Antimicrobial activity of patchouli (*Pogostemon cablin* Benth) citronella (*Cymbopogon nardus*), and nutmeg (*Myristica fragrans*) essential oil and their mixtures against pathogenic and food spoilage microbes

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Abstract. New natural compounds that are effective against pathogenic and food spoilage microbes are needed, especially those that are resistant to conventional antimicrobial compounds. This study aimed to determine the composition and antimicrobial activity of patchouli, citronella, nutmeg essential oil and their mixtures against pathogenic and food spoilage microbes that is *Staphylococcus aureus*, *Shigella* sp, *Candida albicans*, and *Aspergillus niger*. The antimicrobial activity test was carried out by using the disc diffusion method to determine the zone of inhibition. Citronella oil has higher antimicrobial activity in inhibiting the growth of *Aspergillus niger*, *Staphylococcus aureus*, and *Candida albicans*, namely 38.28 mm, 26.22 mm and 16.21 mm, respectively. Meanwhile, for *Shigella* sp, patchouli oil is better able to inhibit the growth of *Shigella* sp, namely 6.80 mm. The combination of the mixture of citronella oil with patchouli oil and citronella oil with nutmeg oil can increase the inhibitory ability of patchouli oil and nutmeg oil respectively against the growth of *Candida albicans*, *Aspergillus niger* and *Staphylococcus aureus*. The sensitivity of pathogenic and food spoilage microbes tested against these three essential oils shows the potential for patchouli, citronella, and nutmeg essential oils to be used as natural preservatives in the food industry.

1. Introduction

Foodstuffs are materials that are prone to microbiological contamination. Microbiological contamination can lead to spoilage and trigger food poisoning. Microbiological contamination is the biggest cause of poisoning in Indonesia [1]. Efforts are needed to prevent microbiological contamination of foodstuffs to avoid food spoilage and poisoning. Essential oils are aromatic and volatile liquids that are extracted from plant materials, such as flowers, roots, bark, leaves [2], seeds, bark, fruits, wood, and whole plants [3]. Essential oil is the result of secondary metabolites. For plants, it functions to defend/protect plants because it has antibacterial properties [4], antiparasitic [5], insecticide [6], antiviral [7], antifungal [8], and antioxidant properties [9]. Various types of plants can be used as a source of essential oils, including patchouli (*Pogostemon cablin* Benth), citronella (*Cymbopogon nardus*) and nutmeg (*Myristica fragrans*).

According to [10]-[12] stated that essential oils contain a very complex mixture of active compounds, so that to determine the most active antimicrobial compounds from essential oils, it is necessary to
identify each of these compounds. The composition of essential oils can also vary depending on the harvest season, and the method used to extract the oil [13]-[15]. The compounds contained in essential oils are a diverse group of organic compounds that have both low molecular weight and high molecular weight, which can cause differences in antibacterial or antimicrobial activity.

The essential oils from the patchouli plant (*Pogostemon cablin* Benth), citronella (*Cymbopogon nardus*), and nutmeg (*Myristica fragrans*) are superior essential oils from Aceh Province and are known to have good quality. So far, the three types of plants have been extracted to obtain their essential oils, but there is still little research to develop them as alternatives to natural preservatives.

Essential oils are effective in inhibiting the growth of Gram-positive, Gram-negative bacteria and fungi, but the use of essential oils in food products in high levels can affect the sensory value of foodstuffs [16]. With the advantages and disadvantages of each essential oil, it is necessary to do a combination treatment of essential oil mixtures which are expected to increase the antimicrobial effectiveness on Gram-positive bacteria, Gram-negative bacteria, and fungi. Also, with the mixed combination treatment, it is expected that the concentration of the two essential oils can be reduced to a smaller amount but will produce the same level of effectiveness when used separately.

Several combinations of essential oils have been shown to produce better antimicrobial activity. The combination of 0.6% thyme essential oil with 1000 IU / g nisin has been shown to be synergistic in reducing the number of *Listeria monocytogenes* at 4 °C [17]. The combination of *Origanum vulgare* essential oil, *Thymus vulgaris* with nisin, and *Satureja montagna* with pediocin respectively showed a synergetic effect in inhibiting the growth of *Listeria monocytogenes*, *Salmonella typhimurium*, and *Escherichia coli* O157: H7 [18].

