Effect citronella oil against bacteria strains: *Escherichia coli* ATCC 10536, *Staphylococcus aureus* ATCC 6538 and *Salmonella typhimurium* ATCC 14028

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**Abstract.** A bactericidal property of *Cymbopogon nardus* oil (CNO) has been investigated in the previous study. This study aimed to confirm the bactericidal activity of *Cymbopogon nardus* against different bacteria strains. CNO with the concentration of 1%, 3% and 8% were injected into 100 mL canned tubes. Each sample was diluted by hard water and added with a bacterial suspension test in the presence of interfering substance. The mixture was maintained at 20 °C for 5 min. Water shall be new distilled water-not demineralized water. At the end of contact time, an aliquot was taken, and bactericidal activity was immediately neutralized by the diluted-neutralization method. Neutralizers used in this study were: peptone 10 g L⁻¹, beef extract 5 g L⁻¹, NaCl 5 g L⁻¹, soy lecithin 1 g L⁻¹ and polysorbate 80 20 g L⁻¹; while the interfering substance was 0.3 g L⁻¹ bovine albumin for clean conditions. The number of cells in the bacterial suspension test per treatment was 1.54 x 10^8 cfu mL⁻¹. It was verified that there was no toxic active chemical compounds found after the application of neutralizer and dilution-neutralization method. The output of this study was a bactericidal against strains: *Escherichia coli* (ATCC® 10536™), *Staphylococcus aureus* subsp. *Aureus* (ATCC® 6538™) and *Salmonella enterica* subsp. enterica serovar *Typhimurium* (ATCC® 14028™).

1. Introduction

The environment has been attacked by various types of bacteria, both gram-negative and gram-positive. Bacteria from gram-negative groups pose more dangerous risk compared to gram-positive groups due to its toxicity membranes to its host. The 3-layered-cellwall in gram-negative bacteria acts as a strong self-defence. The existence of various types of bacteria causes the risk of disease outbreaks and even causes death. Some types of bacteria get particular attention including: *Escherichia coli* (-), and *Salmonella typhimurium* (-) and *Staphylococcus aureus* (+).

Academic or office area was considered as a vulnerable public area to the *E. coli* outbreak. Eating and drinking habits coupled with low hygiene environments were believed to be the main causes of the outbreaks [1]. Academic spots such as canteens, toilets and well water were reported to be the sources of *E. coli* transmission, and even contributed to the spread of hepatitis A virus (HAV) [2]. Predominant transmission source of the *E. coli* outbreak in America was food, followed by personal contact, animal contact and other factors [3]. A study was applied to the *E. coli* outbreaks from bivalve shells [4], vegetables [5], water based recreations [6], beef, poultry, milk, vegetables leaves, nuts, fruit and sprout [3].

Outbreaks of *Salmonella typhimurium* were reported in Spain from February to May 2011. Patients at the Capital Hospital were positively notified of *S. typhimurium* with common symptoms: diarrhea,
vomiting, blood in faeces and abdominal pain, caused by contaminated food [7] and eggs [8,9]. Exceptions to the S. typhimurium outbreak were not contagious to people from the Maghreb (Maroko, Aljazair, Tunisia, Libya, Mauritania) since pork is forbidden for Muslims [7]. Clinical manifestations that were similar: fever, headache, vomiting, and tired and following by diarrhea, colic abdomen, and cough; mentioned in a study at Soerya Hospital, Indonesia. It was identified that 12 patients were suffering from dengue fever and secondary fever salmonellosis more than 10 days, where it usually takes 10 days of S. typhimurium infection [7,10].

The Staphylococcus aureus agent may come from goat milk [11] or cow milk [12]. Milk is a rich source of protein. However, preventive efforts should be taken in consuming dairy products due to the bacteria contamination risks.

Bacteria also exist indoors, thus it should be given particular concern due to its infected risk. The initial study has examined the impact of CNO against bacteria. This study, furthermore, aimed to investigate the impact of CNO in a slight different form, i.e., aerosol products, against the following bacterial strains: Escherichia coli (ATCC®10536™), Staphylococcus aureus subsp. Aureus (ATCC®6538™) and Salmonella enterica subsp. enterica serovar Typhimurium (ATCC®14028™). These products were intended for research purposes only.

