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Effects of combination of alcohol and *Cinnamomum burmannii* essential oil against *Klebsiella pneumoniae* resistance

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ABSTRACT

Alcohol-based antiseptics are widely used in the COVID-19 pandemic to prevent the transmission of infections, including bacterial infections. However, bacterial resistance to the alcohol-based antiseptics is begun reported. *Klebsiella pneumonia* resistance is one of the bacterial resistances that is prioritized by the WHO to be overcome. *Cinnamomum burmannii* essential oil, containing cinnamaldehyde and eugenol, was investigated for antimicrobial activity. This study aimed to evaluate the synergistic effect of the combination of alcohol and *C. burmannii* essential oil in inhibiting bacterial growth. Ethanol 80% in a combination with *C. burmannii* essential oil at concentrations of 1, 2, and 3% v/v were evaluated against *K. pneumoniae* using the Kirby-Bauer disc diffusion method. Test was repeated three times in independent experimental. Inhibition zone diameter (IZD, mm) and antimicrobial index (AI, %) were determined and analyzed using Kruskal-Wallis test continued the Mann-Whitney test. The combination of ethanol and *C. burmannii* essential oil was sensitive to *K. pneumoniae*, meanwhile, ethanol 80% was not more sensitive. The IZD of the combination solution at 1, 2, and 3% concentration were 6.7±0.19, 9.0±0.58, and 11.0±1.15mm, respectively ($p<0.05$). The AI of the combination solution at concentrations of 1, 2, and 3% v/v were 7.04±2.04, 30.53±6.79, and 51.64±12.91%, respectively ($p<0.05$). In conclusion, the combination of ethanol 80% and *C. burmannii* essential oil active against *K. pneumoniae* which resistant to the ethanol.

ABSTRAK

Antiseptik berbasis alkohol digunakan secara luas selama pandemic COVID-19 untuk pencegahan penyebaran infeksi, termasuk infeksi bakteri. Namun, terjadinya resistensi terhadap antiseptik berbasis alkohol tersebut mulai dilaporkan. Resistensi terhadap *Klebsiella pneumonia* merupakan salah satu resistensi bakteri yang diprioritaskan dicegah oleh WHO. Minyak atsiri *Cinnamomum burmannii,* yang mengandung sinamaldehida dan eugenol, telah diteliti aktivitas antimikrobanya. Penelitian ini bertujuan mengkaji efek sinergis kombinasi alkohol dan minyak atsiri dalam menghambat pertumbuhan bakteri. Kombinasi etanol 80% dan minyak atsiri *C. bumannii* pada konsentrasi 1, 2 dan 3% v/v dikaji aktivitasnya terhadap *K. pneumonia* menggunakan metode difusi cakram Kirby-Bauer. Uji diulangi tiga kali secara independent. Diameter zona hambatan (mm) dan indeks antimikroba (%) dihitung dan dianalisis dengan uji Kruskal-Wallis dilanjutkan dengan uji Mann-Whitney. Kombinasi etanol dan minyak atsiri *C. burmannii* sensitif terhadap *K. pneumoniae*, sedangkan etanol 80% tidak sensitif lagi. Diameter zona hambatan larutan kombinasi tersebut pada konsentrasi 1, 2, dan 3% berturut-turut adalah 6,7±0,19, 9,0±0,58, and 11,0±1,15mm ($p<0,05$). Indeks antimikroba larutan kombinasi tersebut pada konsentrasi 1, 2, dan 3% berturut turut adalah 7,04±2,04, 30,53±6,79, dan 51,64±12,91% ($p<0,05$). Dapat disimpulkan, kombinasi etanol 80% dan minyak atsiri *C. burmannii* aktif terhadap *K. pneumoniae* yang resisten terhadap etanol.

