The potency of carica (Carica pubescens Lenne & K. Koch) seed extract as repellent agent against mosquito vector of dengue (Aedes aegypti Linn.)

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Abstract. The spread of Dengue Hemorrhagic Fever (DHF) in some countries around the world is related to the presence of its primary vector, Aedes aegypti. Prevention is generally carried out with the use of chemical insecticides but due to its negative impact, research in bio-insecticides needs to be done. One is in the form of mosquito repellent. This study aimed to determine the percentage repellency from carica (Carica pubescens) seed extract from the value of ED50 and ED99 as a repellent against Ae. aegypti and to identify the content of the compounds by using GC-MS analysis. Carica seed was collected from Dieng Plateau, Wonosobo District, Indonesia. Carica seed extract was made using maceration method with methanol solvents. The doses of extract given to the volunteer’s arm were 0.3, 1, 1.67, 2.33, and 3 mg/cm^2, according to the standard set by WHO. Data were processed using IBM SPSS 23 application with a 95% confidence value. The result showed that carica seed extracts have percentage repellency with 48 ± 0.24, 51 ± 0.34%, 60 ± 0.16%, 68 ± 0.21%, respectively, from the smallest dose given. Statistically, there were no significant differences between all doses tested. Carica seed extract had an ED50 value of 5.241 mg/cm^2 and ED99 value of 99.042 mg/cm^2. The GC-MS test results showed that carica seed extract predominantly contained oleic acid and palmitic acid which were suspected to act as repellent agents against Ae. aegypti, and some other important compounds.

1. Introduction
Mosquitoes can spread any diseases to humans and animals by transmitting viruses, such as dengue and chikungunya when mosquitoes bite the human skin. These viruses are transmitted commonly by 2 species called Aedes aegypti and Ae. albopictus. About half of human population in the world is at risk of dengue infection [1]. The manifestation of its infection includes Dengue Hemorrhagic Fever (DHF) outbreak. It is estimated that there are approximately 50 to 100 million dengue virus infections in humans around the world [2, 3] and 500,000 of them have to be hospitalized due to dengue fever [3]. In Indonesia, DHF has become endemic since the first case was discovered in 1968 [4]. In this country, the widespread of DHF has increased since 1968 where it was only discovered in 2 provinces and quickly spread to other 32 provinces within the next year [5].

Prevention of DHF outbreaks needs to be done by protecting human from contacting with mosquito-borne diseases population by using insecticides [6]. Although synthetic insecticide is quite effective in controlling the Ae. aegypti, it has its side effects such as polluting the environment,
poisoning in humans [7] and causing mosquitoes to become resistant [8]. One example of an insecticide is a mosquito repellent drug. One of the active chemical compounds that have been used for about 40 yr in making repellents is N,N-diethyl-meta-toluamide (DEET) [9]. DEET is a compound which is quite effective as a repellent yet toxic and can cause damage to plastics and synthetic fibers [10].

Research on plants as bio-insecticides to control mosquitoes in larvicidal and repellent forms have been carried out. Bio-insecticides are safer for health and more friendly to the environment than synthetic insecticides. In addition, plants are also easy to get in nature and easy to be processed. There have been a lot of plants were being used as bio-insecticides [9, 11]. Carica (C. pubescens) is one of the potential plants that have medicinal ingredient and phytochemical content that is beneficial to human needs. In Wonosobo, this plant especially its flesh is processed into food products such as syrup, cocktail, and chips. However, seeds from the fruit are not utilized and it ended up to be discarded during the food productions. Several studies on carica seed extract have been carried out, including a test for antibacterial and anti-diarrhea activities [12]. Other research carried out by Supono et al. [13], showed that carica seed extract is potential as larvicidal against Ae. aegypti. In his study, the exposure of carica seed extract causes the death of Ae. aegypti larvae at exposure time of 24 and 48 hr with LC50 values of 148.30 ppm and 103.99 ppm, respectively. However, there has been no study that examines the potency of carica seed as mosquito repellent against Ae. aegypti. Thus, this study aimed to determine the percentage repellency from carica (C. pubescens) seed extract to obtain the value of ED50 and ED99 as a mosquito repellent against Ae. aegypti and to identify the content of its compounds by using GC-MS analysis.