2. Materials and Methods

2.1. Characterization of Cymbopogon nardus oil (CNO)

Characterization of CNO was carried out to observe specific gravity, refractive index, optical rotation, alcohol solubility and viscosity, by using gravimetry, refractometry, polarimetry, volumetric and viscometer, respectively. A gas chromatography was performed to analyse pure compounds as active compounds in CNO (citronella, citronellol and geraniol).

2.2. Experimental design

Three different concentrations of CNO were determined as treatments in this study: 1%, 3% and 8%. It was then injected into 100 mL canned tubes containing 30 g contents/filler and 20 g provelan, for each treatment. In this study, commercial bactericidal was not present in the sample. The industrial standard was applied in the CNO injection process: skilled labour, standardized machinery, safety procedures includes contaminant controls and materials handling. A manufacturing company located in Bogor, was selected to inject the CNO into canned tubes. The company has certified by ISO 9001 in producing aerosol products with rotary filling machines. The content/filler type and provelan were confidential that outlined in the company code of ethic.

| Table 1. Concentrate composition of aerosol product sample |
|----------------------------------------------------------|
| Volume | Expected application | Remarks |
|--------|----------------------|---------|
| CNO-1  | 1%                   | Handwash| All sample contained 30 g filler and 20 g provelan in 100 mL canned tubes packaging |
| CNO-3  | 3%                   | Personal room |
| CNO-8  | 8%                   | Meeting room |

2.3. Test method

A sequence of laboratory test was performed by using the following apparatus: spectrophotometer, autoclave, water baths, incubator, pH meter, stopwatch, mechanical shaker, refrigerator, graduated pipet test, petri dishes, glass beads and volumetric flash. The bactericidal activity (BA) was determined by a dilution-neutralization method [13] on quantitative testing at 80% concentration of product sample.
Selected strains in this study were *Escherichia coli* ATCC®10536™, *Staphylococcus aureus* subsp. *Aureus* ATCC®6538™ and *Salmonella enterica* subsp. *enterica* serovar *Typhimurium* ATCC®14028™. Admittedly, these products were intended for research use only.

2.3.1. The principles of the method. Each sample were sprayed into a sterile tube glass and diluted with hard water. Afterwards, it was added to a test suspension of bacteria in a solution of an interfering substance. The mixture was maintained at 20 °C for 5 min of contact time. Water shall be as new distilled water-not demineralized water. At the end of contact time (5 min), an aliquot was taken, and bactericidal activity (BA) was immediately neutralized by the diluted-neutralization method. The BA was then determined by the reduction logarithm (Log R). The method applies for clean condition. A 0.3 mL bovine albumin was added as interfering substance.

2.3.2. Laboratory practices. The interfering substance was pipetted (1 mL) into a microtube, and added with 1 mL test suspension and mix solution for 2 min in the desired water bath condition. At the end of contact time, the 8 mL of product sample solution was added. After 5 min of contact time, 1 mL of the text mixture was pipetted to a tube containing 5 mL neutralizer and 1 mL water. The tube was then put into water bath at 20 °C for 5 min.

The following was neutralizer composition used in this study: Peptone 10 g L⁻¹, Beef extract 5 g L⁻¹, NaCl 5 g L⁻¹, Soy lecithin 1 g L⁻¹ and Polysorbate 80 20 g L⁻¹. About 1 mL mix solution of product sample solution, interfering substance, neutralizer and test suspension was spread on dried squared glass containing Tryptic Soy Agar (TSA). The incubation time was 24 h at 36 °C. Figure 1 describes the method.

Figure 1. Dilution-neutralization method

Remark: 1=test suspension (N); 2= validation suspension (Nv); 3= interfering substance; 4= hard water; 5= neutralizer (20 °C); 6= diluent; 7= product sample solution; 8= rinsing liquid (20 °C); 9= water; 10= mixture

To validate experimental condition (control A) the following steps were performed. One mL interfering substance was pipetted and added with 1 mL validation suspension. After that, the mixture was put in the water bath for 2 min. At the end of contact time, the obligatory of 8 mL hard water was added. Stopwatch was recalculated for 5 min. Figure 2 illustrates experimental method of control A.
To validate the absence of toxicity of the neutralize (control B), the following method was carried out: 8 mL of the neutralizer was pipetted into a tube, and added with 1 mL of water and 1 mL of the validation suspension just before the end of 2 min contact time at 20 °C. This procedure is illustrated in Figure 3.