Keywords:
alcohol;
Cinnamomum burmannii;
Klebsiella pneumonia;
resistance;
antiseptics

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INTRODUCTION

Nosocomial infection is the most common adverse event during hospitalization that affects patient safety. It contributes to significant morbidity, mortality, and financial burden on patients and healthcare system.[1] *Klebsiella pneumoniae* is one of the Gram-negative bacteria that causes nosocomial infection. Nosocomial *K. pneumoniae* infection affect 46.6% of all hospitalized patients during their stay in ICU at Dr. Cipto Mangunkusumo General Hospital, Jakarta.[2] Nosocomial *K. pneumoniae* bloodstream infection was also associated with 47% of the mortality rate in Istanbul, Turkey.[3] *Klebsiella pneumoniae* is an opportunistic bacterium that often causes pneumonia due to the use of ventilators (ventilator-acquired pneumoniae) in hospitals.[4] During the COVID-19 pandemic, the use of a ventilator significantly increases to help breathing of patients lead to increase of *K. pneumoniae* infection risk.

Antimicrobial resistance due to misuse and overuse of antibiotics is a global health and development threat. More than 2.8 million microbial resistant infections were reported in the Unites States annually resulting more than 35,000 patients death.[5,6] World Health Organization (WHO) lists *K. pneumoniae* as one of the pathogens of high priority and promotes the research and development of new antibiotics due to the growing global problem of antimicrobial resistance.[7] Recently, *K. pneumoniae* is showing a high resistance to a broad spectrum of antibiotics including β-lactams antibiotics, fluoroquinolones and aminoglycosides.[8,9]

One way to control antibiotic resistance is by using antiseptics and disinfectants. They play an important role in the control of infection practices and in the avoidance of nosocomial infections.[9] Alcohol-based antiseptics are widely used in sterilization of medical devices and surgical instruments. However, massive use of the antiseptics might lead to the development of bacteria resistance that eventually causes they become ineffective.[10] Nosocomial bacteria, including *methicillin-resistant Staphylococcus aureus* (MRSA), *Acinetobacter baumannii, Escherichia coli, Klebsiella spp.,* and *Pseudomonas aeruginosa* have become resistant to the antiseptics in many health care centers.[10-12] The CDC recommends alcohol to be used as an antiseptic or disinfectant at a concentration of 70%.[13] An higher concentration, alcohol evaporate more quickly, even before it penetrates the microbial cell membrane and irritates the skin. Alcohol-based antiseptics resistance can be slowed by combination with another kind antiseptics. Some essential oils have been proven to have antibacterial activity and might be used in combination with alcohol as antiseptics.[14]

Essential oil is an oil derived from plant extracted from leaves, flowers, stems, bark, berries, roots, and other parts of plants.[14] The main constituents of cinnamon are cinnamaldehyde, trans-cinnamaldehyde, o-methoxy-cinnamaldehyde, cinnamyl acetate, benzaldehyde, phenylethanol, borneol, eucalyptol, eugenol, coumarin, and cinnamic acid. *Cinnamomum burmannii* essential oil was reported to have an antimicrobial effect. Cinnamaldehyde and eugenol in the essential oil of *C. burmannii* were proven active against *S. aureus, E. coli, A. baumannii,* and *P. aeruginosa*.[15,16] Essential oil from *C. burmannii* showed a better bacterial growth inhibition rate on respiratory tract pathogens than other types of essential oil.[17,18] This essential oil could be used in combination with another antiseptic and expected can slow the bacterial resistance to antiseptics. This study aimed to investigate the antibacterial effect of alcohol in combination with *C. burmannii* essential oil against *K. pneumoniae*.

MATERIALS AND METHODS

Bacterial strain

The study was performed against Gram-negative *K. pneumoniae* bacterium. Standardized *K. pneumoniae* ATCC – BAA 1706 was used in this study. The tested bacteria were cultured in the Clinical Microbiology Laboratory, Central Health Laboratory, Ministry of Health of Republic of Indonesia, Surabaya, East Java, Indonesia. This study was conducted from June to August 2021.

Essential oil preparation

Cinnamomum burmannii was purchased from a company, Purwakarta, Central Java, Indonesia. The essential oil was prepared by extraction the bark of the plant using the steam distillation method. The essential oil contents cinnamaldehyde at concentration of 67%.