2. Methods

2.1. Collection and preparation of C. pubescens seeds extract

Fruit samples were taken from farmer’s field in Wadas Putih Hamlet, Dieng Plateu, Wonosobo District, Central Java, at an altitude of 1830 m asl at 7°13’28 “S 109°55’54” E. Approximately 25 kg of Carica fruits were collected. The fruits were cut into several parts and the seeds were taken out. The seeds were cleaned by using water until there was no membrane attached. The seeds were left air-dried to reduce the water content for 1-2 d at room temperature. The wet weight of the seeds was weighed using a scale. Carica seeds were then dried using an oven for 3-7 d at 40 ºC. Seeds were then weighed again to obtain dry weight data. The dried seeds were ground into powder by using a blender and 200 g ground dried seeds were dissolved into methanol solution with a ratio of 1:5 for 24 hr. The marinate results were then filtered using a filter paper and stored in a container. Carica seeds pulp was then dissolved into methanol in a ratio of 1:4 for 24 hr. The results were then filtered by the same method as before. The filtrate from the first and second maceration was mixed in one container and then evaporated by using a rotary vacuum evaporator at 50 ºC to separate the solvent from the pure extract. This process yielded 8.03 g of viscous extracts. The extract was stored in the 4 ºC refrigerator as a stock. The extraction process was conducted in the laboratory of Biology Department, Bandung Institute of Technology (ITB).

2.2. Preparation of extract solution

The doses of extract applied in volunteer’s arm in this study were 0.3 mg/cm², 1 mg/cm², 1.67 mg/cm², 2.7 mg/cm², and 3 mg/cm². The doses were the concentration of extract per cm² surface area of the arm. A dilution of carica seed extract was made with a concentration of 10% and 20% (w/v) as a stock. The 10% concentration was obtained by diluting 0.1 g of extract in 1 mL of acetone. The number of extracts and solvents needed were adjusted to the number of repetitions and the number of volunteers.
2.3. Repellent activity test
The repellent activity testing was following the method of World Health Organization [14] with modification. The Ae. aegypti eggs were taken from the Toxicology Laboratory, ITB. The eggs were hatched in a plastic tub (28x16x6 cm) that has been filled with water. The eggs which hatched into larvae were then maintained by feeding them with fish pellets. The larvae which grew into pupae were then transferred into a plastic tube filled with water and then transferred into a (30x30x30 cm) cage. The temperature and humidity of the cage placement were measured using a data logger. Mosquitoes were kept with a glucose solution. As much as 50 of 5 d old female blood-starved mosquitoes were placed into a test cage with the same size as the rearing cage. The test cages used in this study were 3 pieces. A total of 50 female mosquitoes that have been isolated from glucose for the previous 24 hr were prepared. The relative temperature and humidity of the room were measured using a digital hygrometer. Volunteer’s arm surface area was measured according to the World Health Organization (WHO) guide [14]. Before starting the repellent test, volunteer’s forearms must be washed using water from the wrist to elbow, then allowed to dry. On the left forearms of volunteers, 0.5 mL of ethyl alcohol (70%) was applied evenly across the ±300 cm² skin surface area as a negative control. Before insertion of arm to the cage, the fingers were protected against mosquito’s bite using rubber gloves. The left-arm then was slowly inserted into the test cage and the number of mosquitoes that landed on the skin during the next 30 sec period was counted. The arm then carefully withdrawn. If for 30 sec the number of mosquitoes landed above or equal to 10, then the repellent test can be continued. The same left-arm then was treated with 0.5 mL of 10% concentration of carica seed extract solution and allowed to dry. The treated arm was inserted into the test cage for another 30 sec period and the number of mosquitoes that landed on the arm were counted and recorded. The procedure was repeated for each additional incremental dose of carica seed extract. Testing at higher doses was done by applying the extract to the same arm and the dose applied is calculated as the sum of the doses to get the cumulative dose at each test. Tests are carried out respectively from the lowest dose to the higher dose without delay. At the end of the test, 0.5 mL of 70 % alcohol was applied to the right of the volunteer’s arm and allowed to dry. The arm was inserted into the same test cage for 30 sec to verify that the mosquitoes landed more than 10 as observed at the beginning of the experiment. Repetition of the repellent test was carried out 3 times on different days with different mosquito samples on 3 volunteers. Repetition was carried out at the same time in daytime (10.30-11.30) and with the same volunteers and guide according to the WHO procedure [14]. The percentage repellency was calculated as follows [14]:

