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Antibacterial activity of Aqueous Garlic (*Allium sativum*) Extracts and Virgin Coconut oil and their combination against *Bacillus cereus* ATCC 14579 and *Escherichia coli* ATCC 8939

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Abstract: Garlic and Virgin Coconut Oil are part of foods containing components found to haveantibacterial activity. The allicin in garlic and medium chain fatty acid in its monoglyceride form (especially monolaurin) in VCO are responsible for their antimicrobial effect by different mechanism. The mixture of free fatty acids and their monoglycerides can be generated by enzymatic hydrolysis of VCO. The aim of this study was to investigate the antibacterial activity of un-hydrolyzed VCO (NVCO), enzymatic hydrolyzed VCO (HVCO), aqueous garlic extracts (AGE) and their combination against pathogenic bacteria causing diarrhea.

The garlic used in this study obtained from traditional the market in Padang Bulan, Medan and VCO from *Palem Mustika VCO*, produced by Siti Nurbaya, West Sumatra. Garlic extracted with bidistilled water and VCO was hydrolyzed by LIPOZIME®TL IM enzyme. Antibacterial activity test carried out on NVCO, HVCO, AGE, and their combination. Tetracycline HCl used as positive control. The test was conducted by diffusion agar method using the paper disc with diameter of 6 mm by observing the zone of inhibition toward Gram positive bacteria: *Bacillus cereus* (ATCC 14579) and Gram negative one: *Escherichia coli* (ATCC 8939). Zone of inhibition data was analyzed by ANOVA method ($\alpha \leq 0.05$), then by Tukey HSD to evaluate the significant difference between the means among the variables.

The results of this study showed that NVCO is not effective but antibacterial activity increased by hydrolysis (HVCO) which is more effective on Gram positive bacteria. AGE is found to have the most effective effect than NVCO and HVCO against Gram positive and Gram negative bacteria. However, the combination of NVCO and HVCO with AGE did not show to be synergisticas antibacterial.
Keywords: antibacterial, virgin coconut oil, aqueous garlic extracts, enzymatic hydrolysis.

Introduction

The most common symptoms of gastrointestinal infection is diarrhea, which if not treated properly may cause death. The main treatment forthis infection is the use of antibiotic and normalization of body fluid. For serious infection accompanied by diarrhea is usually overcome by the use of antibiotic in combination in order to minimize antibiotic resistance.[6] Although many pharmaceutical industries have developed many new antimicrobial drugs, the resistant antibacterial still emerging. So that many efforts have been made to develop antimicrobial drugs from the plants origin which is found to be less in adverse reaction.[6]

In these decades, there have been many studies on garlic and virgin coconut oil (VCO) for their antibacterial activities. The antimicrobial activity of garlic is due to the sulfur organic compounds mainly allicin and its derivatives and the presence of saponin, phenols. Potential antibacterial of VCO is by the presence of short and medium chain free fatty acids, caprylic acid (C8:0), capric acid (C10:0), and myristic acid (C14:0) especially the mixture of lauric acid (C12:0) and its monoglyceride monlaurin.[8,21]

The antibacterial mechanism of garlic apparently results from thiol-disulfide exchange reactions between these sulfur compounds and free thiol groups of bacterial enzymes such as alcohol deshydrogenase, thioredoxin reductase, trypsin, other proteases and RNA and DNA polymerases. This reaction can effect on cell essential metabolism and hence affecting the bacterial growth.[4,20] Garlic is a broad spectrum antibacterial since it is effective against Gram positive and negative bacteria. Antibacterial activity of VCO is through the mechanism of breaking the membrane cell of bacteria caused mainly by the mixture of medium chain free fatty acids and monoglycerides that can be generated from VCO by gastrointestinal lipase.[13]

The combination of garlic containing allicin with antibiotic such as streptomycin and cloramfenicol against *Mycobacterium tuberculosis* was found to be synergistic.[17] Eja, et al. (2011) reported that the synergistic effect of garlic and the conventional antibiotic to some of resistant bacteria giving new hopes for the next research.[12] Several studies reported that the antibacterial activity of hydrolyzed VCO was greater than that without hydrolysis.[13,25,29] The aim of this study was to investigate the antibacterial activity of aqueous garlic extracts and enzymatic hydrolysis VCO and VCO and their combination against gastrointestinal pathogenic bacteria, such as *Bacillus cereus* (ATCC 14579) and *Escherichia coli* (ATCC 8939).

