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Conversion Eugenol to Vanillin: Evaluation of Antimicrobial Activity

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Abstract

Indonesia is known as one of the largest clove producing countries in the world. About 75-90% main component of the clove oil is eugenol. Eugenol can be used as an analgesic, local anaesthetic, and also recognized as antimicrobial. Eugenol is known as starting material to produce synthetic vanillin. Vanillin is a compound that can be isolated from vanilla (Vanilla p lanifolia) or chemically synthesis. Vanillin is widely used for flavouring, fragrance, and also as a precursor of drugs in the pharmaceutical industry. Conversion eugenol to vanillin through several phases including isomerization, acetylation, oxidation, and hydrolysis. From each phase, it produces intermediate products, there are isoeugenol, isoeugenol acetate, and vanillin acetate, and also produce by-product vanillic acid. This present study aims to evaluate the results from the conversion of eugenol to vanillin for its antimicrobial activity against pathogenic bacteria E. coli and S. aureus. The result showed that eugenol, isoeugenol, vanillin acetate, vanillin and vanillic acid had antimicrobial activity. Isoeugenol which is the result of isomerization of eugenol is known to possess the best antimicrobial activity.

INTRODUCTION

Indonesia is known as one of the largest clove producing countries in the world. In the period 2007 until 2011, the production of clove oil, from the distillation of clove, reaches about 2500-4500 tons (Tursiloadi et al., 2015; Riyanto et al., 2015). Cloves (*Syzygium aromaticum, syn. Eugenia aromaticum*) are used in various countries. They are widely used in the fields of food, cosmetics, cigarettes, or used as ingredients for traditional medicines (Tursiloadi et al., 2015). About 75-90% main component of the clove oil is eugenol, depending on which part of the distillation is taken (flower, leaf, or buds) (Nejad et al., 2017).

Eugenol (3- (4-hydroxy-3-methoxyphenyl) propene) is a phenolic compound that is known to have a great effect on biological activities. Eugenol can be used as an analgesic, local anaesthetic, a stimulant, antifungal and also has antibacterial effects (Hemaiswara et al., 2009; Daryono, 2015; Raja et al., 2015; Najed et al., 2017). The many benefits of eugenol in biological activity make eugenol the target molecule to be structurally modified to produce derivative compounds with therapeutics properties (da Silva et al., 2018). Structural modification of eugenol through isomerization and oxidation is known to produce vanillin (Towaha, 2012; Garner et al., 2016).

Vanillin (4-hydroxy-3-methoxybenzaldehyde) is a compound that can be isolated from vanilla (*Vanilla p lanifolia*) or chemically synthesized (Walton et al.,2003). Vanillin is widely used as fragrances, flavourings, and in the pharmaceutical field is used as a precursor of drugs. Vanillin also shows as an antioxidant and antimicrobial activity against bacteria, mold, and yeast (Fitzgerald et al., 2004). In 2010, production of natural vanillin reaches less than 1 % from 15.000.000 kg vanilla pods, that means 1 kg vanillin is being produced from 500 kg vanilla pods (Galage & Moller, 2014). The increasing demand for vanillin, but limited resources and the expensive cost make synthesis or bio-synthesis of vanillin as an alternative way to produce

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vanillin (Walton et al., 2003; Kadorahman et al., 2010; Garner et al., 2016; Galage & Moller, 2014).

Conversion of eugenol to vanillin produce intermediate products. These products are known as isoeugenol, isoeugenol acetate, and vanillin acetate. It also produces a by-product vanillic acid. Eugenol, isoeugenol, vanillin, and vanillic acid is known for its antimicrobial activity (Bezzera et al., 2017; Nielsen et al., 2017; Mourtzinos et al., 2009), however, study about antimicrobial activity of isoeugenol acetate and vanillin acetate are still hard to find. Therefore, this study aims to evaluate the results of the conversion of eugenol to vanillin for antimicrobial activity.

MATERIALS AND METHODS

Chemicals

Eugenol was isolated from the clove tree provided by PT Aroma Essence Prima. Tris (2,4-pentanedionato) ruthenium (III) was purchased from Tokyo Chemical Industry. Sodium hydroxide, potassium permanganate, manganesse (II) sulfate monohydrate, benzyltriethylammonium chloride, acetic anhydride p.a, tert-butyl methyl ether p.a, Dimethyl sulfoxide p.a, and hydrochloric acid 37% p.a were purchased from Merck chemical. Ethanol p.a was purchased from J.T. Baker, ethyl acetate p.a was purchased from Mallinckrodt chemicals, aquadest. *Escherichia coli* (InaCC--B5334) and *Staphylococcus aureus* (InaCC- B4) were used as test bacteria for antimicrobial activity. All strains were provided by the Indonesian Culture Collection (InaCC), Research Center for Biology, LIPI.