Remarks: 1= test suspension (N); 2= validation suspension (Nv); 3= interfering substance; 4= hard water; 5= neutralizer (20 °C); 6= diluent; 7= product sample solution; 8= rinsing liquid (20 °C); 9= water; 10= mixture

Figure 2. Experimental control (A)

To validate the dilution-neutralization method (control C), the subsequent steps were completed: 1 mL of the interfering substance was pipetted into a tube, and added with 1 mL of diluent. It was then mixed in the water bath for 2 min and at the end of time 8 mL sample solution was added. After that, the stopwatch was restarted for 5 min at 20 °C. After 5 min of time, 1 mL of the mix solution was transferred into a tube containing 8 mL of neutralizer and put the solution into water bath for 2 min at 20 °C. Subsequently, 1 mL of the validation suspension was added and the stopwatch was restarted for 30 min at 20 °C. Eventually, 1 mL of the mix (control C) was put in to dried-squared glass and inoculated for 24 h at 36 °C. Method validation (control C) is depicted in Figure 4.

Remark: 1= test suspension (N); 2= validation suspension (Nv); 3= interfering substance; 4= hard water; 5= neutralizer (20 °C); 6= diluent; 7= product sample solution; 8= rinsing liquid (20 °C); 9= water; 10= mixture

Figure 3. Neutralizer control (B)

Remark: 1= test suspension (N); 2= validation suspension (Nv); 3= interfering substance; 4= hard water; 5= neutralizer (20 °C); 6= diluent; 7= product sample solution; 8= rinsing liquid (20 °C); 9= water; 10= mixture

Figure 4. Method validation (control C)
3. Results

3.1. Cymbopogon nardus oil (CNO) profile
Relative gravity of CNO at 20 °C was 0.8862; refractive index 1.4715 (20 °C); optical rotation +0.50°; miscibility alcohol 80% was 1:1 and viscosity was 9.60. Constituents of active compound were citronellal (16.80%), citronellol (15.40%) and geraniol (34.24%).

3.2. Dilution-neutralization method
Diluted-neutralization methods have been used by previous researchers [14–20] to evaluate BA using EN 1276. In this study, product concentration resulting in a $10^5$ or greater reduction in the number of viable cells was bactericidal (log R ≥ 5 or % R ≥ 99.999 %). The samples were bactericidal legitimated by following condition (Table 2):

1. N is between $1.5 \times 10^6$ cfu mL$^{-1}$ and $5 \times 10^7$ cfu mL$^{-1}$;
2. Nv is between $1.5 \times 10^5$ cfu mL$^{-1}$ and $5 \times 10^6$ cfu mL$^{-1}$;
3. Nv is between 300 cfu mL$^{-1}$ and 1600 cfu mL$^{-1}$;
4. Nvo is between 30 cfu mL$^{-1}$ and 160 cfu mL$^{-1}$;
5. A, B, C are greater than or equal to 0.5 Nvo.
Table 2. Validation and controls of bactericidal test

| Strain              | Test Bacterial Suspension | Validation and Control | A  | B  | CNO-1 | CNO-3 | CNO-8 |
|---------------------|---------------------------|------------------------|----|----|-------|-------|-------|
|                     |                           |                        |    |    |       |       |       |
| *Escherichia coli*  |                           |                        | 79 | 74.5| 39.5  | 43.5  | 43.5  |
| N                   | $= 1.98 \times 10^8$      | Nv = 610               |    |    |       |       |       |
| No                  | $= 1.98 \times 10^7$      | Nvo = 61               | 37 | 47.5| 35    | 32.5  | 32    |
| Log No              | $= 7.3$                   |                        |    |    |       |       |       |
| *Salmonella typhimurium* | $= 1.54 \times 10^8$   | Nv = 360               |    |    |       |       |       |
| No                  | $= 1.54 \times 10^7$      | Nvo = 36               |    |    |       |       |       |
| Log No              | $= 7.19$                  |                        |    |    |       |       |       |
| *Staphylococcus aureus* | $= 1.25 \times 10^8$   | Nv = 1175              | 129| 136.5| 78    | 81    | 74    |
| No                  | $= 1.25 \times 10^7$      | Nvo = 117.5            |    |    |       |       |       |
| Log No              | $= 7.35$                  |                        |    |    |       |       |       |

