well as a negative control, while tetracycline (1000 ppm) served as the positive control.

Antibacterial activity was determined by observing the clear zone formed around the hole and measuring with a caliper. Furthermore, inhibition zone was calculated by measuring the diameter of the clear zone, subtracting the value, and multiplying the result by the diameter of the hole. The analysis identified fraction with the most potent antibacterial activity (Harijani & Nazar, 2013).

Determination of Minimum Inhibitory Concentration (MIC)

MIC against *E. coli* was determined only for fraction F2 with the highest antibacterial activity. The method was performed using the same method mentioned previously, with concentration ranging from 0.5, 1, 3.125, 6.25, 12.5, 25, 50, and 100%.

Formulation of Hand Sanitizer Gel

A total of 3 different versions of hand sanitizer gel were created, as detailed in **Table 1**. Formulation process commenced by combining 25 mL of hot distilled water with carbomer while stirring. Methylparaben was then added to the mixture until homogeneous. Glycerin, citronella oil fraction F2 (for formulation II and III), TEA, and distilled water (to a total volume of 50 mL) were gradually incorporated into the mixture. Stirring and heating were maintained until homogeneity was achieved. The addition of lime fragrance was exclusively conducted for formulation III to study the difference between the scented and unscented hand sanitizer.

Characteristic Test of Hand Sanitizer

The feasibility assessment of the gel comprised test for homogeneity, pH, dispersive power, and consistency. The homogeneity test was conducted by placing 0.5 grams of hand sanitizer gel on 2 pieces of glass media. The sample was considered to be homogeneous when no grains or granules were observed at the point of smearing on the glass (Wijayanto et al., 2013).

The pH test was conducted by dissolving 0.5 grams of hand sanitizer gel in 10 mL of distilled water and measuring the value with a meter. The sample was proposed safe and comfortable for application to the skin when the pH ranged from 4.5 to 6.5 (Sayuti & Winarso, 2015).

The dispersive power or spreadability test commenced by placing 0.5 g of hand sanitizer gel between 2 spherical glasses. The dispersion diameter was measured using a caliper after 1 minute. Subsequently, the diameter was remeasured after applying an additional load of 150 g to the glasses. According to the SNI standards, good dispersibility in the gel typically falls within the range of 50-70 mm (Arista et al., 2013). The consistency test aims to determine the uniformity of the prepared hand sanitizer by observing possible separation between the mixed ingredients (water-oil phase) during formulation step. This test was conducted by examining the physical changes in 1.5 g of hand sanitizer gel after being centrifuged at 3000 rpm for 10-15 minutes.

Table 1. Formulation of hand sanitizer gels (Ningsih et al., 2019).

Materials	I	II	III
Fraction F2	-	0.3125 mL	0.3125 mL
Carbomer	0.15 g	0.15 g	0.15 g
TEA	50 μL	50 μL	50 μL
Glycerin	0.5 mL	0.5 mL	0.5 mL
Methyl Paraben	0.01 g	0.01 g	0.01 g
Fragrance	-	-	0.1 mL
Distilled water		Added up to 50 mL	

Antibacterial Assay of Hand Sanitizer Gel

Antibacterial activity was assessed using the well diffusion method, as previously described. Hand sanitizer gel formulation without the addition of citronella oils fraction (formulation I) served as the negative control, while the positive control was a commercial hand sanitizer.

Hedonic Test of Hand Sanitizer

Hedonic test was performed with 10 panellists by rubbing the prepared hand sanitizer into palms (Komala Sari & Dominica, 2022). The parameters evaluated include color, scent, form, texture, ease of wiping, moisturizing effect, non-sticky impression, sensation on the skin, and evaporation rate. The hedonic test was evaluated in the range of 1 (low/not preferred) to 5 (high/preferred).

RESULTS AND DISCUSSIONS

Antibacterial Activity Assay of Fractionated Citronella Oils

Antibacterial activity assay was performed to determine which fractions (F1, F2, F3) of citronella oils have the most potential as antibacterial agent. Based on **Figure 1**, fraction F2 had highest value of the inhibition zone diameter at 10.76 mm, followed by F3 and F1, at 8.30 mm and 7.40 mm, respectively. The larger the transparent area (clear zone), the more potent the inhibitory effect of antibacterial substance on the tested bacteria (Kurang, 2023).

The composition of each fraction of citronella oils from *Cymbopogon nardus* L has been reported to be 67.07% limonene (F1), 92.39% citronellal (F2), and 47.03% geraniol (F3) (Rastuti et al., 2019). Citronellal was the main composition in fraction F2 and served as the proposed active compound for antibacterial efficacy. Furthermore, it is formed from the elements of carbon (C), hydrogen (H), and oxygen (O) with the formula of $C_{10}H_{18}O$, with O atom being classified as the monoterpene (C10) of terpenoid compounds. Most of antibacterial activity of the plant was related to the secondary metabolites identified as phenolic and terpenoid groups in fractionated essential oil. The phenolic group in citronellal acts as antibacterial by damaging membrane permeability and causing damage or destruction of the cell walls (Ergüden, 2021).