Preparation of solution combination of ethanol 80% and *C. burmannii* essential oil

Five tested solutions were prepared against *K. pneumoniae* bacterium. They consisted of gentamycin 10 µg as antibiotic control group (C1), ethanol 80% control group (C2), and treatment group consisting ethanol 80% in combination with *C. burmannii* essential oil 1 (T1), 2 (T2), and 3% (T3). The ethanol 80% was prepared by diluting ethanol 96% with aquadest and glycerin 8% solution. Whereas the *C. burmannii* essential oils were prepared by diluting isolated essential oils with aquadest and glycerin 8% to obtained final concentration of 1, 2 and 3%.

Preparation of bacterial suspension

A standard McFarland suspension was prepared by mixing 0.5 mL of $BaCl_2$ with 99.5 mL of H_2SO_4. The bacterial suspension was prepared by mixing several bacterial colonies from cultured *K. pneumoniae* ATCC – BAA 1706 on Mac Conkey agar into a 0.9% NaCl solution. The bacterial turbidity is expected to be equal to the turbidity of the standard 0.5 McFarland suspension containing 1.5 x 10^8 CFU/mL.[19]

Preparation of the bacterial culture media

For the antibacterial susceptibility testing, the MHA (Muller–Hinton Agar) was used as bacterial culture media. The culture media were prepared in 1 L distilled water by dissolving 9.5 g of MHA. The obtained amber color solution was mixed thoroughly and boiled with frequent agitation to dissolve agar powder completely and a clear to slightly opalescent gel was obtained. The culture media were then sterilized by heating in an autoclave under pressure of 15 psi at 121 ^oC for 15 min. The sterilized culture media were then allowed to cool at room temperature in laminar flow hood.

Antibacterial susceptibility testing

The Kirby Bauer disc diffusion method was used for antibacterial susceptibility testing of different combination of ethanol 80% and *C. burmannii* essential oil. Twenty five mL of the cool sterilized culture media were poured into each Petri plate and were leaved for few minutes to allow the culture media to solidify. After solidification, the bacterial suspension were spread on the culture media by using cotton swab and cover the whole media with turn 90° degree rotation without leaving any gap. Five bores in diameter of 6 mm were made using a sterile cork borer in each Petri plate separated from each other by 2.5 cm distance. Thirty µL of each tested combination and control was poured in

the first three bores (T1-T3), gentamycin in the second last bore (C1), ethanol 80% in the third last bore (C2), and solvent in the last bore. All Petri plates were incubated in an incubator at 35 °C for 16-18 hr. Inhibition zone diameter (IZD) observed in the following day was measured to interpret antimicrobial susceptibility. This study has been approved by the Health Research Ethic Committee, Faculty of Medicine, Widya Mandala Surabaya Catholic University.

Data analysis

The inhibition zone diameter for each tested combination and control measured were presented as mean ± standard error of the mean (SEM). The tested combination or control is considered sensitive if the inhibition zone diameter > 6 mm, and considered resistant if the inhibition zone diameter ≤ 6 mm.[19-21] Furthermore, the antimicrobial index (AI, %) was calculated based on the following formula: $(1\text{-}Da/Dk) \times 100$ where Da is inhibition zone diameter in the experimental disc (cm) and Dk is inhibition zone diameter in the control disc (cm). The Kruskal-Wallis test continued the Mann-Whitney test were used to compare IZD and AI of each treatment and control. A p value < 0.05 was considered significant.

RESULTS

Antibacterial activity of various solutions tested against *K. pneumoniae* are presented in FIGURE 1 and TABLE 1 summarized the IZD of all solutions tested against *K. pneumoniae.*

