Synthesis of Organonitrogen Compounds from Eugenol through The Ritter Reaction and The Toxicity Test on Artemia salina Leach.

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Abstract. Eugenol or 4-allyl-2-methoxyphenol is the main component in clove oil which can be used as a starting material in organic synthesis. One of the example is synthesis of organonitrogen compound through the Ritter reaction. Eugenol as a carboxication precursor is reacted with acetonitrile, in the presence of a strong acid catalyst H₂SO₄, to form organonitrogen compounds. The number of moles in the reaction of eugenol:acetonitrile:H₂SO₄ is 0.1:0.5:0.1 moles. The reaction was carried out at several conditions namely at ice temperature (0°C) for 4-hours reaction time, at room temperature (27°C) for 4 hours reaction time, and at reflux temperature (90°C) for 4, 8 and 16 hours reaction times. The products were characterized using UV-Vis, IR, and GC-MS. The 4-hours (0°C) and 4-hours (27°C) reactions did not produce any organonitrogen compounds. Meanwhile the 4-hours (90°C) reaction produces organonitrogen compounds, eugenyl acetamide. The 8- and 16-hours reflux temperature (90°C) reactions are also gives organonitrogen compounds, namely eugenyl acetamide (8% yield in 8-hours reaction time and 13% yield in 16-hours reaction time) and isoquinoline (7% yield in 8-hours reaction time and 11% yield in 16-hours reaction time), in which the yield was increased as the reaction time increased. The toxicity level of the crude obtained from the 16-hours reflux temperature (90°C) reaction time was tested using the Brain Shrimp Lethality Test method to Artemia salina. The result shows that LC₅₀ value is 16 ppm (higher than that of eugenol) and is classified as having a strong toxicity activity.

Keywords: Eugenol, Ritter reaction, Brain Shrimp Lethality Test, Artemia salina.

1. Introduction

People in Indonesian have already use natural materials since long ago, especially natural materials derived from plants in medical practice as an illness treatment medium. Plants that are found in nature can be extracted from its leaves, flowers, stems, or seeds in order to get its essential oil. These kinds of plants are widely grown in Indonesia. Essential oil contains metabolite compounds that act as producers of pharmacological activities, including as medicine, antioxidants, colouring agents, aroma enhancers in food, perfume, and even as insecticides [1].

One of the Indonesia's natural resources which is widely grown and used by the community is the clove plants (Syzygium aromaticum). The production of the clove plants (Syzygium aromaticum) oil through a steam distillation is commonly performed in Indonesia. Clove oil has several biological activities including as antiseptic, antispasmodic, stimulant, and also local anaesthetics. The main components contained in clove oil are eugenol, acetyl eugenol, beta caryophyllene, and vanillin. Other
compounds contained in clove oil are tannin, methyl salicylate, eugenine flavonoids, kaempferol, krategolat acid, and triterpenoid oleaonic acid, stigmasterol, and small amount of campesterol [2].

The main component of clove oil is eugenol (4-allyl-2-methoxyphenol) for about 80-95%. Eugenol belongs to the benzene group of phenol compounds. Based on pharmacological studies, eugenol has anticonvulsant effects, local anaesthesia, anti-stress, bacteriostatic, bactericidal, anti-candide, antifungal, antigenotoxic, and has the potential to be anti-cancer [3]. Based on its structure, eugenol has the potential as a starting material of aromatic organonitrogen compounds. The synthesis of aromatic compounds such as eugenol can be carried out in one-step reaction through the Ritter reaction, thus the synthesis stage is more efficient [4].

Eugenol, which reacted with nitrile compounds such as acetonitrile or benzonitrile with an acid catalyst, can forms organonitrogen compounds. Phi (π) bond breaking reaction in the eugenol alkene group and the formation of the C-N amide bond are taken place [5]. Eugenol will form carbocation when dehydrated with acid due to protonation. The carbocation will be attacked by nitrogen atoms nucleophilic from nitrile compounds and finally organonitrogen compounds was formed.