$$\%\text{Repellency} = \frac{C - T}{C} \times 100\%$$

Where $C$ is the number of mosquitoes collected from the forearm of the controls and $T$ is the number collected from the treated forearm.

2.4. GC-MS Test
The relative content of compounds in carica seed extract was analysed using the GC-MS method which adopted from previous study [15] in the Chemical Engineering Laboratory, ITB. The carrier gas used in the GC-MS test is Helium gas with an ion source temperature of 250°C and an interface temperature of 300 °C. The solvent cut time of 3 min. The first step was diluting the carica seed extract as much as 100 mg in 5 mL of 70% ethanol. The extract then was injected as much as 1 µL with a split ratio of 1:5 to the heated injection port. Previously the initial GC column temperature was 100°C and slowly increased to reach 150°C with an increase of 5°C per min. Then at 150°C the temperature is held for 5 min. The temperature then was increased to 160°C with an increase of 2°C per min and increased again to reach 270°C with an increase of 10°C per min.

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2.5 Data analysis
Data were carried out with Probit analysis for calculating ED$_{50}$ and ED$_{99}$. In addition, the Kruskal-Wallis Test was used to determine the significance of differences in the effect of given extract dose. The statistical analysis was carried out using the IBM SPSS Statistics 23 application. Results with p<0.05 are considered statistically significant.

3. Results and discussion
The drying process by oven from 1.562.51 g of wet seeds resulted in 519.87 g of dry seeds. The amount of extract obtained from this study was 8.03 g of 200 g dry weight. Thus, the yield produced was 4.015%.

The result of the repellent activity of *C. pubescens* extract was presented in Table 1. The result showed the lowest level of repellency percentage was seen at the exposure of 0.3 mg/cm$^2$ and the repellency percentage tended to increase along with the increase of dose exposure, although at the exposure of 2.3 mg/cm$^2$ the value decreased. Whilst, the higher dose tended to give greater protection, this increase was not statistically significant (p > 0.05). The result of this study also showed that *C. pubescens* seed extract had an ED$_{50}$ value of 5.241 mg/cm$^2$ and an ED$_{99}$ value of 99.042 mg/cm$^2$. This ED value, especially ED$_{99}$ is important for further study such as research on determining the complete protection time of repellent against *Ae. aegypti* with the same extract[14].

| Application Sequences | Cumulative amount of repellent (mg/300cm$^2$ area) | Average mosquito landing to skin | Percentage Repellency (%) |
|-----------------------|--------------------------------------------------|---------------------------------|--------------------------|
| Left arm control      | Pre-treated with alcohol only                     | 12.56 ± 2.403                   | Average control (Left and right) 13 (C) |
| Left arm dose 1       | 100                                              | 6.89 ± 3.92                     | 48 ± 0.24                |
| Left arm dose 2       | 300                                              | 7.38 ± 4.34                     | 51 ± 0.34                |
| Left arm dose 3       | 500                                              | 5.44 ± 3.00                     | 60 ± 0.16                |
| Left arm dose 4       | 700                                              | 5.78 ± 2.22                     | 56 ± 0.13                |
| Left arm dose 5       | 900                                              | 4.2 ± 3.23                      | 68 ± 0.21                |
| Right arm control     | Pre-treated with alcohol only                     | 13.33 ± 2.60                    | -                        |