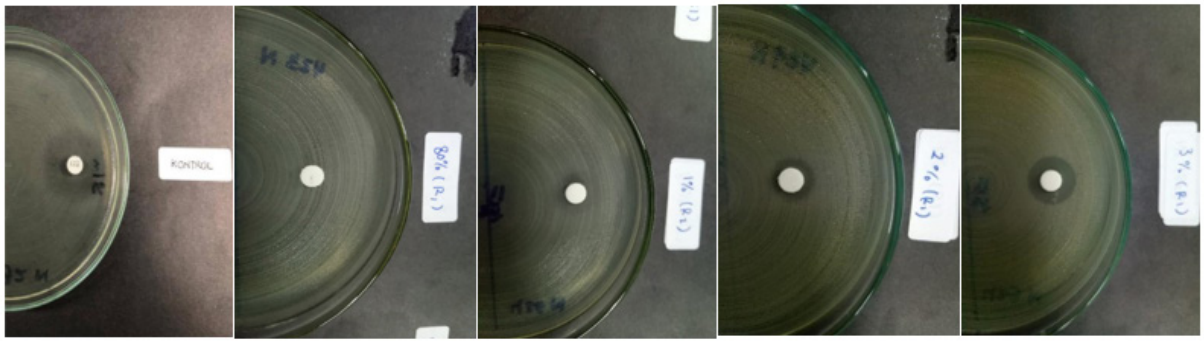

FIGURE 1. Antibacterial activity of various solutions tested against *K. pneumoniae.* C1:gentamycin; C2: ethanol; T1: ethanol + 1% essential oil; T2: ethanol + 2% essential oil; T3: ethanol + 3% essential oil.

TABLE 1. Results of disk diffusion test and AI

Groups	n	IZD (mean ± SEM mm)	Interpretation	AI (%)
C1	3	17.0 ± 0.88	Sensitive	100.0 ± 0.0
C2	3	< 6 ± 0	Resistant	0.0 ± 0.0
T1	3	6.7 ± 0.19	Sensitive	7.04 ± 2.04
T2	3	9.0 ± 0.58	Sensitive	30.53 ± 6.79
T3	3	11.0 ± 1.15	Sensitive	51.64 ± 12.91

n: replications; IZD: inhibition zone diameter; AI: antimicrobial index (%); C1:gentamycin; C2: ethanol; T1: ethanol + 1% ssential oil; T2: ethanol + 2% essential oil; T3: ethanol + 3% essential oil

Gentamycin 10 µg (C1) as antibiotic control and ethanol 80% (C2) as control had an IZD average of 17.0 ± 0.88 mm and < 6 ± 0.0 mm, respectively. Therefore, ethanol 80% was considered as had no antibacterial activity against *K. pneumoniae*. Furthermore, the ethanol 80% in combination with *C. burmannii* at concentrations of 1 (T1), (T2), and 3% (T3) had IZD average of 6.7 ± 0.19 mm, 9.0 ± 0.58 mm, and 11.0 ± 1.15 mm, respectively. Significantly different was observed between groups of this study ($p<0.05$). With a zone diameter < 0.6 mm, the ethanol 80% (C2) could not inhibit the *K. pneumoniae* growth. Therefore, it was considered that the *K. pneumoniae* is resistant to ethanol 80%. The combination of ethanol and *C. burmannii* essential oil could increase its sensitivity to *K. pneumonia* as indicated by the increase of the IZD. Furthermore, concentration-dependent in antibacterial activity of the combination of ethanol and *C. burmannii* essential oil was also observed. TABLE 1 also presented the AI of all solutions tested against *K. pneumoniae*. Gentamycin 10 µg (C1) as antibiotic control had an AI of 100%, whereas ethanol 80% did not have AI (0%). Furthermore, the ethanol 80% in combination with *C. burmannii* at concentrations of 1 (T1), (T2), and 3% (T3) had AI average of 7.04 ± 2.04%, 30.53 ± 6.79%, and 51.64 ± 12.91%, respectively. Significantly different was observed between groups of this study ($p<0.05$).

DISCUSSION

Alcohol-based antiseptic is widely used due to it is easy to find and does not require water for rinsing. It is designed as a hand antiseptic available in some formulations either in liquid, gel, or foam preparations to inactive microorganisms or temporarily inhibit their growth.[22] Antimicrobial activity of alcohol is well understood through its ability to denature and coagulate protein of microorganism.[23] Alcohol-based antiseptics have been used for cleaning routines in hospital such as hand rubs, positioned in and around hospital wards. However, due to its routine and massive use a number of bacteria species are already resistant to alcohol such as *S. aureus, A. baumannii, E. coli, Klebsiella spp.,* and *P. aeruginosa.*[9,10,24-26] In order to slow or stop bacterial resistance, new antiseptics or alcohol-based antiseptic combinations should be applied.