MIC Test

MIC value determines the lowest concentration of compounds capable of inhibiting bacterial activity. In this study, test was performed on fraction F2 using the wells diffusion method against *E. coli* bacteria. Typically, the smaller concentration of the active compound required to inhibit or kill bacteria, the better its activity (more toxic) (Kowalska-Krochmal & Dudek-Wicher, 2021). The result of the MIC assay of fraction F2 was presented in **Figure 2**.

Based on **Figure 2**, an increase in the concentration (%) of the active compound can lead to the wider inhibitory diameter zone. (Kowalska-Krochmal & Dudek-Wicher, 2021) stated that higher concentration of antibacterial compound administered over a certain period lead to more bacteria eradication. The negative control, ethanol, had zero inhibitory zones, showing no effect of antibacterial activity during test. Additionally, concentration of 0.5 and 1% of fraction F2 did not show antibacterial activity.

Significant antibacterial activity was obtained at 3.125% of fraction F2, which was determined as the MIC. Statistical analysis using ANOVA showed that F2 with 3.125% essential oil formed a distinct subset compared to 0.5% and 1%, having zero antibacterial activity. This fraction was ranked as the second highest subset with a value of 2.1350, showing the lowest MIC. The result was quite excellent compared to the previous study by Rizkita (2017) who reported MIC value ranging from 7-20%. It is established that the MIC value of antibacterial agents is inversely proportional to antibacterial activity (Handayani et al., 2021). Therefore, the remarkable efficacy of 3.125% fraction F2 as an antibacterial agent against *E. coli* led to its selection for inclusion in hand sanitizer gel formulation.

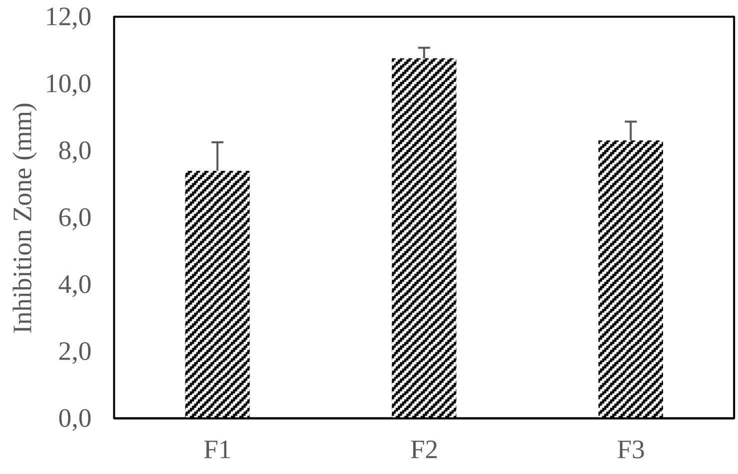

Figure 1. Antibacterial potential test of fractions (F1, F2, F3) of citronella oils

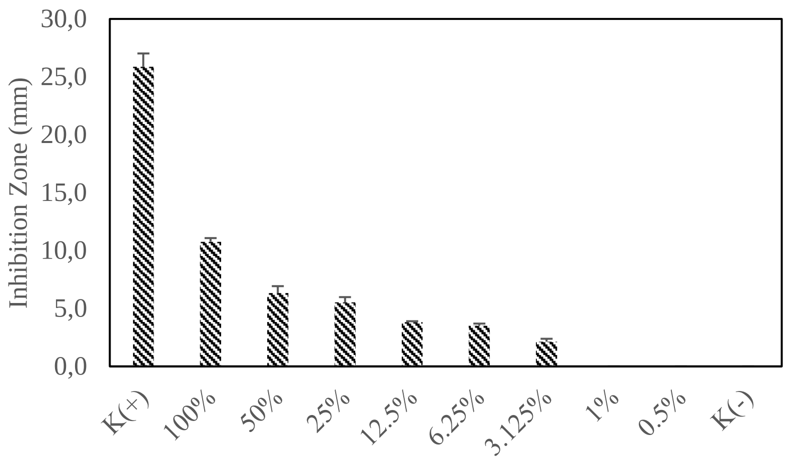

Figure 2. Determination of MIC on fraction F2

Gel Characteristic Test

A total of 3 hand sanitizer formulas were prepared by combining distilled water, carbomer, methylparaben, glycerin, and TEA. Fraction F2, at a concentration of 3.125%, was incorporated into formulations II and III, while lime fragrance was exclusively included in formulation III.