The results of GC-MS analysis on *C. pubescens* seeds extract showed that there were 17 chemical compounds successfully identified by GC-MS analysis (list of compounds were presented in Table 2). Three compounds with the largest relative content of *C. pubescens* seeds extract based on GC-MS test were oleic acid, methyl oleate and palmitic acid with the percentage area of 18.73, 14.77 and 12.98%, respectively. Methyl oleate is a group of Fatty Acid Methyl Ester (FAME). Another FAME group compounds found were methyl linoleate, methyl stearate and methyl palmitate. In addition, stearate acid was also found. The other compounds consisted of FAME group, tocopherol, bombykol and so on.

According to previous research conducted by examining the content of *Jatropha curcas* as a plant that can protect skin against *Ae. aegypti*, it was found that oleic acid, palmitic acid, and stearic acid act as biting deterrent against *Ae. Aegypti* [16, 17]. This result showed that there might be a relation between the biting deterrent activity with the repellent activity. Hence more extensive study needs to be done. Another study also concluded that oleic acid in soybean oil has repellency against *Ae. aegypti*.
as much as 52% after 1 hr of application [18]. Thus, it can be suspected that the fatty acid content found in C. pubescens is thought to act as repellent agents for Ae. aegypti.

Besides having a function as a repellent agent, C. pubescens seed extract has another benefit because it contains compounds that act as larvicidal agents. It is known that the linoleic fatty acid and oleic fatty acid can act as larvicidal agents on Ae. aegypti, Anopheles stephensi, and Culex quinquefasciatus [19, 20]. Another compound found in C. pubescens seed extract was 1,6,10,14,18,22-Tetracosahexaen-3-ol, 2,6,10,15,19,23-hexamethyl-, (all-E) or Geranylgeraniol which is a compound that acts as a larvicide, based on research conducted by Shazad, et al [21] that found in Ocimum sanctum plant extracts. Geranylgeraniol is an intermediate in the formation of mevalonate acid compounds that plays a role in juvenile hormone biosynthesis in Ae. aegypti [22] and can act as an analogous compound or a mimic agent in the formation of juvenile hormones in mosquitoes which will affect the growth and development of mosquitoes [21]. In addition, another research mentioned that palmitic acid can act as a larvicide in 4-instar larvae of Ae. Aegypti, An. stephensie and Cx. quinquefasciatus [23]. Another study conducted by Mathivanan et al. [24], showed that the content of palmitic acid in petroleum ether extract of Andrographis echioides plants has activity as a larvicide for Ae. aegypti and Cx. quinquefasciatus. The same result was reported by Ragavendran, et al. [10]. The information mentioned before supports the research by Supono et al. [13] which stated that C. pubescens seeds extract has the potential as a larvicidal against Ae. aegypti.