Remarks:
N = number of cfu mL$^{-1}$ of the test bacterial suspension
No = N/10
Nv = number of cfu mL$^{-1}$ of validation suspension
Nvo = Nv/10
A = validation of experimental and/or the absence of any lethal factor in test condition
B = the absence of toxicity in the neutralizer
C = sample study (CNO-1, CNO-3 and CNO-8)

Table 3. Laboratory test report on bactericidal activity (BA)

| Sampel | Strain           | Test bacterial suspension | Result at concentration 80% (v/v) |
|--------|------------------|---------------------------|----------------------------------|
| CNO-1  | *Escherichia coli* |                           | Na $< 140$; Log Na= 2.15         |
|        | N                | $= 1.98 \times 10^8$      | % R = 99.9993%; Log R= 5.15     |
|        | No               | $= 1.98 \times 10^7$      |                                   |
|        | Log No           | $= 7.3$                   |                                   |
|        | *Salmonella typhimurium* | $= 1.54 \times 10^8$   | Na $< 140$; Log Na= 2.16         |
|        | No               | $= 1.54 \times 10^7$      | % R = 99.9991%; Log R= 5.04     |
|        | Log No           | $= 7.19$                  |                                   |
|        | *Staphylococcus aureus* | $= 1.25 \times 10^8$   | Na $< 140$; Log Na= 2.15         |
|        | No               | $= 1.25 \times 10^7$      | % R = 99.9994%; Log R= 5.2      |
|        | Log No           | $= 7.35$                  |                                   |
| CNO-3  | *Escherichia coli* |                           | Na $< 140$; Log Na= 2.15         |
|        | N                | $= 1.98 \times 10^8$      | % R = 99.9993%; Log R= 5.15     |
|        | No               | $= 1.98 \times 10^7$      |                                   |
|        | Log No           | $= 7.3$                   |                                   |
|        | *Salmonella typhimurium* | $= 1.54 \times 10^8$   | Na $< 140$; Log Na= 2.16         |
|        | No               | $= 1.54 \times 10^7$      | % R = 99.9991%; Log R= 5.04     |
|        | Log No           | $= 7.19$                  |                                   |
|        | *Staphylococcus aureus* | $= 1.25 \times 10^8$   | Na $< 140$; Log Na= 2.15         |
|        | No               | $= 1.25 \times 10^7$      | % R = 99.9994%; Log R= 5.2      |
|        | Log No           | $= 7.35$                  |                                   |
| CNO-8  | *Escherichia coli* |                           | Na $< 140$; Log Na= 2.15         |
|        | N                | $= 1.98 \times 10^8$      | % R = 99.9993%; Log R= 5.15     |
|        | No               | $= 1.98 \times 10^7$      |                                   |
|        | Log No           | $= 7.3$                   |                                   |
|        | *Salmonella typhimurium* | $= 1.54 \times 10^8$   | Na $< 140$; Log Na= 2.16         |
|        | No               | $= 1.54 \times 10^7$      | % R = 99.9991%; Log R= 5.04     |
|        | Log No           | $= 7.19$                  |                                   |
|        | *Staphylococcus aureus* | $= 1.25 \times 10^8$   | Na $< 140$; Log Na= 2.15         |
|        | No               | $= 1.25 \times 10^7$      | % R = 99.9994%; Log R= 5.2      |
|        | Log No           | $= 7.35$                  |                                   |

Remarks: Interfering substances: 0.3 g L$^{-1}$ bovine albumin (clean conditions); contact time: 5 min; test temperature: 20 °C; incubation temperature: 36 °C.
N = number of cfu mL\(^{-1}\) of the test bacterial suspension
No = N/10
Log No = logarithmic No
Na = number of cfu mL\(^{-1}\) in the test mix
R = reduction in number of viable cells
Log R = logarithmic reduction in number of viable cells (log No – log Na)

All samples in this study were bactericidal against strains: *Escherichia coli* (ATCC\(^{\text{®}}\)10536\(^{\text{™}}\)), *Staphylococcus aureus* subsp. *Aureus* (ATCC\(^{\text{®}}\)6538\(^{\text{™}}\)) and *Salmonella enterica* subsp. *enterica* serovar *Typhimurium* (ATCC\(^{\text{®}}\)14028\(^{\text{™}}\)) as noted in Table 3.

