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Research Article

EFFECT OF VIRGIN COCONUT OIL ON *MYCOBACTERIUM SMEGMATIS* AND *STAPHYLOCOCCUS AUREUS* TREATED WITH EXTRACTS OF *ZANTHOXYLUM ACANTHOPODIUM* FRUIT

HEDDY JULISTIONO*, INTAN PERMATASARI SUSENO, NURUL HANDAYANI, RINI HANDAYANI, PUSPA DEWI LOTULUNG

Research Center for Chemistry LIPI, Jakarta, Indonesia. Email: hedd001@lipi.go.id

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ABSTRACT

Objectives: To understand the potency of herbal formulation of virgin coconut oil (VCO) and andaliman (*Zanthoxylum acanthopodium*) fruit activity against microbes, effects of ethylene acetate and hexane extracts of fruit of andaliman on viability and ions leakages of *Mycobacterium smegmatis* dan *Staphylococcus aureus* treated with VCO has been investigated.

Methods: Antibacterial activity of extracts of andaliman fruit, or VCO, or andaliman and VCO against *M. smegmatis* and *S. aureus* was investigated using MTT assay method. Membrane disruption of bacterial cells treated with the plant extract and VCO was determined by measuring potassium and sodium ions leakages using Atomic Adsorbtion Spectrophotometer.

Results: VCO of 512 μg/ml did not have antibacterial activity. In *M. smegmatis* treated with andaliman hexane extract, presence VCO decreased both ions leakage whereas in *S. aureus* treated with ethyl acetate extract only sodium ion was decreased. In both microorganisms, VCO could not protect cells of both *M. smegmatis* and *S. aureus* from death caused by andaliman extracts.

Conclusions: VCO prevented ions leakages of the bacteria treated with extract of andaliman but did not protects cells from death.

Key words: *Mycobacterium smegmatis, Staphylococcus aureus,* Virgin coconut oil, *Zanthoxylum acanthopodium,* Ions leakages.

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INTRODUCTION

Traditional coconut oil, which is widely known before virgin coconut oil (VCO), is an oil that is processed from dried coconut (*Cocos nucifera*) called as copra. However, nowadays, the VCO, refined coconut oil, has been becoming more popular due to its unchanging oil content and vitamins content such as provitamin A, Vitamin E, phytosterol, and polyphenol. VCO is also free of aflatoxin and not rancid. The concept of VCO production was inspired by virgin olive oil (VOO). Virgin oil is produced from olive oil in the Mediterranean region [1]. VCO is processed from fresh and ripe coconut flesh mechanically or naturally (without heat), by chemical distillation, bleaching process, and deodorization to maintain oil content [2]. Some examples of VCO health benefits were immunomodulators in chickens [3] and antibacteria used directly [4,5] and through pretreatment to release bound fatty acid content [6]. VOO could be enriched with herbal mixture that has certain ability such as antioxidants to obtain health product that can be used both orally and topically [7]. One of the advantages of this method is the application of herbal maceration via "green process" in oil.

In the effort of discovering the potential of VCO to be used with medicinal plants in a combined-formula, a preliminary study of the toxicity of the mixture of VCO and fruit extract of Andaliman (*Zanthoxylum acanthopodium*) on *Mycobacterium smegmatis* and *Staphylococcus aureus* was carried out. The andaliman fruit essential oil is notorious to have anti-bacterial [8] and anti-mycobacterial activities [9]. *M. smegmatis* is a type of a non-virulent *Mycobacterium* genus that was used as a model to study pathogenic *Mycobacterium* responses including *Mycobacterium tuberculosis* [10-12].

According to Yoon et al. [13], the cell membrane of bacteria is one of the targets of fatty acids, as well as essential oils, antibacterial action [14] that resulted in the cell membrane damage. Therefore, in this research, the study of VCO and fruit andaliman effects on Gram-positive bacteria *M. smegmatis* and *S. aureus* was emphasized on the destruction of membranes characterized by the leaking of K+ dan Na+ ions.

METHODS

Bacterial and mycobacterial cultures

The cultures of *S. aureus* InaCC-B4 and *M. smegmatis* NBRC 3082 were obtained from INACC LIPI, Indonesia. Laboratory collection of *Lactobacillus plantarum* was used for coconut oil fermentation.