Organonitrogen compounds are heteroatom compounds in the presence of N groups. The heteroatom group causes the organonitrogen compounds, with their affinity and polarity, to make the biochemical mechanisms that cause the molecular interactions in the body. The interaction that occur makes the organonitrogen to have biological activities as antivirus, analgesic, anti-inflammatory, anti-depressant, anti-tumor, and anticonvulsant [6].

A research involving organonitrogen compounds has been carried out by Rahman et al. (2006) by synthesizing organonitrogen compounds through Ritter reaction from methyl eugenol with variations of reflux temperature and room temperature, in which the yields from reflux temperatures was greater than that of the room temperature [7]. Another study conducted by Fatma, et al. (2013,) regarding the synthesis of α-pinene based organonitrogen compounds through the Ritter reaction, showed that the yield was increased along the reaction time [8].

The synthesized organonitrogen compounds can be tested for their toxicity using the Brain Shrimp Lethality Test method on Artemia salina larvae. The Brain Shrimp Lethality Test method is sensitive, inexpensive, fast, and simple. Through Brain Shrimp Lethality Test, the median lethal concentration or LC50 is obtained from the dose-response relationship curve equation [9]. If the LC50 value of a compound is below 100 μg/ml, it is categorized as high toxicity activity. If the LC50 value is between 100 μg/ml - 500 μg/ml, it is classified as medium toxicity activity, and if the LC50 value is between 500 μg/ml - 1000 μg/ml, it is considered as low toxicity activity [10].

This paper exploring further research on organonitrogen compounds synthesis from eugenol through the Ritter reaction and focuses on the effect of various temperature and reaction time treatments. A toxicity testing on Artemia salina Leach through the Brain Shrimp Lethality Test method is also discussed.

2. Materials and Method

2.1. Chemicals and instrumentation

Eugenol (Merck), acetonitrile (Merck), sulfuric acid (Merck), saturated sodium bicarbonate, Artemia salina cysts (Crystal), sea water, and dimethyl sulfoxide (Merck). UV-Vis spectrophotometer instrument (double beam Shimadzu 1600 series), IR spectrophotometer (Shimadzu 8400), and Gas Chromatograph-Mass Spectrometer (Shimadzu QP2010S).

2.2. Synthesis of organonitrogen compounds with temperature variations.

A total of 0.1 mol (16.4 g, 16 ml) eugenol and 0.5 mol (20.5 g, 26 ml) acetonitrile were placed in Erlenmeyer (for ice temperature (0°C) and room temperature (27°C)) or round bottom flask (for reflux temperature (90°C)) and then stirred with a magnetic stirrer. Next, sulphuric acid was added dropwise (0.1 mol, 9.81 g, 5 ml). The mixture kept stirred for 4 hours. The mixed solution then neutralized by adding saturated sodium bicarbonate solution to pH 7-8 and separated using a separating funnel. The
organic layer was washed with 50 ml of distilled water. The products weight then measured and the product was characterized using UV-Vis spectrophotometer, IR spectrophotometer, and GC-MS.

2.3. Synthesis of organonitrogen compounds with reaction time variations.
A total of 0.1 mol (16.4 g, 16 ml) eugenol and 0.5 mol (20.5 g, 26 ml) acetonitrile were added into the round bottom flask. The mixture then stirred with a magnetic stirrer, added with sulphuric acid dropwise (0.1 mol, 9.81 g, 5 ml) and refluxed (90°C) for 8 and 16 hours. The mixture was neutralized by adding saturated sodium bicarbonate solution to pH 7-8 and separated using a separating funnel. The organic layer was washed with 50 ml of distilled water. The products weight then measured and the product was characterized using UV-Vis spectrophotometer, IR spectrophotometer, and GC-MS.