Materials and Methods

Instruments used including Laminar Air Flow Cabinet (Astec HLF 1200L), autoclave (Express), incubator (Memmert), oven (Fisher), separating funnel and necessary laboratory glassware (Pyrex). The VCO sample used in this study was *Palem Mustika VCO-Virgin Coconut Oil*, product of Siti Nurbaya, West Sumatra; garlic was purchased from the traditional market in Padang Bulan, Medan. The bacteria tested were *Bacillus cereus* (ATCC 14579), and *Escherichia coli* (ATCC 8939). The chemicals were pro-analysis grade product of E. Merck (Germany), including ethanol, n-hexane, potassium hydroxide, tris-(hydroxymethyl) aminomethan (Tris-buffer), hydrochloric acid, calcium chloride, anhydrous sodium sulfate, potassium bipthalat, phenolpthalein indicator (1% in alcohol) and LIPOZIME®TL IM (Novozymes). The culture media used were nutrient agar (Oxoid), Mueller Hinton Agar (Oxoid), sterile bidestilled water, NaCl 0,9%, and DMSO as solvent in antibacterial test, paper disc with diameter of 6 mm (Oxoid), and Tetracycline HCl as standard reference (BPOM, Medan).

Procedures

Enzymatic hydrolysis of VCO

VCO was hydrolyzed by lipase enzyme LIPOZIME®TL IM which is active at sn-1 and sn-3 position in the triacylglycerol molecule. 30 g of oil placed in a Erlenmeyer to which 50 ml water, 12.5 ml $CaCl_2$ of 0.063M, 25 ml buffer solution Tris-HCl and 500 mg LIPOZIME®TL IM were added. The mixture was stirred with magnetic stirrer for 10 minutes to homogenize. Then it was allowed to stand (incubated) for 14 hours at temperature of 55 ± 0.5^{o}C, and the mixture shaken for 10 minutes in every one hour during incubation. After hydrolysis was completed, the mixture was transferred into separating funnel, acidified with dilute HCl, extracted with 50 ml n-hexane.[13,25,32,35]The filtrates were combined to which then 50 mg anhydrous Na_2SO_4 added and allowed to stand for 15 minutes. It was then evaporated on a water bath to dryness. This enzymatic hydrolyzed of oil was called HVCO. Acid value was determined by titration with potassium hydroxide standard solution using phenolpthaleinas indicator.[39] Antibacterial activity test was conducted on this recovered HVCO.

Preparation of Garlic extracts

Garlic bulb was washed in tape water. A total amount of 100 grams of cleaned garlic were cut into small pieces by sterile knife and blended with 100 ml bidistilled water using sterile warring blender at medium speed for 5 minutes. The macerate was filtered using sterile funnel and whatman filter. The result obtained was called Aqueous Garlic Extracts (AGE).

Antibacterial Test

Bacterial inoculums was prepared by transferring bacterial colony by the help of a sterile inoculating loop and suspending in Nutrient Broth Agar solution in 10 ml isotonic solution of sodium chloride and turbidity was equivalent to McFarland 0.5 turbidity standard (1.5 x 10^8 cfu/ml).[40] The volume of 0.1 ml bacterial inoculums was mixed with 15 ml MHA in a petri dish, allowed to stand until the media solidified. Antibacterial test was conducted as previously described[13,25] by diffusion agar method using paper disc with diameter of 6 mm. Bacterial test was conducted against *Bacillus cereus* (ATCC 14579) and *Escherichia coli* (ATCC 8939). Firstly, the antibacterial effect of un-hydrolyzed VCO (NVCO) was determined, followed by HVCO, and AGE in different concentration in DMSO v/v (10%, 25%, 50%, 75%, 100%). Then test performed onthe combination between NHVO with AGE and the combination between HVCO and AGE at different ratio (50%:50%, 75%:25%, 25%:75%). As the comparison, Tetracycline HCl (5 mg/ml, 2.5 mg/ml, 1 mg/ml, 0.5 mg/ml, 0.1 mg/ml, 0.05 mg/ml, 0.01 mg/ml) were used. Paper disc was dipped in the tested materials for 5 minutes and then incubated in prepared media for 24 hours at temperature of 37^{o}C. Antibacterial activity was determined by measuring the diameter of transparent area around the paper disc which was recorded as zones of inhibition. The test was carried out in five replicates and the data collected was statistically analyzed by ANOVA method then by Tukey HSD test using SPSS®17.