Plant extract

The plants and green andaliman fruits were obtained from North Sumatra about 24 h after the harvest. Plants were identified in Herbarium Bogoriense, Indonesia. Before the extraction, andaliman fruits were stored at 4°C overnight. As many as, 366 g of fruits was extracted with 1500 ml of hexane; and then the residue was extracted with ethyl acetate.

Coconut milk was fermented by *L. plantarum* for 24 h for VCO preparation. The oil formed was separated from the water, and then filtered with filter paper [4]. Fatty acids content of this VCO is presented in Table 1.

The growth of target microbes

The cultures of *M. smegmatis* and *S. aureus* InaCC-B4 were grown on 100 ml of liquid media (Nutrient Broth, NB, HIMEDIA) in 300 ml Erlenmeyer flask, incubated on a shaker at 100 rpm and room temperature for 72 h (*M. smegmatis*) and 24 h (*S. aureus*).

Minimum inhibition concentration (MIC) measurement

Anti-mycobacterial or antibacterial activity was measured as MIC using MTT (Thiazolyl Blue Tetrazolium Blue) according to.[15-17] MIC

determination was performed on "microplate" with 96 wells at room temperature. The measurement of hexane extract on *M. smegmatis* was done by inoculating the culture (final concentration was 1 % v/v) with as much as 100 µl (with one series of extract concentration on media ranging from 521 µg/ml and 1% (v/v) Tween 80) on NB media. It was then diluted twice and continued until the final concentration of 1 µg/ml, shaken at 100 rpm for 72 h. The initial concentration of *S. aureus* started on the concentration after 24-h incubation. After incubation, as much as 10 µl of MTT solution (5 mg/ml) was added to each suspensions and the suspension was incubated for 2 h. After incubation, a total of 11 µl of propanol containing 0, 04 M HCl was added to the cell suspension and it was incubated for 2 h. The reduction of MTT by the cell enzymes produced fomazan. It then was measured using a microplate reader at the wavelength of 595 nm. MIC was determined at the same absorbance of the NB media, the absorbance at the time of reduction of MTT into formazan since there was no activity of the reducing enzyme detected. As the positive control, *M. smegmatis* culture was added with 8 µg/ml of rifampicin while *S. aureus* was added with 32 µg/ml of amoxicillin.

The effect of the mixture of VCO and andaliman fruits extract on the viability of *M. smegmatis* or *S. aureus*

Principally, the method of studying the effect of VCO and fruit extract of andaliman on microbes viability was carried out as in MIC measurement. The treatments included; the effect of 512 µg/ml of VCO on both tested microbes, the mixture of 512 µg/ml VCO and 64 µg/ml of hexane extract of andaliman fruit on *M. smegmatis* or 2048 µg/ml of ethyl acetate extract on *S. aureus*.

Measurements of cell leakage

The effects of VCO and andaliman extracts on the damage of cell membrane were observed indirectly by inspecting the ion leakage on cell membrane [18]. About 2 × 100 ml of 1 day old *S. aureus* culture and 3 days old of *M. smegmatis* culture were harvested through centrifugation at 15.000 g for 10 min. The pellets were washed with sterile distillation water and centrifuged. Sterile physiology solution was added into the pellet until the volume was 10 ml. About 1 ml of the suspension was taken for 512 µg/ml VCO treatment, 64 µg/ml of the hexane extract of the fruit andaliman (*M. smegmatis*) treatment or 2048 µg/ml of ethyl acetate extract (*S. aureus*) treatment, mixed VCO 512 µg/ml and 64 µg/ml of hexane extract of andaliman fruit (*M. smegmatis*) treatment, mixture of 512 µg/ml VCO and 2048 µg/ml of ethyl acetate extract (*S. aureus*) treatment and without treatment (control). The culture was incubated at room temperature on a shaker at 100 rpm. The supernatant of *S. aureus* was taken after one day incubation by centrifugation and 3 days incubation for (*M. smegmatis*). The amount of K+ and Na+ ions in the supernantan were measured using Atomic (Atomic Adsorbtion Spectrophotometer, Shimadzu AA - 6800).