The combination of patchouli essential oil, citronella and nutmeg has never been done, even though they have great potential, both from their antimicrobial activity and availability. This combination is expected to be used to prevent microbiological contamination of foodstuffs. This study was conducted to determine the antimicrobial activity of patchouli, citronella and nutmeg essential oil and their mixtures on some spoilage and pathogenic microorganisms in food.

2. Material and methods

2.1. Material

The material used in this study were patchouli oil, citronella oil, nutmeg oil, Mueller Hinton Agar (MHA) media (Oxoid), bacterial cultures of *Staphylococcus aureus*, *Shigella* sp, *Candida albicans*, and *Aspergillus niger*. The chemicals used were distilled water, NaCl, antibiotic gentamicin and nystatin as a positive control, and ethanol as a negative control.

2.2. Antimicrobial activity testing [19]

The media for bacteria was prepared using Mueller Hinton Agar (MHA), while Potato Dextrose Agar (PDA) was used as a medium for mold. 11.4 grams of MHA and PDA each were dissolved into 300 ml of distilled water, then homogenized and sterilized in an autoclave at a temperature of 121 °C for 15 minutes. It was then allowed to cool until the temperature was approximately 45 °C then poured into 12 sterile petri dishes as much as 25 ml / petri dish, and allowed to stand until solid.

Antibacterial activity testing was carried out using the Disc Diffusion method (Kirby-Bauer test). Preparation of bacterial suspension is done by inoculating 1 loop of bacterial culture into 9 ml of NaCl. Then each NaCl containing bacteria is vortexed, and the level of turbidity is equalized with Mc. Farland 0.5. Petri dishes that already contain MHA media are labeled as needed. A sterile cotton swab was dipped in each of the tested bacterial suspensions, then flattened in a petri dish containing MHA solid media, then let stand for 10-30 minutes. Disc paper was immersed in each sample with a volume of 10 ml for ± 5 minutes. Disc paper was placed on the surface of the test media in accordance with the desired position and then incubated at 37 °C for 24 hours. The diameter of the clear zone (zone of inhibition) that appears is measured using a caliper which is expressed in units of millimeters. The positive control used was gentamicin and nystatin, while the negative control used was ethanol.
3. Result and discussion

3.1. The composition of the chemical components of essential oils

The essential oils used in this study were patchouli oil, lemongrass oil, and nutmeg oil. The results of the chemical composition analysis of the three essential oils using Gas Chromatography-Mass Spectra (GCMS) can be seen in Table 1, Table 2, and Table 3.

Table 1. The chemical components of patchouli oil with a percentage of each component > 1%.

| R.Time (min) | Component          | Area (%) |
|--------------|--------------------|----------|
| 10.374       | Beta-patchoulene   | 3.15     |
| 11.121       | Trans-caryophyllene| 4.22     |
| 11.397       | Alpa-guaiene       | 14.56    |
| 11.862       | Seychellene        | 8.18     |
| 12.125       | Alpha-patchoulene  | 5.64     |
| 12.299       | Aromadendrene      | 1.60     |
| 12.778       | Alpha-guaiene      | 3.21     |
| 12.920       | Delta-guaiene      | 18.23    |
| 15698        | Alpha-guaiene      | 1.71     |
| 16387        | Delta-guaiene      | 2.50     |
| 16.816       | Patchouli alcohol  | 29.12    |

Table 2. Chemical components of citronella oil with a percentage of each component > 1%.

| R.Time (min) | Component                | Area (%) |
|--------------|--------------------------|----------|
| 13.625       | Cyclehexene, 1-methyl-4-(1-methylene) | 4.28     |
| 21.484       | Citronellal              | 43.02    |
| 26.337       | Beta-Citronellol         | 10.99    |
| 27.961       | Nerol                    | 16.21    |
| 34.229       | Citronellyl acetate      | 2.72     |
| 36.048       | Linalyl acetate          | 2.09     |
| 36.583       | Beta elemene             | 2.12     |
| 42.095       | Germacrene-D             | 2.19     |
| 44.449       | Delta cadinene           | 1.86     |
| 46.188       | Elemol                   | 3.70     |
| 51.982       | Gamma gurjunene          | 1.45     |