### 4. Discussions

#### 4.1. Profile Cymbopogon nardus oil (CNO)

Essential oil (EO) has a distinctive aroma and biological property, due to its terpenoids and phenylpropanoids. Essential oil has long been used as a traditional medicine in various parts of the world. An article review describes the application of essential oils, including: antifungal [21], antibacterial, anticancer, antimutagenic, antidiabetic, antiviral, anti-inflammatory and antiprotozoal properties [22]. Cymbopogon nardus oil was part of the list, representing Poaceae grasses family.

Citronella oil derived from *Cymbopogon nardus* (L.) Rendle, however, it does not have its own quality standards yet. Species in Cymbopogon genus have distinctive characteristics. Some quality standards have been established for *Cymbopogon nardus* (L.) W. Watson var. lenabatu Stapf (MS ISO 3849: 2008), *Andropogon nardus* de jong (SNI 06-3953-1995), *Cymbopogon winterianus* Jowitt syn and *Cymbopogon nardus* (L.) Will. Watson var. maha-pengiri Winter (ISO 3848: 2001). It was agreed that the active compounds commonly found in species were citronellal, citronellol and geraniol compounds. Characteristics and chromatographic profile of CNO was depicted in Table 4.

#### Table 4. Characteristics and chromatographic profile of CNO compare to particular standard

| Parameter                          | Present study | MS ISO\(^{a}\) 3849:2008 | SNI 06-3953-1995\(^{b}\) | ISO 3848:2001\(^{c}\) |
|------------------------------------|---------------|-----------------------------|---------------------------|------------------------|
| Relative density at 20 °C          | 0.8662        | 0.891-0.910                 | 0.880-0.922               | 0.880-0.893            |
| Refractive index at 20 °C          | 1.4715        | 1.479-1.490                 | 1.466-1.475               | 1.467-1.473            |
| Optical rotation at 20 °C          | +0.50°        | -25° to -12°                | -5° - 0°                  |                        |
| Miscibility in ethanol, 80 %       | 1:1           | 1:2                         | 1:2                       | 1:2                    |
| Citronellal, %                     | 16.80         | 3 to 6                      | Min 35                    | 31-39                  |
| Citronellol, %                     | 15.40         | 3 to 8.5                    | 8.5-13                    |                        |
| Geraniol                           | 34.24         | 15.0 to 23.0                | Min 85                    | 20-25                  |

Remarks: \(^a\)Cymbopogon nardus (L.) W. Watson var. lenabatu Stapf; \(^b\)Andropogon nardus de jong, \(^c\)Cymbopogon winterianus Jowitt syn and Cymbopogon nardus (L.) Will. Watson var. maha-pengiri Winter

#### 4.2. Calculation and validation of dilution method

Bactericidal activity test in this study was valid, as shown in Table 2. A method is accurate according to the following factors: the number of cfu mL\(^{-1}\) of test bacterial suspension (N), the number of cfu mL\(^{-1}\) of validation suspension (Nv), control of experimental condition (A), control of non-toxicity of neutralizer (B) and sample study (CNO). The N value of 1.5 x 10\(^8\) cfu mL\(^{-1}\) to 1.9 x 10\(^8\) cfu mL\(^{-1}\) while Nv value of 360 cfu mL\(^{-1}\) to 1175 cfu mL\(^{-1}\), within the range of EN 1276 standard. The value of experimental control (A), neutralizer control (B) and method control (C) were greater than 0.5 x Nvo. In the described condition, the neutralization method was validated for the tested bacterial, when a concentration of 80% or the highest concentration of the sample was used.
4.3. Bactericidal activity