RESULTS AND DISCUSSION

MIC of andaliman fruit extract and VCO on *M. smegmatis* or *S. aureus*

As shown in Table 1, the MIC of VCO was undetected up to the concentration of 512 µg/ml. This result corresponded to the absence of the inhibition area on a paper disc with 100% VCO (data was not shown). Table 1 shows that the andaliman fruit extracts antimicrobial activity depended on the target microbial. *M. smegmatis* was more susceptible to hexane extract of andaliman fruits; while *S. aureus* was vulnerable to ethyl acetate extract. The MICs of hexane and ethyl acetate extracts against *M. smegmatis* and *S. aureus* were 64 µg/ml and 2408 µg/ml respectively. Hexane extract of 64 µg/ml and ethyl acetate extracts of 2408 µg/ml was then applied to investigate the effect of VCO and andaliman fruit extracts on the viability and ions leakage on *M. smegmatis* and *S. aureus*.

Table 1: The fatty acids composition of VCO sample analyzed using gas chromatography

Fatty Acids	Percentage
Caprylic acid	9.028
Capric acid	6.825
Lauric acid	41.693
Myristic acid	17.121
Palmitic acid	9.092
Oleic acid	11.148
Linoleic acid	3.839
Linolenic Acid	Not detected

VCO: Virgin coconut oil.

The effect of the mixture of VCO and andaliman fruit extracts on the viability of *M. smegmatis* or *S. aureus*

Fig. 1a shows that VCO did not have antimicrobial activity of *M. smegmatis*, whereas andaliman hexane extract of 64 µg/ml caused cell death. The mixture of VCO and fruit andaliman extract treatment also resulted in cell death. This phenomenon implied that the VCO did not possess protective properties against the toxicity of andaliman fruit extract.

Fig. 1b also shows that the effect of the mixture of VCO and andaliman fruit extract on the viability of *S. aureus* showed the same pattern as the effect on mycobacteria.

The effect of the mixture of VCO and andaliman fruit extract on the ion leakage

Bacterial cell membrane or cytoplasm could be a target for antibacterial action of essential oil [14]. Ion leakage on target mycobacteria and bacteria treated with VCO and andaliman fruit extract was observed to obtain initial description of membrane as the target of the VCO and fruit extract of andaliman. However, the release of ions from the cytoplasm was not solely a result of membrane destruction but it might be caused by the changes in "ion channel" activity without any membrane damage [19].

In *M. smegmatis*, hexane extract of andaliman caused the increase of K+ and Na+ ions in supernatant however VCO decreased the ions content (Fig. 2a and b). Data presented in Fig. 2a and b demonstrate that VCO was able to attenuate ions leakage induced by andaliman extract.

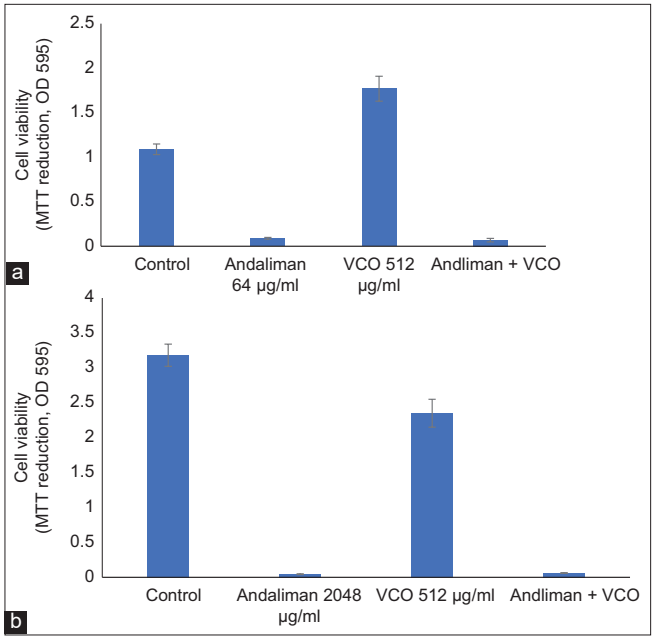

Fig. 1: (a) Effect of hexane extract of andaliman fruit and Virgin coconut oil on viability of *Mycobacterium smegmatis*. Bars represent mean ± standard error of three independent experiments. (b) Effect of ethyl acetate extract of andaliman fruit and virgin coconut oil on viability of *Staphylococcus aureus*. Bars represent mean ± standard error of three independent experiments