Conversion of Eugenol to Vanillin

Conversion of eugenol to vanillin through several phases. The first is the isomerization of eugenol to isoeugenol, the second is acetylation of isoeugenol, the third is oxidation of isoeugenol acetate to vanillin acetate, and the last step is hydrolysis of vanillin acetate to vanillin (Lampmann et al., 1977; Garner et al., 2016).

Isomerization Eugenol to Isoeugenol. The method that we used in this step following Riyanto et al., (2019). A total of 30 grams of eugenol was added 30 mg of Tris (2,4-pentanedionato) ruthenium (III) catalyst according to a ratio of 1: 1000 without solvents. The mixture is then sonicated for 30 minutes at an amplitude of 30%. The results then analyzed qualitatively with Thin Layer Chromatography (TLC) and component analysis by Gas Chromatography-Mass Spectrometry (GC-MS).

Acetylation of isoeugenol. The method used in this step according to Garner et al. (2016). A total of 3 ml isoeugenol was mixed with 100 ml solution of sodium hydroxide 1 M. The mixture was stirred for 2 minutes and cooled off with ice bath for 15 minutes. A total of 5 ml of acetate anhydride was added drop by drop to the mixture then stirred for 15 minutes in cold condition. The solids that formed during the reaction is separated and purified by recrystallization in a 1:1 ethanol-water mixture. The yields then analyzed qualitative with TLC and component analysis by GC-MS.

Oxidation of isoeugenol acetate. A total of 3,8 mg potassium permanganate, 3.8 mg manganese (II) sulfate monohydrate, 0.2 g of benzyltriethylammonium chloride, 75 ml aquadest, and 75 ml Methyl tert-buthyl eter are mixed together, after that, 2 g of isoeugenol acetate is slowly added to the mixture. The mixture is stirred for 15 minutes without heated. The solution formed in two phases, where the organic phase is separated from the water that contained brown -solid phase. The brown-solid phase is rinsed twice with 20 ml methyl tert-buthyl eter and collected with the organic

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phase. The organic solvent then removed by rotary evaporator. The compound that obtained as brownish-yellow oil is Vanillin acetate and analyzed by GC-MC.

Hydrolysis of vanillin acetate and purification. Vanillin acetate is boiled at 100 °C with 30 ml half-concentrated hydrochloric acid under reflux for 20 minutes. After that, the solution is cooled off and vanillin is extracted twice with 15 ml methyl tert-buthyl eter. Organic phase then removed by rotary evaporator. Vanillin is obtained as greenish colour, then purified by recrystallizing with adding water and stirred-heated for at least 20 minutes. The compound is extracted again with 20 ml ethyl acetate and remove the organic solvent by rotary evaporator. The vanillin was obtained as brownish-yellow and analyzed by GC-MS.

Antimicrobial Activity

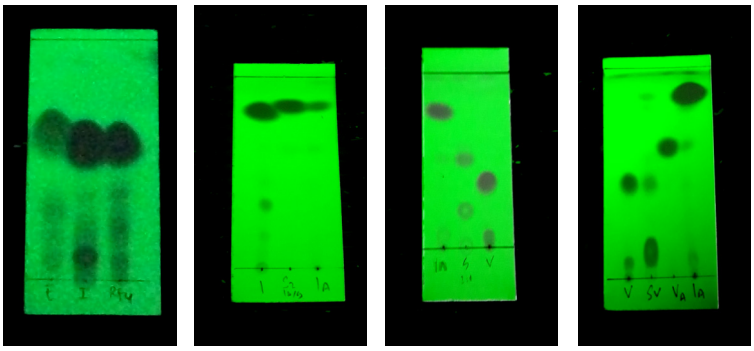
Preparation of Sample. Each compounds was evaluated at different concentration from 10%, 5%, 2.5%, 1%, and 0.5% in dimethyl sulfoxide (DMSO). A total of 10 mg compound was dissolved in 100 ml DMSO to obtain 10 % of concentration, afterwards a serial diluted was performed to obtain 5%, 2.5%, 1%, and 0.5% concentrations.