A homogeneity test was conducted to identify when all the components in the gel formulation are finely mixed. Test was performed during the storage time of day 1, 6, 11, and 16. The results showed no changes during the storage time, implying that the prepared hand sanitizer gel complied with SNI 16-4399-1996 standards.

The pH value was measured to assess the acidity or alkalinity of the prepared hand sanitizer gels. All hand sanitizer formulation had relatively stable pH value during the storage time in the range of 5.6 to 6.2. This range was categorized safe by falling within the skin pH of 4.5 to 6.5 (Ali & Yosipovitch, 2013). A lower value shows acidity, which can potentially cause dry skin due to its impact on stratum corneum in the epidermis (Sayuti & Winarso, 2015).

Formulation I showed no changes until 11 days of storage, maintaining a pH of 5.7, which decreased to 5.6 after 16 days. Formulation II and III demonstrated change from 5.6 to 6.0 and 5.9 to 6.0, respectively, after 16 days of storage. These change was tolerable and remained within the skin pH range. Statistical analysis of the relationship between storage time and the pH value of each formulation expressed the significant value of 0.37 (> 0.05). The result shows that there was no significant correlation between the 2 parameters.

The dispersive power describes the ability of the gel to spread evenly when applied to the skin. Sayuti & Winarso (2015) stated that the semisolid or gel is considered comfortable for skin use at a dispersion value of 50-70 mm, while Wijaya et al. (2022) proposed a value of more than 4 cm (40 mm). The results of the dispersive power test are presented in Figure 4. Generally, the 3 formulations showed dispersive power value in the range of 56 to 64 mm. The dispersive value of the prepared hand sanitizer gels did not experience a constant decrease or increase during the storage period, showing no significant change over time.

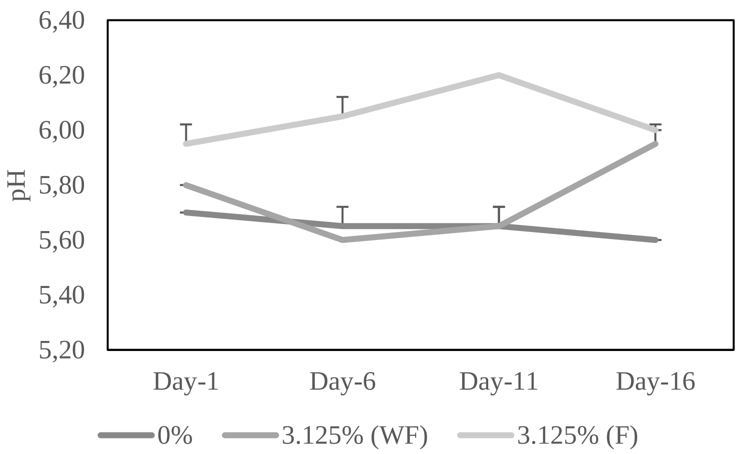

Figure 3. The pH value of the prepared hand sanitizer

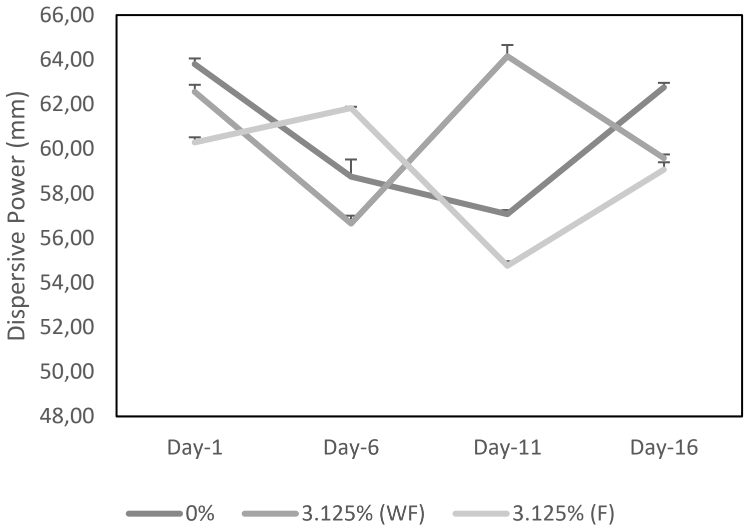

Figure 4. The dispersive power test of the prepared hand sanitizer

The consistency test is a qualitative measurement obtained through sensory observation and without numerical data. Test was conducted to determine the physical characteristics of the semisolid gel formulation. According to Mappa et al. (2013), gel consistency is demonstrated by a stable formulation, where no changes are observed, indicating thorough mixing of all ingredients. Furthermore, it is determined through the centrifugation process. In this study, the prepared formulation produced gel with no syneresis present. Separation of materials was not observed after centrifugation, even during the storage time. This implied that the prepared hand sanitizer gel formulation has been completely mixed and have a stable consistency.