The results of GC-MS analysis showed that patchouli essential oil, citronella and nutmeg had different constituent components. These components are composed of terpenes, in the form of monoterpenes and sesquiterpenes. The identified components of patchouli oil were 35 components, while the components with a percentage of > 1% were 11 components (Table 1). The identified constituent components of citronella oil were 30 components, while the components with a percentage of > 1% were 11 components (Table 2). The identified components of nutmeg oil were 40 components, while the components with a percentage of > 1% were 14 components (Table 3). The main component that has the highest percentage in each of the three essential oils is patchouli alcohol (29.12%) in patchouli oil, citronellal (43.02%) in citronella oil, and sabinene (22.12) in nutmeg oil. Differences in constituent components and the percentage of each component in essential oils can be caused by several factors such as genetics (type), cultivation, environment, harvest and post-harvest handlings such as withering and refining processes [20], land, climate and nutrients [21].
According to Dantas et al. [22], patchouli oil originating from Ceará-Mirim, Rio Grande do Norte, Brazil has been studied and has major components, namely patchouli alcohol (66.08%), α-humulene (8.87%), α-guaiene (4.95%), α-patchoulene (4.95%) and β-elemene (2.19%). According to Su et al. [23], patchouli oil originating from Guangzhou, China has 5 main components, namely patchouli alcohol (31.18%), δ-guaiene (24.92%), α-longipinene (22.21%), β-patchoulene (19.80%) and longipinocarrone (31.58%). Aisyah [24], has also conducted research with patchouli oil sourced from Tapaktuan, South Aceh, and has main components, namely patchouli alcohol (32.60%), δ-guaiene (23.07%), α-guaiene (15.91%), seychellene (6.95 %), and α-patchoulene (5.47%).

Table 3. The chemical components of nutmeg oil with a percentage of each component > 1%.

| R.Time (min) | Component               | Area (%) |
|-------------|-------------------------|----------|
| 8.719       | Alpha thuene             | 3.01     |
| 9.057       | Alpha pinene             | 20.63    |
| 10.920      | Sabinene                 | 20.24    |
| 11.106      | Beta pinene              | 16.80    |
| 11.771      | Beta myrcene             | 3.17     |
| 12.626      | Delta 3 carene           | 3.14     |
| 13.046      | Alpha terpinene          | 3.15     |
| 13.412      | Benzene                  | 3.53     |
| 13.673      | Limonene                 | 4.38     |
| 15.136      | Gamma terpinene          | 3.22     |
| 16.480      | Alpha terpinolene        | 3.33     |
| 21.152      | 3 cyclohexene-1-ol-methyl| 3.53     |
| 26.247      | 1.3 benzodioxole         | 3.57     |
| 36.174      | Myristicin               | 7.17     |

The chemical components in citronella oil are quite complex, but the most important components are citronellal, citronellol, limonene, linalool [25]. The content of the main chemical components of lemongrass oil is not fixed and depends on several factors. The results of the identification of [26] reported that citronellal, geraniol, citronellol, citronellal, and citronellal compounds are found in citronella, while Indian citronella contains citronellal, geraniol, α-terpineol, cis-sabinene hydrate, nerolidol, β-caryophyllene, germacrene-4-ol [27]. According to [20], the main components of citronella oil are geraniol (C_{10}H_{15}O), citronellol (C_{10}H_{15}O) and citronellal (C_{10}H_{15}O).

Nutmeg essential oil contains aromatic components in the form of α-pinene (8.7%), 3-karene (3.54%), D-limonene (8%), α-terpinene (3.69%), 1,3,8-mentatrien (5.43%), α-terpinene (4.9%), α-terpinol (11.23%), safrole (2.95%), and myristicin (23.37%) [28]. Apart from having a high aromatic component, nutmeg oil is also known to be a source of several active components such as alkaloids, steroids, tannins, flavonoids, phenolics, and glycosides [29].

3.2. Antimicrobial activity

The antimicrobial activity of essential oils is influenced by these constituent compounds. The constituent components that have hydroxyl ions have an antimicrobial activity which tends to be higher than other constituent components [30]. However, according to O’Bryan et al., [31], hydroxyl ions are not an absolute requirement in determining the antimicrobial activity of essential oil. Besides, trace components may also affect the bacteriostatic, bactericidal, fungistic, and fungicidal activity of essential oils.