Antimicrobial activity. All tested bacteria (*E. coli* and *S. aureus*) were cultured in nutrient agar for 24 hours at 37 °C. The overnight cultures were diluted into 1 ml sterile aquadest until having an optical density (OD) at 600 nm around 0.4. This OD_{600} represent the growth of the bacteria or equivalent to 1. 10^8 CFU/ml according to 0.5 Mc. Farland standart. Antimicrobial activity were determined using disk diffusion method (Balouiri et al., 2016)). 100 ml suspension of bacteria tested were spread on agar plates. Sterile disk with 6 mm were placed on the agar plates, then the samples were loaded into the disk. The plates were incubated for 24 hours at 37 °C. The clear zone that showed after overnight incubating were counted as inhibition zone. Antimicrobial activity was evaluated by measuring zone of inhibiton against the microorganism.

RESULTS AND DISCUSSION

Conversion Eugenol to Vanillin

Conversion of eugenol to vanillin through several phases, including isomerization of eugenol, acetylation of isoeugenol acetate, oxidation of isoeugenol acetate to vanillin acetate, and hydrolysis of vanillin acetate to vanillin. The result of qualitative screening with TLC showed in Figure 1 meanwhile, the analysis of component using GC-MS can be seen in figure 2. The yield of the reaction is comparable to the isoeugenol standard provided by PT. AEP (Figure 1.a). Analysis from GC-MS showed 90,10 % component from the yields are isoeugenol (Figure 2.a) in the form of *cis*-isoeugenol at retention time 12,63 minutes and *trans*-isoeugenol at retention time 13.21 minutes. Isomerization of eugenol to isoeugenol is a process wherein the double bond in the alkenyl group was migrated to a position conjugated with the benzene ring (Sharma et al., 2006). Study by Kadorahman et al (1999) showed that isomerization eugenol with catalyst KOH at high temperature (140–190 °C) for 5 to 7 hours reaction can produce 93,71 % isoeugenol. Meanwhile, Riyanto et al (2015) reported that isomerization of eugenol with sonication method could produce almost 100 % isoeugenol with condition ratio of eugenol and catalyst 1:2000, amplitudo 70%. Therefore, sonication method is carried out to shorten the reaction time and produce the product in an optimal amount.



(a) (b) (c) (d)

Figure 1. Qualitative screening with TLC; (a) Isomerization eugenol to isoeugenol, (b) acetylation of isoeugenol, (c) Oxidation isoeugenol acetate to vanillin acetate, and (d) Hydrolisis of vanillin acetate.