Result and Discussion

Acids Value of Hydrolyzed VCO

The acid value of the un-hydrolyzed VCO (NVCO) is 0.43 ± 0.05 which is far below that of hydrolyzed VCO (HVCO) that is 137.29 ± 1.4. The acid value at very low level in VCO without hydrolysis is to indicate that free fatty acids may be formed or generated during oil extraction or storage. In this study, VCO was hydrolyzed by enzymatic method using lipozim (LIPOZIM® TL IM) which is active specifically at sn-1,3 positions in a triacylglycerol molecule. Therefore, hydrolysis of a triglyceride by this specific enzyme will generate two free fatty acid molecules and one 2-monoglyceride, so that this enzymatic hydrolysis is called as partial hydrolysis. This is the reason why the acid value of hydrolyzed VCO in this study is about 30 % lower than saponification value of coconut oil or VCO, since during the saponification, oil will be completely hydrolyzed into glycerol and free fatty acids as soap.[1,32,33,34]

VCO or coconut oil is the oil composed of medium chain fatty acids dominated by lauric acid which is about 50 % out of the total fatty acids present in VCO. By hydrolysis of VCO will produce the mixture of free fatty acids and their 2-monoglycerdies, and hence this mixture is dominated by the mixture of lauric acid and its 2-monoglyceride as 2-monolaurin. According to Kabara, et al. (1972), free fatty acids and its monoglyceride are active as antibacterial, but lauric acid and monolaurin proved to be more active than the other fatty acids and monoglycerides.[9,21] Triglyceride and diglyceride are not active as antibacterial. It is also reported that hydrolyzed VCO by enzyme is more effective than partial hydrolyzed VCO by alkaline during saponification.[29]

Antibacterial Activity

The results of antibacterial activity test of NVCO, HVCO, AGE, their combination, and Tetracycline against tested bacteriaare shown in Figure 1-2, and the data in Table 1.