The use of patchouli, citronella and nutmeg oil either partially or mixed, is proven to inhibit the growth of pathogenic and spoilage microbes on testing with three replications (triplicate) (Table 4).
Table 4. Antibacterial activity of essential oils.

| Sample                      | S. aureus (mm) | Shigella sp (mm) |
|-----------------------------|----------------|-----------------|
| Patchouli Oil              | 11.36±1.85     | 6.80±0.10       |
| Nutmeg Oil                 | 21.13±4.33     | 0               |
| Citronella Oil             | 26.22±2.65     | 0               |
| Patchouli Oil + Nutmeg Oil | 16.45±1.05     | 0               |
| Patchouli Oil + Citronella Oil | 22.65±2.23   | 0               |
| Nutmeg Oil + Citronella Oil | 25.54±2.20    | 0               |
| Gentamicin                 | 19.24±1.59     | 38.49±0.22      |
| Ethanol                    | 6.15±0.03      | 0               |

All of the essential oil samples (Table 4) inhibited the growth of Gram-positive *Staphylococcus aureus* bacteria. The highest diameter of inhibition in the citronella oil sample is 26.22 mm. This shows that citronella oil works more effectively than other essential oil samples. When compared to gentamicin which is a commercial antibacterial, only patchouli oil has a lower diameter of the inhibition area. However, the combination of patchouli oil with nutmeg oil and citronella oil has a higher diameter of the inhibition area than gentamicin.

The test results for Gram-negative *Shigella* sp. showed that only patchouli oil partially inhibited the growth of Gram-negative *Shigella* sp., although the inhibitory area diameter was lower than that of Gram-positive bacteria. Meanwhile, nutmeg oil and citronella oil were not able to inhibit the growth of Gram-negative *Shigella* sp bacteria either partially or in combination. This is because Gram-negative bacteria have better resistance to antibacterial compounds than Gram-positive bacteria. According to Pelczar and Chan [32], the different responses of the two groups of bacteria to this compound are due to differences in the sensitivity of Gram-positive and Gram-negative bacteria to the antibacterial compounds contained. Gram-positive bacteria tend to be more sensitive to antibacterial components. This is because the structure of the Gram-positive bacteria cell wall is simpler, making it easier for antibacterial compounds to enter the cell and find targets to work on. On the other hand, the cell wall structure of Gram-negative bacteria is more complex and three-layered: the outer layer is a lipoprotein, the middle layer is peptidoglycan and the lipopolysaccharide layer.

According to Nazzaro et al. [33], the cell wall of Gram-positive bacteria allows hydrophobic molecules such as essential oils to penetrate the cells and damage the cell wall and cytoplasm. Gram-positive bacteria have a cell wall containing 90% peptidoglycan and a thin layer of teichoic acid. Peptidoglycan plays a role in regulating the diffusion of molecules into cells, while teichoic acid is a part of peptidoglycan. The cell wall of Gram-positive bacteria contains more amino acid alanine, which functions to maintain protein stability in hydrophobic bacteria so that it is better able to bind non-polar compounds such as essential oils compared to Gram-negative bacteria.

The outer membrane of Gram-negative bacteria has hydrophilic properties, that is, it easily binds with water and other polar groups, but not with essential oils which are non-polar compounds. The outer membrane also acts as a barrier to the entry of compounds that are not needed by cells, including bacteriocins, enzymes, and other hydrophobic compounds [34]. In addition, in Gram-negative bacteria, there is a channel made of protein, namely porin, which functions as a place for the entry of hydrophilic components such as sugars (oligosaccharides, monosaccharides) and amino acids which are essential for the nutritional needs of bacteria. This also causes compounds with large molecules cannot enter the cell walls of Gram-negative bacteria so that the bacteria are more resistant to being inhibited by essential oils which on average have large molecular constituents.