2.4. Toxicity test using the Brain Shrimp Lethality Test method (Hatching of Artemia salina shrimp larvae cysts).
Artemia salina cysts (0.03g) soaked with 1L sea water in a container. The container was closed and a 40-watt lamp was placed next to the container. Aeration was given through a hose that connects the aerator. After 48 hours of immersion, the eggs were hatched and the larvae was used for the test. The Eugenol compound and other synthesized compounds were made with concentrations of 0, 10, 25, 50, 75, 100, 125 and 150 ppm from 500 ppm by diluting the test solution (eugenol and other synthesis products obtained from 16-hours reflux temperature (90°C) reaction) using sea water and added with 1% dimethyl sulfoxide. Each test solution (5 ml) was put in the sample bottle, followed by the addition of 30 shrimp larvae. The solution was left for 24 hours, and the percent mortality of each sample was calculated using equation 1:

\[
\text{Mortality (\%)} = \frac{N_t}{N_o} \times 100\% \tag{1}
\]

\(N_t\) is the number of larvae that died after treatment, and \(N_o\) is the total number of larvae. The calculation of \(LC_{50}\) values was done by online system through the four parameters logistic equation, which corresponds to the dose-response curve with the slope of the variable slope [11].

3. Result and Discussion
The 4-hours reactions give an orange solution (for ice temperature, 15.82g), a brownish orange solution (for room temperature, 16.31g) and an oily brown solution (for reflux temperature, 12.60g, 2%). Meanwhile the 8-hours and 16-hours reflux temperature reactions produces 11.41g (7%) and 8.36g (11%), respectively. The products were then characterized using UV-Vis spectrophotometer, IR spectrophotometer, and GC-MS.

The UV-Vis analyses of the products that obtained from the three 4-hours reactions found that they all had an absorption identical to eugenol with a maximum wavelength at 282 nm based on the electron transition that occurred, namely \(\pi \rightarrow \pi^*\). This is probably due to the 4-hours space time was not enough for the mixture to undergo reaction as expected, hence most of the products remains as the starting material. Meanwhile, the 8 and 16-hours reflux temperature reactions shows a maximum wavelength of 280 and 281.5 nm in which the adsorptions were lower than that of the three 4-hours reactions. This indicates that there is another compound beside eugenol that present dominantly in the sample. In addition, a blue shift in the wavelength suggest the presence of an auxochrome group or due to solvent effect.

As indicated from UV analyses, the 4-hours reactions at 0° and 27°C did not undergo reaction and the solution remains as eugenol. This is supported by FTIR analyses in which the infrared spectra of the products obtained from the 4-hours reactions at 0° and 27°C is identical to that of eugenol (standard). The 4-hours reactions at 0° C display infrared bands at 3445.39 cm⁻¹ (O-H stretch), 1638.21 cm⁻¹ (C=O stretch), 1431.85 and 1464.63 cm⁻¹ (C-C stretch in ring), 1514.76 and 1613.14 cm⁻¹ (aromatic compounds stretch), and 1235.12 cm⁻¹ (C-O stretch). Similarly, the 4-hours reactions at 27° C display infrared bands at 3445.39 and 3501.32 cm⁻¹ (O-H stretch), 1638.21 cm⁻¹ (C=O stretch), 1433.77 and
1464.63 cm\(^{-1}\) (C-C stretch in ring), 1514.78 and 1607.36 cm\(^{-1}\) (aromatic compounds stretch), and 1235.12 cm\(^{-1}\) (C-O stretch).

Slightly different to two previous samples, the infrared spectra of the 4-hours reactions at 90º C shows peaks at 2255.39 cm\(^{-1}\) (C≡N stretch), 1636.29 cm\(^{-1}\) (C=O stretch), 1429.92 and 1456.92 cm\(^{-1}\) (C-C stretch in ring), 1516.71 and 1615.07 cm\(^{-1}\) (aromatic compounds stretch), and 1233.19 cm\(^{-1}\) (C-O stretch). An additional peak was appeared in the 8 and 16-hours reactions at 90º C spectra that correspond to imine, and the spectra of the 8 and 16-hours reactions at 90º C did not differ significantly to one another except the adsorption intensities. The infrared spectra of the 8-hours reactions at 90º C has bands at 1640.14 cm\(^{-1}\) (C=N stretch in ring), 1150.26 cm\(^{-1}\) (C-N stretch), 1431.85 and 1464.63 cm\(^{-1}\) (C-C stretch in ring), 1516.71 and 1605.43 cm\(^{-1}\) (aromatic compounds stretch), 1368.20 cm\(^{-1}\) (CH\(_2\)-CH\(_3\) bend), 3437.68 and 3510.97 cm\(^{-1}\) (O-H stretch), and 1235.12 cm\(^{-1}\) (C-O stretch).