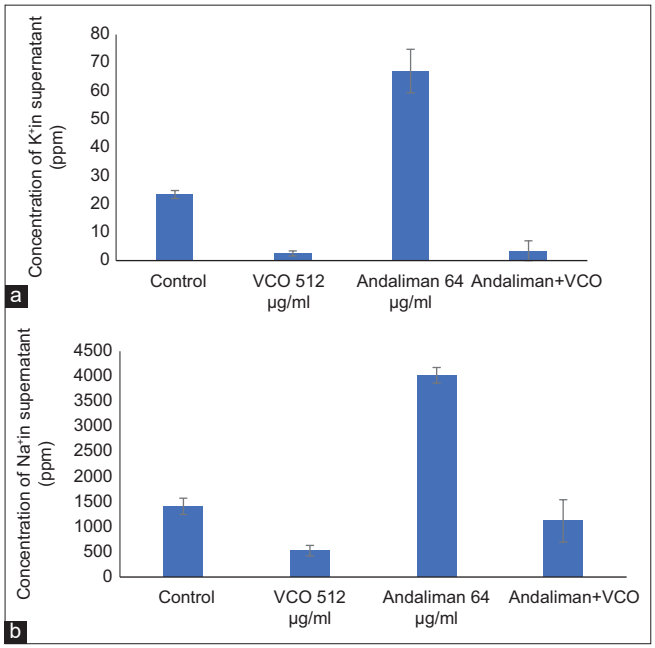

Fig. 2: (a) Effect of hexane extract of andaliman fruit and virgin coconut oil on K^+ ion leakage in *Mycobacterium smegmatis*. Bars represent mean ± standard error of three independent experiments. (b) Effect of hexane extract of andaliman fruit and virgin coconut oil on Na+ ion leakage in *Mycobacterium smegmatis*. Bars represent mean ± standard error of three independent experiments

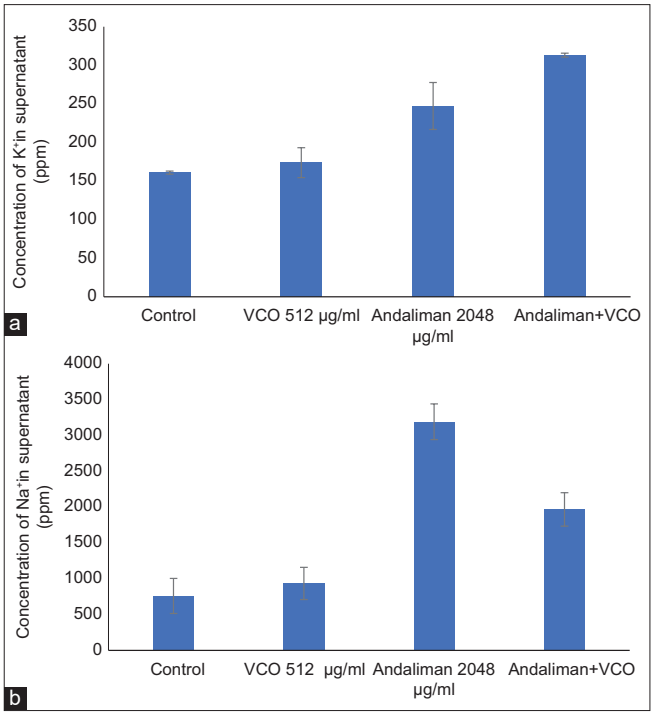

Fig. 3: (a) Effect of ethyl acetate extract of andaliman fruit and virgin coconut oil on K^+ ion leakage in *Staphylococcus aureus*. Bars represent mean ± standard error of three independent experiments. (b) Effect of ethyl acetate extract of andaliman fruit and virgin coconut oil on Na^+ ion leakage in *Staphylococcus aureus*. Bars represent mean ± standard error of three independent experiments

In *S. aureus* treated with andaliman extract, the increase of both K+ and Na+ ions was also observed but VCO had no effect on ions leakage (Fig. 3a and b). Although VCO has no effect on the movement of ions across cell membrane, it could attenuate Na+ leakage in cells treated with andaliman extract (Fig. 3b) not K+ (Fig. 3a). Therefore, compared to *M. smegmatis, S. aureus* responded differently to VCO.