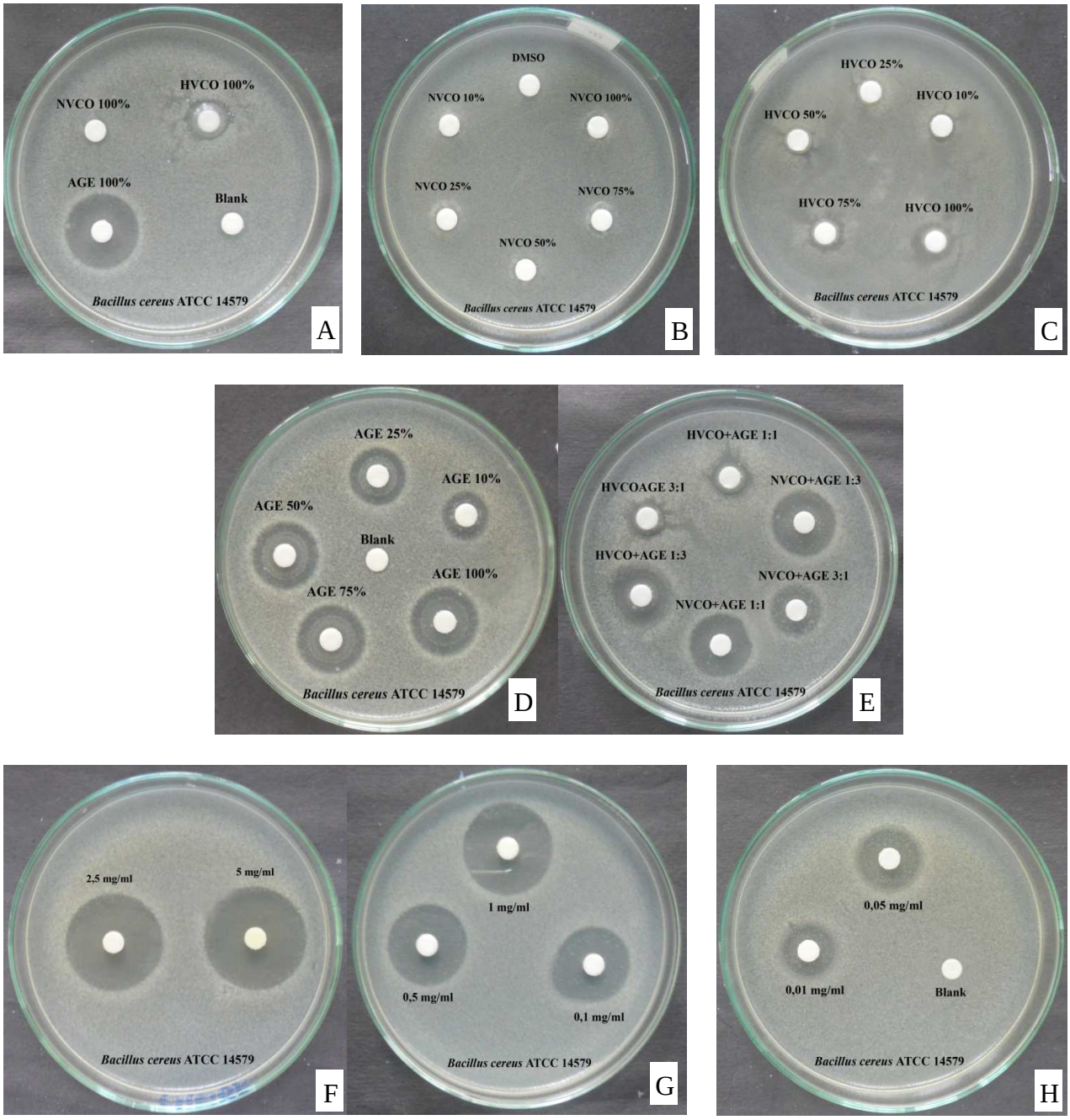

Figure 1. The zone inhibition of NVCO, HVCO, AGE, and Tetracycline HCl on *Bacillus cereus* ATCC 14579

Note: diameter of paper disc 6 mm; zone inhibition of (A,B,C,D) Blank, NVCO, HVCO, AGE in various concentration; (E) combination HVCO-AGE and NVCO-AGE; (F,G,H) Tetracycline HCl in various concentration