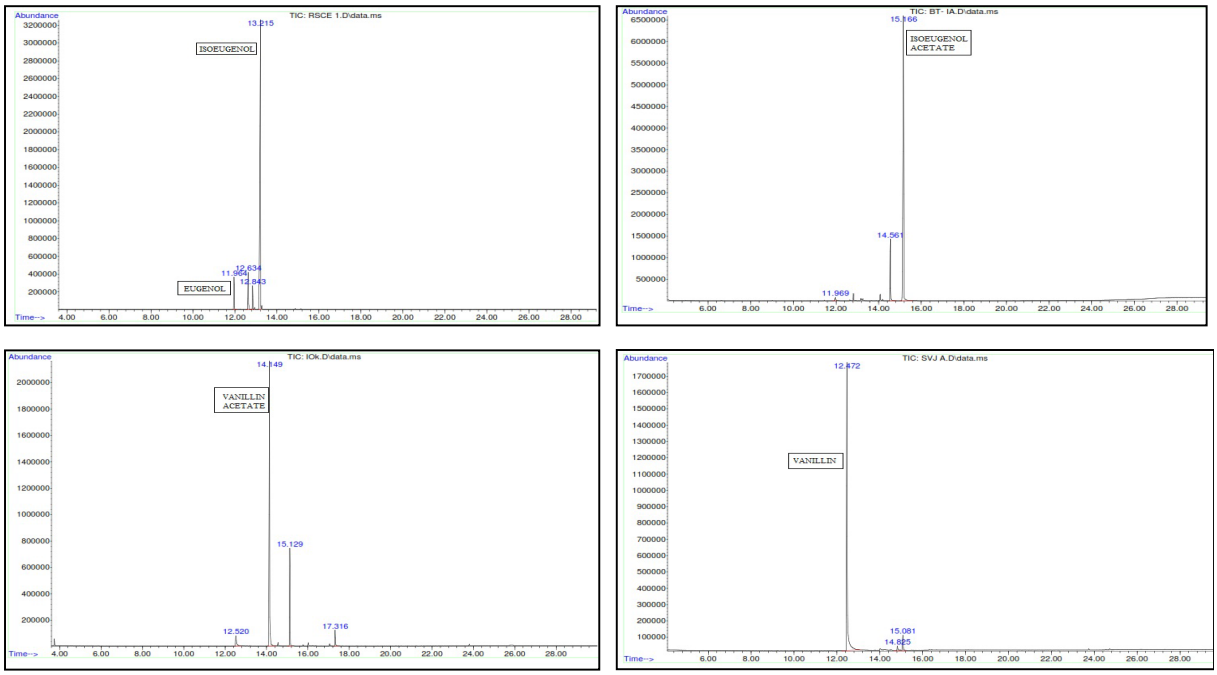


Figure 2. Chromatogram of GC-MS (a) Isoeugenol; (b) Isoeugenol acetate; (c) Vanillin acetate; (d) Vanillin

Acetylation of isoeugenol is carried out to increase the yield product during oxidation and also reduce the formation of unwanted by-compounds (Garner et al., 2016). Addition of an acetyl group into the structure could protects the hydroxyl group from oxidation because of its stabilize properties against nucleophile and base (Garner et al., 2016). Figure 1.b showed that isoeugenol acetate has a retention factor higher than isoeugenol. Analysis from chromatogram GC (Figure 2.b) showed that 86,21 % of isoeugenol is converted to isoeugenol acetate.

Oxidation isoeugenol acetate to form vanillin acetate is carried out by adding potassium permanganate as an oxidator agent. Manganesse (II) sulfate was added to maintained pH-neutrality and also reduced the probability of acetyl group is being removed by hydrolysis. Benzyltriethylammonium chloride worked as a phase-transfer catalyst. The yields that formed from this process is a yellow oily-form. Figure 1.c showed that vanillin acetate is formed has a

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retention factor above the natural-vanillin standard. Anylisis from GC-MS (Figure 2.c) showed that the 71.44 % isoeugenol acetate is converted to vanillin acetate. Hydrolysis of vanillin acetate to produce vanillin is carried out by adding hydrochloric acid under reflux conditions. Figure 1.d showed qualitative screening by TLC from the reaction. It showed that the compound obtained is comparable to the natural-vanillin standard. Analysis GC-MS showed that 93% of vanillin acetate is converted to vanillin (Figure 2.d). Beside vanillin, there is a by-product that formed during the reaction that is vanillic acid. Vanillic acid is a form of over-oxidation of vanillin and is formed when there is a long heat treatment of vanillin (Mourtzinos et al.,2009).

Antimicrobial Activity

In this present study, conversion of eugenol to vanillin produce intermediate compounds.This compounds are isoeugenol, isoeugenol acetate, vanillin acetate, and by-product from the reaction is vanillic acid. Each of this compounds were evaluated for antimicrobial activity against gram negative bacteria (*E. coli*) and gram positive bacteria (*S. aureus*) using disk diffusion method. The diameter zone of the inhibition can be seen in table 1. The results showed that apart of isoeugenol acetate, the compounds obtained from the conversion eugenol to vanillin have an antimicrobial activity. The activity of eugenol, isoeugenol,vanillin acetate, vanillin and vanillic acid were comparable to the standar antibiotic streptomycin against the tested bacterial strains.

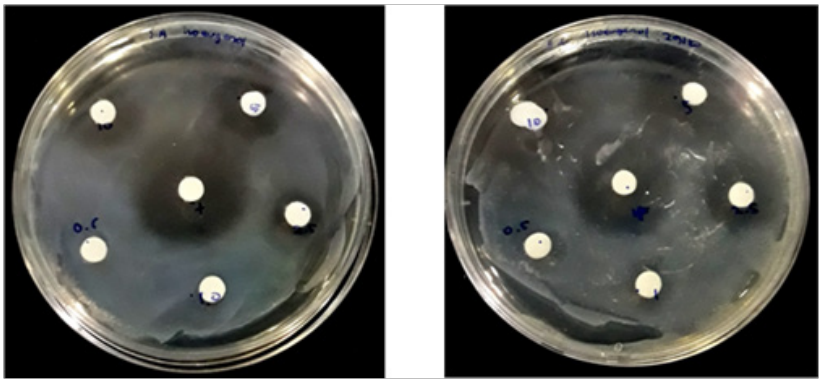

Figure 3. Growth inibition of *E. coli* and *S. aureus* strains by different concentration of isoeugenol using disk diffusion method