GC-MS analysis (Table 1) shows that there was only one absorption peak on the chromatogram of 4-hour (0ºC) product with a retention time of 21.588 and on the chromatogram of the 4-hour (27ºC) product with a retention time of 21.59, which is likely correspond to eugenol, based on the mass spectra with a M\(^+\) (m/z) of 164. For the 4-hour (90ºC), there were two absorption peaks, with a retention time of 21.86 (peak 1) and 34.809 (peak 2). The mass spectrum of peak 1 has a M\(^+\) (m/z) of 164, correspond to eugenol, and the mass spectrum of peak 2 has a M\(^+\) (m/z) of 223, correspond to eugenyl acetamide. Meanwhile, GC-MS analyses of the 8 and 16-hour (90ºC) products were insignificantly different (base peak on mass spectrum is 205), with retention time of 12.98 (eugenol), 19.99 (eugenyl acetamide), and 18.65 (isoquinoline). The area (%) produced from each peak of the chromatogram showed that there was a decrease in the area of eugenol as the starting material, while the area of eugenyl acetamide and isoquinoline compound were increased. This means that increasing reaction time resulting in more eugenol to be converted into the products. The activation energy, fulfilled by both longer reaction time and higher temperature, transforms the reactants into products. The product yields will increase (in quantity and variety of compounds) as reaction time increases until it reaches its optimum conditions of temperature and or reaction time.

### Table 1. Results of GC-MS from various reaction conditions.

| Temperature (ºC) | Reaction time (h) | % Area | Eugenol | Eugenyl acetamide | Isoquinoline |
|------------------|-------------------|--------|---------|------------------|--------------|
| 0                | 4                 | 100    | 0       | 0                | 0            |
| 27               | 4                 | 100    | 0       | 0                | 0            |
| 90               | 4                 | 96.06  | 3.94    | 0                | 0            |
| 8                | 67.76             | 15.44  | 12.85   | 12.85            | 26.96        |
| 16               | 19.85             | 34.21  | 26.96   | 26.96            | 26.96        |

Toxicity test using the Brain Shrimp Lethality Test method was performed on *Artemia salina* Leach using eugenol and the crude oil obtained from the 16-hours (90ºC) reaction (Figure 1). The concentration of the test solution is proportional to the mortality percentage of *Artemia salina*. The eugenol test solution has a LC\(_{50}\) value of 27 ppm, which is categorised to has strong toxicity activity, whereas the crude oil test solution has a LC\(_{50}\) value of 16 ppm, which is also categorised to has strong toxicity activity. This was indicated by the large number of dead *Artemia salina* in low test solution concentrations. The crude oil test solution shows higher toxicity level than that of eugenol due to the presence of heteroatom groups from organonitrogen compounds with their affinity and polarity which can potentially become drug compounds.
Figure 1 (a) Toxicity Test of eugenol compounds on *Artemia salina* and crude oil and (b) Toxicity Test of crude oil from the reaction product at reflux temperature (90°C) with 16 hours reaction time on *Artemia salina*.

4. Conclusion
Synthesis of organonitrogen compounds from eugenol through the Ritter reaction for 4 hours at 0°C and 27°C did not produce any organonitrogen compound products. The synthesis works at reflux temperature of 90°C, in which the reaction time affect the organonitrogen compositions. Eugenyl acetamide and isoquinoline were produced when 8 and 16-hours reaction time was used, but at 4 hours (90°C) reaction, only eugenyl acetamide was formed. At 90°C, the longer reaction time used, the higher concentration of eugenyl acetamide and isoquinoline yielded, in which the product obtained from 16-hours reaction time has composition (%) of eugenol, eugenyl acetamide, and isoquinoline of 19.85, 34.21, and 26.96 respectively. The results of the toxicity test through the Brain Shrimp Lethality Test method on *Artemia salina* showed that the crude product, obtained from the 16-hours (90°C) reaction, has a LC$_{50}$ of 16 ppm, which is higher than that of eugenol with a LC$_{50}$ value of 27 ppm, and categorised as strong toxicity activity.

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