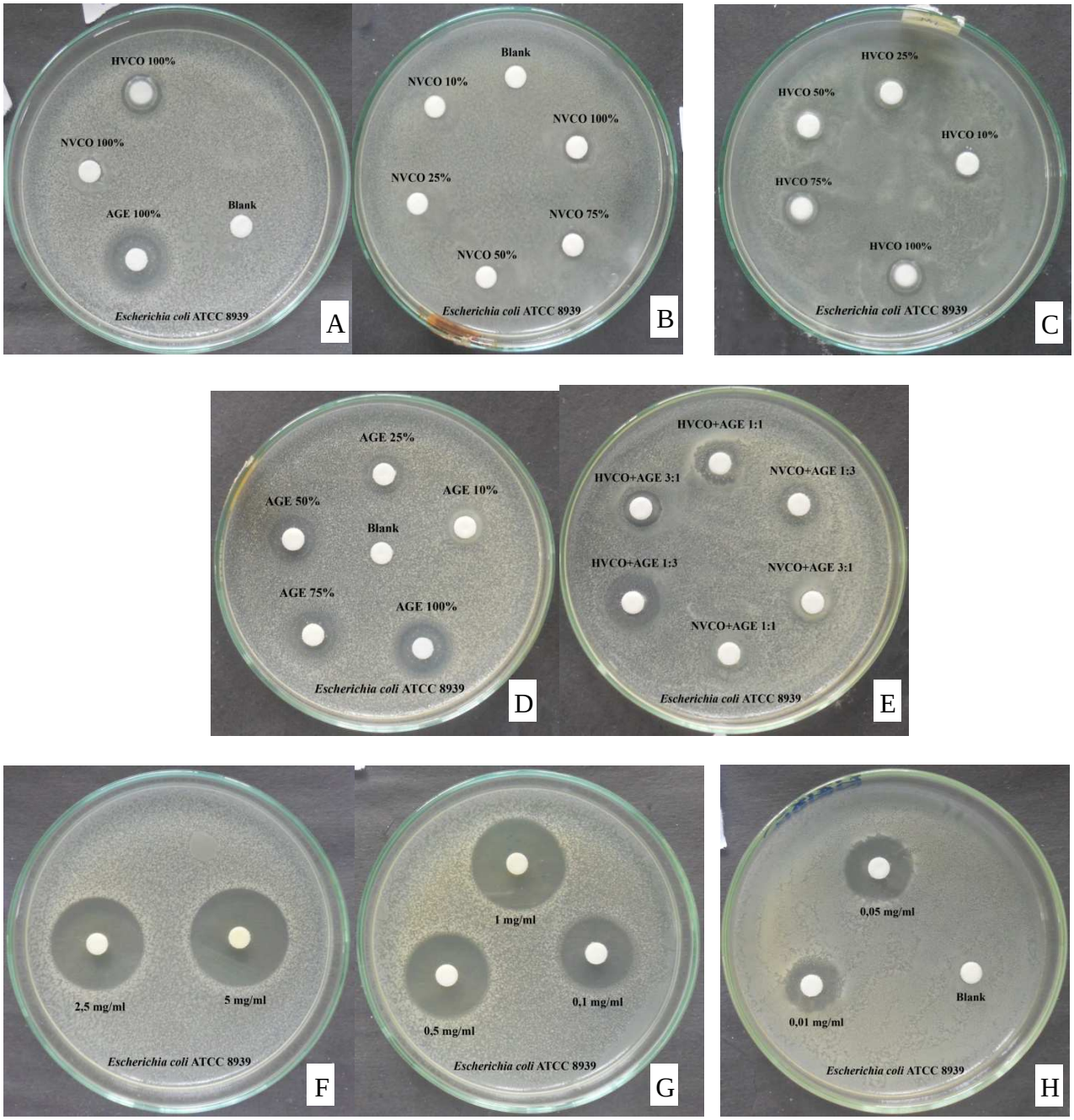

Figure 2. The zone inhibition of NVCO, HVCO, AGE, and Tetracycline HCl on *Escherichia coli* ATCC 8939

Note: diameter of paper disc 6 mm; zone inhibition of (A,B,C,D) Blank, NVCO, HVCO, AGE in various concentration; (E) combination HVCO-AGE and NVCO-AGE; (F,G,H) Tetracycline HCl in various concentration

Table 1. Zone of inhibition of NVCO, HVCO, AGE, its combination, and Tetracycline HCl

Sample	Concentration	Mean of zone inhibition ± SD (mm)	
		B. cereus	*E. coli*
Blank		-	-
NVCO	10%	-	-
	25%	-	-
	50%	6.04 ± 0.08	-
	75%	6.18 ± 0.26	6.06 ± 0.13
	100%	6.29 ± 0.56	7.70 ± 0.38
HVCO	10%	7.82 ± 0.64	8.44 ± 0.43
	25%	8.70 ± 0.21	8.86 ± 0.11
	50%	9.54 ± 0.48	9.86 ± 0.30
	75%	11.42 ± 0.48	10.16 ± 0.23
	100%	13.64 ± 0.40	10.98 ± 0.22
AGE	10%	13.64 ± 0.51	9.48 ± 0.47
	25%	16.60 ± 0.50	11.60 ± 0.80
	50%	18.16 ± 0.20	13.94 ± 0.19
	75%	19.34 ± 0.23	15.06 ± 0.54
	100%	21.86 ± 0.35	18.26 ± 0.39
HVCO 50% + AGE 50%	1:1	10.86 ± 0.56	11.57 ± 0.41
HVCO 75% + AGE 25%	3:1	10.06 ± 0.59	9.87 ± 0.69
HVCO 25% + AGE 75%	1:3	17.32 ± 0.82	13.32 ± 0.57
NVCO 50% + AGE 50%	1:1	15.78 ± 1.19	10.62 ± 0.83
NVCO 75% + AGE 25%	3:1	12.12 ± 1.58	7.70 ± 0.62
NVCO 25% + AGE 75%	1:3	18.70 ± 0.43	11.62 ± 0.89
Tetracycline HCl	5	29.26 ± 0.35	28.29 ± 0.36
	2.5	27.44 ± 0.32	26.90 ± 0.15
	1	27.10 ± 0.29	26.14 ± 0.23
	0.5	24.72 ± 0.20	23.94 ± 0.32
	0.1	21.54 ± 0.35	20.02 ± 0.19
	0.05	17.88 ± 0.17	19.02 ± 0.27
	0.01	14.22 ± 0.24	14.62 ± 0.21