| No. | Name of Compound                      | Retention Time (min) | Molecular formula | Area (%) |
|-----|--------------------------------------|----------------------|-------------------|----------|
| 1.  | Octadec-9-Enoic Acid                 | 32.177               | C_{18}H_{36}O_{2} | 18.73    |
| 2.  | Methyl oleate                        | 31.620               | C_{19}H_{38}O_{2} | 14.77    |
| 3.  | Palmitic acid                        | 29.303               | C_{16}H_{32}O_{2} | 12.98    |
| 4.  | E,E-3,13-Octadecadien-1-ol           | 39.433               | C_{17}H_{34}O     | 10.99    |
| 5.  | Stigmast-5-en-3-ol, (3β)-             | 50.754               | C_{20}H_{40}O     | 10.64    |
| 6.  | 1,6,10,14,18,22-Tetracosahexaen-3-ol, 2,6,10,15,19,23-hexamethyl-, (all-E) | 39.281 | C_{19}H_{38}O | 6.66 |
| 7.  | Benzene, (isothiocyanatomethyl)-      | 8.263                | C_{3}H_{7}NS      | 6.33     |
| 8.  | Methyl palmitate                     | 28.404               | C_{17}H_{34}O_{2} | 5.18     |
| 9.  | E,E,Z-1,3,12-Nonadecatriene-5,14-diol | 40.828              | C_{19}H_{38}O_{2} | 2.33     |
| 10. | Octadecanoic acid                    | 32.456               | C_{18}H_{36}O_{2} | 2.03     |
| 11. | Benzyl isocyanate                    | 4.082                | C_{6}H_{12}O        | 1.92     |
| 12. | Methyl linoleate                     | 31.531               | C_{19}H_{38}O_{2} | 1.90     |
| 13. | Bombykol                             | 32.092               | C_{19}H_{38}O_{2} | 1.60     |
| 14. | gamma-Tocopherol                     | 43.242               | C_{28}H_{56}O_{2} | 1.58     |
| 15. | Methyl stearate                      | 31.983               | C_{19}H_{38}O_{2} | 1.26     |
| 16. | Benzene acetonitrile                 | 4.311                | C_{3}H_{7}N        | 0.75     |
| 17. | Methyl elaidate                      | 31.698               | C_{10}H_{20}O_{2} | 0.36     |

The compound Stigmast-5-en-3-ol, (3β) - is a group of phytosterols that is commonly found in plants [25]. Several studies showed that these compounds have a role as a cholesterol-lowering agent [26], antiproliferation (anticancer substances) [27] and antidiabetic substances [25].
Benzene (isothiocyanatomethyl) or benzyl isothiocyanate is commonly found in papaya seeds [28]. Research showed that this compound in papaya seeds was believed to have an activity as an anthelmintic substance [29]. This substance is very much needed in the animal husbandry to eradicate worms in ruminant animals [30]. Benzene cyanide (phenylacetonitrile) is not found in papaya seeds [28] but can be found in desert grasshoppers and is used as a pheromone issued by male grasshoppers to indicate that they are mature enough to mate and at the same time prevent the mating of the same sex in male [31].

The compounds of bombykol and Υ-Tocopherol were found in relatively small amounts. Bombykol is a sex pheromone that is secreted by silkworms, Bombyx mori [32]. The presence of this compound in C. pubescens seeds extract is possible because of the presence of palmitic acid as a precursor of intermediate in bombykol biosynthesis [33, 34]. Υ-Tocopherol is a type of tocopherol, an organic compound commonly known as vitamin E in foods often consumed in America. According to a study conducted by Shin et al. [35] it is known that Υ-Tocopherol acts as a supplement that can modulate the hyperglycemia process induced by acute kidney inflammation. Bombykol and Υ-Tocopherol activities have not been found as compounds related to attractants or repellents for mosquitoes, especially Ae. aegypti.

There are 2 other compounds namely E, E, Z-1,3,12-Nonadecatriene-5,14-diol and compound E, E-3,13-Octadecadien-1-ol which the function is still unknown. E, E, Z-1,3,12-Nonadecatriene-5,14-diol is a compound found in jasmine flowers (Jasminum sp.), flamboyant (Delonix regia) (in the petal section) and moringa (Moringa oleifera) [36].

4. Conclusion

Carica pubescens seed extract from Dieng Plateu, Wonosobo, Indonesia, is predominantly contained oleic acid and palmitic acid which are thought to act as an important repellent compound against Ae. aegypti. The C. pubescens seed extract has repellency percentage against Ae. aegypti at doses of 0.3; 1; 1.67; 2.33; and 3 mg/cm² and able to protect with 48 ± 0.24%; 51 ± 0.34%; 60 ± 0.16%; 68 ± 0.21%; respectively. C. pubescens seed extract also has an ED₅₀ value of 5.241mg/cm² and ED₉₀ value of 99.042 mg/cm². However, further research needs to be conducted to understand the amount of the repellency percentage if only using isolated fatty acid (oleic, palmitic, and stearic acid) from the extract and its complete protection time in protecting humans from Ae. aegypti bites.

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