In this study, a combination of ethanol 80% with *C. burmannii* essential oil was evaluated against *K. penumoniae*. This combination can inhibit the *K. pneumoniae* growth which resistant to ethanol 80%. The IZD and AI of the combination significantly increased compared to that ethanol 80% alone indicating a synergic effect of both of them (TABLE 1).

The antibacterial activity of *C. burmannii* and other *Cinnamomun* sp. were reported by some authors. The *C. burmannii* essential oil had a high antifungal and antimicrobial activities against *A. flavus* and *K. pneumoniae*.[26] The methanol extract of *C. zeylanicum* was reported active against multidrug resistant (MDR) Gram-negative bacteria over expressing active efflux pumps including *K. pneumoniae* ATCC.[27] Another study reported that essential oils from *C. verum* and *C. camphora* actives against *A. flavus* and *K. pneumoniae* isolated from respiratory tract.[28] Zhang *et al.*,[21] have proven the antibacterial activity of cinnamon essential oils against *E.coli* and *S. aureus*, whereas Elcocks *et al.*[29] reported the antibacterial activity of cinnamon essential oils against *P. aeruginosa.* The active constituents of cinnamon essential oils as antibacterial dan antifungal have been also identified and isolated. The major constituents are found to be cinnamaldehyde (65-80%), cinnamyl acetate (2.5-16%), cinnamyl alcohol (2.25-4.6%), cinnamic acid (3-8%). Other abundant constituents are compounds containing endocyclic double bond

as α-thujene, α-terpineol, α-cubebene, unconjugated exocyclicdouble bond eugenol, β-caryophyllene, terpinolene and hydroxyl-substituted aliphatic compounds.[30-31]

The mechanism of actions as antibacterial both ethanol and cinnamon essential oil have been investigated. The antibacterial activity of ethanol is due to the ability to lyse cell membranes, denature and coagulate proteins from microorganisms.[23,32] Whereas, cinnamon essential oil acts by inhibit the ATPase lead to bacterial membranes damages as showed by irregular, invaginated, and abnormality of the bacteria membranes structure.[26,33-35] The combination of ethanol and cinnamon essential oil may result a synergistic effect as illustrated in FIGURE 2. This combination cause bacterial cell membrane disruption that lead to ethanol and active constituents facilitate enter into the bacterial cells and interact with ATPase. This interactions inhibit DNA synthesis and protein denaturation lead to bacterial metabolic disruption.

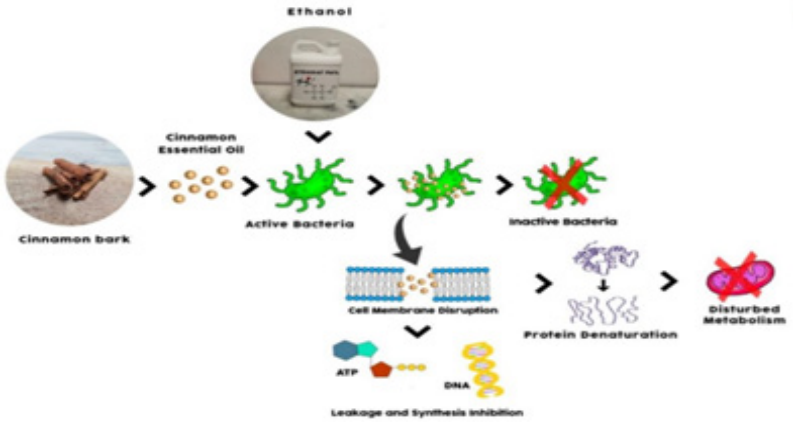

FIGURE 2. Illustration of mechanism of actions of the antibacterial activity of the combination of ethanol and cinnamon essential oils.

CONCLUSION

The combination of ethanol and *C. burmannii* essential oil has an antibacterial activity against *K. pneumonia* which resistant to the ethanol.

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