Our environment has become vulnerable to the presence of bacteria since it poses negative effects to health. The outbreaks of *E. coli* [1–6], *S. typhimurium* [7,8,10] and *S. aureus* [11,12] showed the evidence of pathogenic bacterial infections risks. Preventive efforts should be made to lessen the hazardous level. Office area as a public space is vulnerable to sanitary behavior. Personal and meeting rooms in the office are considered to be a bacteria-free area. This study aimed for investigating CNO product as a bactericidal for hand, private and meeting room hygiene. The CNO has been widely used as floor cleaner, and the collaboration of CNO floor cleaner and spray CNO would be increase the efficacy of CNO as bactericidal. This study included CNO concentrates in can spray tubes, for hand hygiene purposes, personal rooms and meeting rooms. The method was used to determine whether or not a product sample has bactericidal activity [14–18,20].

It should be noted that bactericidal activity was expressed in the logarithm of reduction (Log R), must be more than or equal to the value of 5. Aerosol containing CNO at a concentration of 1%, 3% and 8% was stated to have effective bactericidal activity against selected strains (Table 3). The Log R value at each concentration was above 5, this value showed a remarkable result for each concentration. Nevertheless, this study has slightly distinctive results with previous studies [22,23]. This may due to the concentration of active compounds determined by soil, local climate, extraction methods and material conditions during distillation. Furthermore, a very test methods and target bacterial strains could become an argument. This heterogeneous result is a necessity.

The earlier study [24] showed different result from this study who argued that CNO could not kill *E. coli O157: H7 ATCC 35150*, *E. coli O157: H7 S0575*, and *S. typhimurium ATCC 14028* strains, by using paper disk diffusion method, except for *S. typhimurium S0584*. Similarly, Acheampong et al. [25] reported that *C. nardus* hydrosol dried leaves by agar-diffusion method had lack of impact for *E. coli ATCC 25922* and *S. aureus ATCC 25923*.

This study, nevertheless, confirmed the agar dilution method through the Minimum Inhibitory Concentrations (MIC) for *E. coli NCTC 10418* and *Salmonella enterica subsp. enterica serotype typhimurium ATCC 13311* [26]. Correspondingly, another study by Ratananikom et al. [27] reported similar data against the following strains: *Staphylococcus aureus* (TISTR 746), *Escherichia coli* (TISTR 117) and *Salmonella typhimurium* (TISTR 1469) evaluated by a measurement of the diameter of inhibition (MIC) zone. *E. coli O157: H7* strains, *Salmonella typhimurium SL 1344* and *Staphylococcus aureus* were reported to have a negative response to CNO [28]. Also, CNO was effective to kill *S. aureus ATCC 25923*, *S. Typhimurium ATCC 14028* and *E. coli O157: H7 NCTC 12900* [29]. The efficacy of CNO was also demonstrated against *E. coli strains CIP 105182*, *S. enterica CIP 105150*, *S. typhimurium ATCC 13311* and *S. aureus ATCC 9144* [30].

The initial study found that CNO had bactericidal activity against *E. coli*, *S. aureus* and *S. typhimurium*, with the absence of strain identification [31–34]. CNO is even reported to be effective against *Pseudomonas putida* [35], *Aspergillus*, *Penicillium* and *Eurotium* [36], *Listeria monocytogenes ATCC 19117* [37], *Listeria monocytogenes 2812* [28], *L. monocytogenes*, *B. cereus* and *P. aeruginosa* [29].

5. Conclusion

This study demonstrated the efficacy of CNO against *Escherichia coli ATCC 10536*, *Staphylococcus aureus ATCC 6538* and *Salmonella typhimurium ATCC 14028* through the value of Log R algorithm above 5. Typical compounds responsible for bactericidal activity were citronell, citronellol and geraniol. The prospect of using CNO for hand hygiene, private and meeting rooms in office area is possible. The discrepancy between the existing data and study results, however, requires further discussions. Materials used for active compounds concentration and strain types should be revealed in details.
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**Conflict Statement**
Authors state that there is no conflict of interest in this study.

**Acknowledgement**
The authors are gratefully to Forest Product Research and Development, Mr. Erik Dahlan and Mr. Amri for their support in conducting this study. All authors had equal contribution to this paper.