Eugenol is known for having a good antimicrobial effect. Eugenol has phenyl propane structure, that is lipophilic molecules, therefore this compounds could damage the cell by penetrating into the lipid membrane and damage the permeability of the membrane. Compared to the eugenol, isoeugenol shows a greater inhibition against bacterial strain (Figure 3). According to Zhang et al. (2017), isoeugenol had a greater antibacterial effect than eugenol because structurally, the double bond in isoeugenol is closer to benzene ring that can lead a better biological activity. Meanwhile, isoeugenol acetate had no sign of antimicrobial activity. this might be happened because of structural changes, the free hydroxyl group in isoeugenol replaced by an acetyl group. According to Nazzaro et al. (2013), antimicrobial activity of phenyl propane compounds such as eugenol or isoeugenol, conferred by their free hydroxyl group. The type and substitution of the aromatic ring also affect antimicrobial activity. Study by Vanin et al. (2018) also shows the decrease of antibacterial effect on eugenol after esterification process.

According to literature, compounds that have zone of inhibition less than 7 mm, between 7 to 16 mm and greater than 16 mm are considered inactive, moderately active, and have a potential

antimicrobial., respectively (da Silva et al., 2018). Vanillin acetate shows a moderate activity against tested bacteria strain (Table 1). This might because the presence aldehyde group in vanillin acetate. It is known that aldehyde groups are reactive and can form covalent bonds with DNA and protein, therefore it can potentially interfering cells function (Fitzgerald et al., 2005). Meanwhile, vanillin also shows moderate activity to inhibit bacterial growth, but it is slightly greater than vanillin acetate and vanillin acid on gram-negative bacteria. Vanillin is known for antimicrobial activity depending on the time of exposure, the concentration used, and the organism's target (Fitzgerald et al., 2004). Study by Zou et al. (2014), vanillin as a cross-linker between polyvinylalcohol and lysin showed a good antibacterial effect against bacterial gram-negative or gram-positive. Vanillin associated with antibiotic gentamicin and impinem also showed synergistic effect against *E.coli*, and *S.aureus* (Benzerra et al., 2017).

Generally, the destruction of cell walls in gram-positive bacteria will be easier than in gram-negative bacteria due to complex components of the gram-negative cell wall bacteria (composed of lipopolysaccharides) (Nazzaro et al 2013). In this present study, we and others showed that the compounds obtained from the conversion are more effective against gram-negative bacteria than gram-positive bacteria (Hyldgaard et al., 2015; Nielsen et al., 2017; Pathirana et al., 2019). According to Hyldgaard et al (2015), isoeugenol has a detergent-like mechanism which causes damage to the cell walls and damages the fluidity of the membrane. Gram-negative bacteria may be more susceptible because inner and outer membranes represent target sites for isoeugenol, and the cytoplasmic membrane is easily accessible once the outer membrane disrupted (Nielsen et al., 2017).

Table 1. Zone of inhibition at different concentration using disk diffusion method.

Compound	Concentration (%)	Zone of inhibiton (mm)	
		E.coli	*S.aureus*
Eugenol	10	16	10
	5	11	4
	2.5	5	-
	1	-	-
	0.5	-	-
Isoeugenol	10	22	13
	5	19	10.5
	2.5	13.5	5
	1	8	-
	0.5	-	-
Isoeugenol acetate	10	-	-
	5	-	-
	2.5	-	-
	1	-	-
	0.5	-	-
Vanillin acetate	10	11	12
	5	9	4
	2.5	8	-

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Compound	Concentration (%)	Zone of inhibiton (mm)	
		E.coli	*S.aureus*
	1	-	-
	0.5	-	-
Vanillin	10	13	11.5
	5	7.5	6
	2.5	-	-
	1	-	-
	0.5	-	-
Vanillin acid	10	11	11
	5	8	5
	2.5	-	-
	1	-	-
	0.5	-	-
Streptomycin (positive control)	0.1	20.8	23

*Value minus disc diameter 6 mm

CONCLUSION

The conversion of eugenol to vanillin produce intermediate compounds, they are isoeugenol, isoeugenol acetate, vanillin acetate, and also side-product that form during the reaction is vanillin acid. All these compounds showed moderate activity against gram-negative bacteria (*E. coli*) and gram-positive bacteria (*S. aureus*), except isoeugenol acetate. The loss of a free hydroxyl group in its structure causes the loss of its antimicrobial ability. Meanwhile, in vanillin acetate, the presence of aldehyde group from vanillin causes antimicrobial activity, but not as great as vanillin. However, more detailed research needs to be conducted, such as determining the minimum inhibitory concentration (MIC) and also disclosure the mechanism of action against tested bacteria to understand how these compounds structurally can affect antimicrobial activity.

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