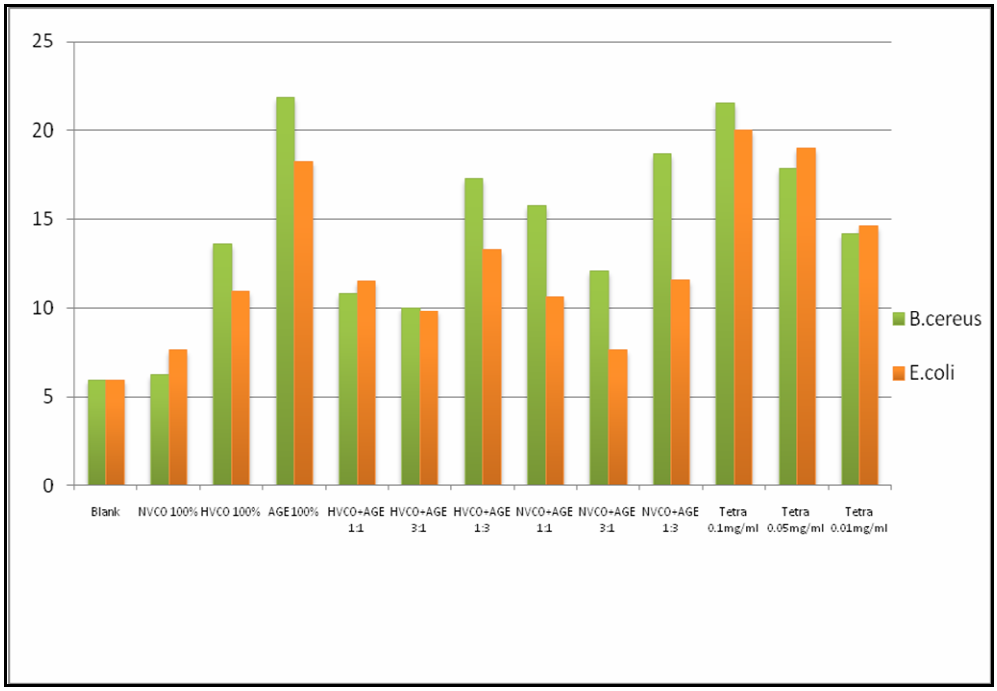

Figure 3. Zone of inhibition by tested materials on *Bacillus cereus* (ATCC 14579)and *Escherichia coli*(ATCC 8939)

From Table 2 can be seen that un-hydrolyzed VCO is almost not active but activity significantly increased by hydrolysis. Low antibacterial activity shown by NVCO may be due to the presence of free fatty acids at low level as represented by low acid value. The activity of NVCO could result from releasing fatty acids from VCO by lipase enzyme produced by bacteria which may hydrolyze VCO to produce fatty acids[26] in addition to the free fatty acids measured as acid number (0.43 ± 0.05). Enzymatic hydrolysis of VCO will increase free fatty acids (higher acid value) in combination with their monoglycerides, and therefore increase antibacterial activity. Similar results also reported previously by Silalahi, et al. (2014), but other studies reported that un-hydrolyzed VCO also showed to be active although the activity found to be lower than hydrolyzed VCO.[13,15,25,35] It is also shown that the higher the concentration the greater the antibacterial of the tested materials. As the activity of antimicrobial agents based on zone of inhibition can be classified into three categories; inhibition zone above 11 mm would belong to be "active and very active"; 6–11 mm as "medium active", and less than 6 mm as "inactive".[28] In this study, the mean of inhibition zone of AGE alone against all tested pathogens isvery active and significantly greater than HVCO and NVCO; HVCO was greater than NVCO. HVCO is more effective against Gram positive than Gram negative. It was proved that hydrolysis of triglycerides could improve its antibacterial properties. Because hydrolysis especially by enzyme used in this study will generate free fatty acids and monoglycerides mainly lauric acid and monolaurin may be responsible for the antibacterial activity by disrupting the lipid membrane of bacteria.[5,9,11,14,21,24]

AGE is the most active antibacterial agent among the three materials tested against tested bacteria characterized by inhibition zone ranged from 16-21 mm far above HVCO ranging from 11-13 mm and the lowest is NVCO (6-9 mm). The mean inhibition zone of AGE 100% against *Bacillus cereus* 21.86 ± 0.35 and *Escherichia coli* 18.26 ± 0.39. Antibacterial effect of garlic is caused of its organic sulfur (allicin).[2,3,7,8,10,12,30,23,31] Saponins and flavanoids in garlic could also contribute to its antibacterial effect. That's why garlic was effective against both on Gram positive and negative bacteria (broad spectrum).[16] Allicin is generated by crushing or cutting garlic cloves, then alliin (allicin precursor which is present in garlic cloves) is metabolized by alliinase enzyme become allicin and other thiosulfinates, and most of the sulfur compound derivatives from allicin which are potential antimicrobial agents dissolve in water.[23,28,29]