DISCUSSION

Our results show that VCO had no inhibitory capability to *M. smegmatis* or *S. aureus* Loung *et al*. [6]. reported that the VCO was not active against *S. aureus*, *Salmonella typhi,* and *Escherichia coli*. However, free lauric acid showed antibacterial activity toward those microbes. Although VCO contained lauric acid (Table 2), it did not have anti-bacterial activity unless the lauric acid was released from the glycerol of VCO. In *S. aureus*, Chen and Alonzo [20] demonstrated a secreted lipase of *S. aureus* that could inactivate bacterial-derived lipoproteins and change the local inflammatory environment [17]. Lipolytic enzymes of the bacteria that could be able to release free lauric acid from VCO could, therefore, harmful for bacteria itself.

The essential oils contained in the fruit of andaliman that possess antibacterial activity were reported by [8]. Our previous research showed that geranyl acetate of andaliman extract was responsible for cell death in *M. smegmatis* exerting ion leakage [9]. The presence of andaliman fruit hexane extract resulted in the leakage of K^+ ions and Na^+ while the presence of VCO attenuated this harmful effect of the extract (Fig. 2a and b). This data indicated that toxicity of andaliman extract in *M. smegmatis* involved not only the cell wall or membrane but also its effect on the cytoplasm as described by Nazzaro *et al*. [14]. Interestingly, in *M. smegmatis* VCO decreased potassium and kalium ions in supernatant whereas cell viability was not affected. Note that VCO is emulsified by Tween 80. Mechanism by which VCO inhibits ions movement out of the cells is not known. However, it is known that lipids in cell membrane could affect ion channel activity [21,22].

Table 2: MIC of VCO and extracts of andaliman fruit on *M. smegmatis* and *S. aureus*

Target microbe	MIC of andaliman extracts (µg/ml)		MIC of VCO (µg/ml)
	hexane	Ethyl acetate	
M. smegmatis	64	>2408	>512
S. aureus	>2408	2408	>512

MIC: Minimum inhibition concentration, VCO: Virgin coconut oil.

Since VCO was not toxic (Fig. 2) and did not induce leakage, the presence of VCO was suspected to affect ion exchange activity. To the best of author knowledge, the effect of VCO on mycobacterial membrane and cell wall is still unclear. According to Chen and Alonzo [17], the olive oil interacted with the cell wall of *Mycobacterium brumae* and affected the cell physiology. Although this study did not have the data on the membrane characters dynamics or the changes on *M. smegmatis* cell walls treated with VCO, the effect on the cell wall may turn out to be similar to that in *M. brumae*. Further study on the role of cell wall or membrane on the effects of VCO affecting ionic exchange on *M. smegmatis* was required.

Contrary to *M. smegmatis*, the exchange of K^+ dan Na^+ ions in *S. aureus* was not affected by VCO (Fig. 3a and b). The ethyl acetate extract of andaliman resulted in ions leakage and the death of *S. aureus* cells (Fig. 2). VCO was not able to restore the intracellular and extracellular ion balance.

CONCLUSIONS

VCO had no antibacterial activity but affected the balance of intracellular and extracellular ions in cells of *M. smegmatis.* The extract of the

andaliman fruits resulted in the leakage of K+ dan Na+ ions and the death of both *M. smegmatis* and *S. aureus.* VCO attenuated ions leakage in cells treated with fruit andaliman extract but could not prevent cells death.

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CONFLICT OF INTEREST STATEMENT

The authors had no conflict of interest regarding this manuscript.

AUTHOR CONTRIBUTION STATEMENTS

HJ: Conceived and planned the experiments, and wrote the manuscript; NH and IPS performed anti-microorganisms experiments; RH wrote manuscript and prepared VCO; DPL performed chemical analysis.

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