Antibacterial Activity of Hand Sanitizer Gel

Antibacterial test was conducted to determine the effect of formulation on the bioactivity of fractionated citronella oils against *E. coli*. In this assay, 3 formulation hand sanitizer gels were compared with the commercial hand sanitizer (brand X) using the wells diffusion method. Antibacterial test was performed on the 1st and 16th days to investigate the impact of storage time on bioactivity. **Figure 5** showed the inhibitory zone diameter (mm) of the samples. Formulation I, without the addition of fractionated citronella oils (fraction F2) and fragrance, showed no antibacterial activity. However, the inclusion of fractionated citronella oils (fraction F2), increased the inhibitory zone diameter to 19.56 and 15.82 mm at 1st and 16th day storage time, respectively. The presence of lime fragrance in formulation III showed an inhibitory zone diameter of 29.56 and 30.13 mm at 1st and 16th day. **Figure 5** proposed that the prepared hand sanitizers have better antibacterial activity toward *E. coli* than the commercial counterparts (<10 mm). Antibacterial activity experienced slight difference during the storage time, and this condition could be caused by other factors such as bacterial contamination, time of measurement, and sterilization problems (Li et al., 2017). Therefore, the presence of fractionated citronella oils (fraction F2) and lime fragrance in formulation was beneficial to increase antibacterial activity against *E. coli.*

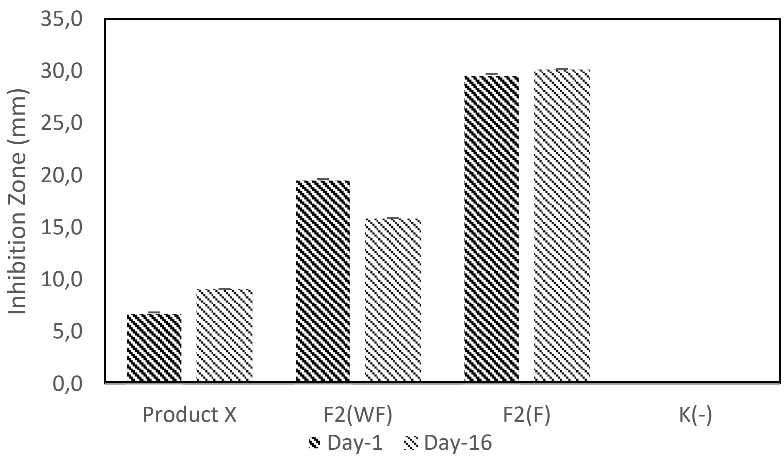

Figure 5. Antibacterial activity of the prepared hand sanitizer

Hedonic Test

Hedonic test is a sensory evaluation method used to measure product acceptance based on the preference, liking level, or impression. The panelists assessed the product on a scale ranging from 1 to 5. In the process of testing, formulation II and III were adopted. Formulation II contains 3.125% of fraction F2 without fragrance, while formulation III included lime fragrance. The results showed a significant preference for the smells of both prepared hand sanitizer. The hedonic values of the unscented and scented formulation were 1.8 and 4.5, respectively. The value was significantly different, showing that panelists dislike the distinct aroma of lemongrass, considering it too pungent and less suitable. Conversely, formulation III, with its typical lime fragrance, was more preferred.

The lowest value was obtained from the non-sticky impression and the volatilization power parameters with both score of 3.6. This value was quite good and above the average score. The addition of glycerin as a moisturizer was a contributing factor to the sticky impression that lasts longer. Formulation using natural ingredients is different compared with the commercial alcohol-based hand sanitizer. Furthermore, the absence of alcohol was also suggested to contribute to the sticky impression and low volatilization power. Alcohol is relatively volatile and evaporates easily, even at room temperature. Therefore, in the prepared non-alcohol-based hand sanitizer gel with fractionated citronella oils and glycerin, evaporation occurs at a slower rate, resulting in a a sticky impression on the skin.

CONCLUSIONS

In conclusion, among the 3 fractions of citronella oils, F2 had the highest antibacterial activity, as demonstrated by an inhibitory zone diameter of 10.76 mm and a MIC value of 3.125%. The prepared hand sanitizer formulation had great consistency and homogeneity during the storage period. Results from the pH and spreadability test confirmed that the gel formulation met the safety standards for application on the skin. Based on the hedonic test, formulation containing F2 and lime fragrance was preferred over the unscented version. Furthermore, antibacterial activity test for formulation II and III showed the inhibitory zone diameter of 17.69 and 29.84 mm, respectively, better than the commercial hand sanitizer with 7.92 mm.

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