From the data obtained from the combined tested materials is not as previously predicted to happen. It was assumed that the combination between AGE and HVCO would be synergistic due to the different mechanism of action of AGE from HVCO on bacteria, but it was found that activity of the combination is lower than that of AGE alone. The results also show that combination of HVCO-AGE is more effective on *Escherichia coli* ATCC 8939 than *Bacillus cereus* ATCC 14579. However the zone inhibition of HVCO-AGE combination and NVCO-AGE (plate E in Figure 1 and 2) did not show any increasing activity of antibacterial if it was compared with the single sample. According to the ratio on the combination, data shows that the amount of AGE is more influential to give the antibacterial effect of the combination.

Based on the active compound of garlic and VCO, the reduction in antibacterial activity shown by combination can suggest explanation. According to Volk and Weller (1989), saponin is a molecule that can interact either with hydrophilic and lypophylic molecules.[37] In the combined AGE and NVCO, and saponin in garlic may interact with fatty acid from NVCO, and therefore decrease bacterial activity.

In the present study, activity of standard antibiotic tetracycline of different concentrations also tested and compared with NVCO and AGE. The test was conducted against Gram positive: *Bacillus cereus* (ATCC 14579) and Gram negative: *Escherichia coli* (ATCC 8939). Based on the results of this study (Table 2), the inhibition zone increased significantly which directly proportional to the increasing concentrations. Table 2 and Figure 3 show that there is not significant different in inhibition zone between 50% of AGE and tetracycline HCl 0.05 mg/ml, and 100% HVCO equivalent with tetracycline HCl 0.01 mg/ml against *Bacillus cereus* ATCC 14579.

Tetracycline HCl is a broad-spectrum antibiotic, inhibiting almost all Gram positive and negative bacteria. Antibacterial activity of tetracycline is caused by binding the bacterial 30S ribosomal sub unit and directly inhibit peptidyl transferase activity thereby disrupting protein synthesis.[22,38] While the wide spectrum antibacterial mechanism of garlic was caused by synergy of allicin, saponin and flavanoid.[16] Whereas hydrolyzed VCO inactivate bacteria by medium chain free fatty acids and its monoglycerides generated from VCO by affecting the bacterial membrane.[5,11,14,24]

Tetracycline HCl does have excellent antibacterial activity against pathogenic bacteria.[19] However, the frequent use of this antibiotic could develop bacterial resistance, then adversely affect kidney and liver function.

In addition, the use of oral antibiotics in the long term will reduce the normal flora of the colon and affect the body's levels of calcium ions. Tetracycline also cannot be used for treatment of diarrhea in baby.[22, 29,38] Natural antibacterial agents from plant origin like HVCO and garlic were also effective against several pathogenic bacteria, including the bacteria that cause diarrhea as reported in this study. According to Hasibuan (2012), VCO inhibit effectively the growth of *Salmonella thypi* but less effective toward probiotic *Lactobacillus casei* .[18] Similarly, garlic is more active against *E.coli* ten times than *Lactobacillus*.[27,36] In addition, the use of natural antibacterial compound provide other favorable biological activity, and they have lower side effects..

Conclusion

In the present study, it is shown that un-hydrolyzed VCO does prove to show low antibacterial activity, but enzymatic hydrolysis of VCO will generate fatty acids and monoglycerides significantly increase antibacterial properties. Enzymatic hydrolyzed VCO is more effective against Gram positive (*Bacillus cereus* ATCC 14579) than Gram negative (*Escherichia coli* ATCC 8939). However, antibacterial effect of AGE is greater than HVCO and NVCO, and effective against both Gram positive and negative bacteria. Antibacterial activity of HVCO-AGE combination and NVCO-AGE neither did show synergism effect nor addictive, but decrease activity compared to the activity of single tested material. This might be as a result of the interaction between saponin present in garlic and fatty acids in hydrolyzed VCO. Further clinical study and safety of garlic and VCO, as well as effective dosage are necessary conducted.

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