The work activity of essential oils in inhibiting growth or killing bacteria is by interfering with the process of forming a membrane or cell wall so that the membrane or cell wall is not formed or formed imperfectly. Essential oils from terpenoid components contained in a plant can damage bacterial cell membranes by binding to enzyme proteins and damaging cell membranes so that they can inhibit...
bacterial cell growth. The plasma membrane is also a site of action for several antimicrobial agents, so it is suspected that the antibacterial activity of essential oils against Gram-positive bacteria is by damaging the membrane or bacterial cell wall. In Gram-negative bacteria, a more complex cell wall structure is thought to make the active compounds in essential oils unable to damage the bacterial cell membrane or cell wall [35].

Table 5. The antifungal activity of essential oils.

| Sample                        | C. albicans | A. niger |
|-------------------------------|-------------|----------|
| Patchouli Oil                 | 0           | 0        |
| Nutmeg Oil                    | 8.38±0.76   | 9.12±0.36|
| Lemongrass Oil                | 22.00±0.79  | 38.28±2.53|
| Nutmeg Oil + Patchouli Oil    | 6.23±0.13   | 6.41±0.35|
| Nutmeg Oil + Lemongrass Oil   | 13.23±0.71  | 30.81±0.79|
| Patchouli Oil + Lemongrass Oil| 9.62±0.32   | 31.20±1.05|
| Nystatin                      | 16.21±0.04  | 9.94±2.48|
| Ethanol                       | 7.31±0.23   | 6.93±0.22|

In Table 5, it can be seen that citronella oil can inhibit the growth of Candida albicans with the largest diameter of the inhibition area, namely 22 mm, and larger than the diameter of the nystatin inhibition area as a positive control, namely 16.21 mm. The combination of citronella oil and nutmeg oil had a higher diameter of the inhibition area compared to other essential oil combinations, but still lower than nystatin.

The combination of each of patchouli oil, citronella oil, and nutmeg oil was done not only to reduce the concentration of the use of each antimicrobial compound but also to obtain inhibitory activity in the same level. The test results show that the combination of each of these essential oils on the growth of Candida albicans has not been proven to reduce the concentration of the use of antimicrobial compounds compared to the use of each antimicrobial compound separately (Table 5). However, the diameter of the inhibition area is lower. The destruction of mold cells occurs due to the hydrophobic nature of essential oils that disrupt transglycosylation in cell wall biogenesis and increase pore formation activity in cells. This results in increased cell permeability so that cells are damaged [36][37].

In Aspergillus niger, citronella oil also works more effectively than patchouli oil, and nutmeg oil, which is 38.28 mm in diameter. When compared with the diameter of the nystatin inhibition area, which is a commercial antifungal (9.94 mm), citronella oil has a larger diameter of the inhibition area. The combination of citronella oil and patchouli oil, as well as the combination of citronella oil and nutmeg oil, results in effective inhibition of Aspergillus niger. The combination can reduce the concentration of one or both antimicrobial compounds (Table 5). The combination of citronella oil and patchouli oil and the combination of citronella oil and nutmeg oil work synergistically in inhibiting the growth of Aspergillus niger resulting in a decrease in the concentration of use. The diameter of the inhibition zone of these two combinations was greater than that of nystatin (diameter of the inhibition zone was 9.94 mm).

In the study of Hu et al., [38], turmeric essential oil can inhibit the germination of Aspergillus flavus spores besides that it can also inhibit ergosterol metabolism. Ergosterol is a sterol that maintains the function and robustness of cells. Temulawak (Curcuma zanthorrhiza) and Temu Putih (Curcuma zedoaria) essential oils are possible to inhibit the same mechanism by inhibiting spore germination and inhibiting the ergosterol metabolism of Aspergillus niger.

4. Conclusion
From this study, it can be concluded that citronella oil has a higher ability to inhibit the growth of Staphylococcus aureus with a diameter of the inhibition area of 26.22 mm, Candida albicans with a diameter of the inhibition area of 16.21 mm, and Aspergillus niger with a diameter of the inhibition area
of 38.28 mm. As for the growth of Shigella sp, patchouli oil is better able to inhibit the growth of Shigella sp with an inhibition area diameter of 6.80 mm.

In general, the combination of a mixture of citronella oil, patchouli oil, and nutmeg oil has not been able to match the inhibitory ability of each essential oil. However, the combination of citronella oil with patchouli oil and citronella oil with nutmeg oil can increase the inhibitory ability of patchouli oil and nutmeg oil respectively against the growth of Candida albicans, Aspergillus niger and Staphylococcus aureus.

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