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Address: Sri Agus Sudjarwo1*, Ngadino2, Koerniasari2, Setiawan2, Gifta Wardani Sudjarwo3 1Department of Pharmacology, Faculty of Veterinary Medicine, Airlangga University, Surabaya, Indonesia 2Study Program of Environmental Health, Polytechnic of Health, Surabaya, Indonesia 3Faculty of Pharmacy, Hang Tuah University, Surabaya, Indonesia *Corresponding Author Published In: Volume - 10, Issue - 4, Year - 2017 Keywords: ? Extract Pinus merkusii leaf ? Larvicidal ? Aedes aegypti. Cite this article: Sri Agus Sudjarwo, Ngadino, Koerniasari, Setiawan, Gifta Wardani Sudjarwo. Larvicidal Activity of Ethanol Leaf Extract of Pinus merkusii on Aedes aegypti larvae. Research J. Pharm. and Tech. 2017; 10(4): 1011-1014. doi: 10.5958/0974- 360X.2017.00182.2
Larvicidal Activity of Ethanol Leaf Extract of Pinus merkusii on Aedes aegypti larvae Sri Agus Sudjarwo1*, Ngadino2, Koerniasari2, Setiawan2, Gifta Wardani Sudjarwo3 1Department of Pharmacology, Faculty of Veterinary Medicine, Airlangga University, Surabaya, Indonesia 2Study Program of Environmental Health, Polytechnic of Health, Surabaya, Indonesia 3Faculty of Pharmacy, Hang Tuah University, Surabaya, Indonesia *Corresponding Author E-mail: ags158@yahoo.com
ABSTRACT: The mosquito species, Aedes aegypti L. is a vector of major diseases such as dengue haemorrhagic fever, chikungunya and yellow fever. Therefore, it is necessary to find new bioinsectiside which is expected to have larvicidal effects. This study investigates ethanol leaf extracts of Pinus merkusii for efficacy against Aedes aegypti larvae. This experiment using a completely randomized design with 8 treatment groups. Each group contained five times repetition using 20 third instar larvae of Aedes aegypti with concentrations of Pinus merkusii leaf extract are 0, 10, 20, 40. 80, 160, 320, 640 ppm. Larval mortality was observed for 24 h, LC50 and LC90 were analyzed using Probit analysis. Ethanol leaf extract of Pinus merkusii showed highest larvae mortality against the larvae of Aedes aegypti with LC50 = 145.7 ppm; LC90 = 379.4 ppm after 12 h, and LC50 = 86.3 ppm; LC90 = 173.6 ppm after 24 h. These findings suggest that ethanol leaf extracts from Pinus merkusii have larvicidal activity that can be exploited in development of new bioinsecticides. KEYWORDS Extract Pinus merkusii leaf, Larvicidal, Aedes aegypti.

INTRODUCTION: The mosquito species, Aedes aegypti L. is a vector of major diseases such as dengue haemorrhagic fever, chikungunya and yellow fever. A. aegypti is reported to infect more than hundred million people every year in more than 110 countries in the tropics .1 The present resurgence of these diseases is due to the higher number of breeding places in today's throwaway society. 2A. aegypti is very closely associated with the human habitat. The geographical range of A. aegypti is increasing in part due to rapid urbanization and increased global movement of people and cargo. 3 The technique in controlling mosquitoes depends on the larval stages (egg, larvae, pupae, and adult) on target. Mosquito control includes targeting the adult mosquito through spraying chemical insecticides or by killing the mosquito larvae before they emerge into adults via using synthetic larvicides or botanical extracts as an alternative larvicide. The indiscriminate use of synthetic insecticides is creating multifarious problems like environmental pollution, insecticide resistance, and toxic hazards to humans. 4 Globally, there have been conscientious efforts to overcome these problems, and great emphasis has been placed recently on enviro- friendly and economically viable methodologies for pest control. Natural products of plant origin are preferred over synthetic insecticides due to their eco-friendly nature. Current research trends use plant extracts as alternative larvicides because they contain various phytochemicals that are specific in killing mosquito larvae without harming other organisms and the environment. 5,6 Instead of using synthetic larvicides, the use of these plant-derived products in controlling mosquito larvae is inexpensive and environment-friendly. In most parts of the world, Synthetic chemical larvicides continue to be applied for controlling mosquitoes but many of these chemicals are toxic to human, animal and plant life and resistance can be problematic in regulating the control. Phytochemicals obtained from the huge diversity of plant species are important source for safe and biodegradable chemicals, which can be screened for mosquito repellent, larvicidal, and insecticidal activities; and tested for mammalian toxicity. Therefore, researchers are currently exploiting natural substances to be used as insecticides for controlling larval mosquitoes.7,8 The phytochemicals of the plants serve as huge storage of compounds that have biological action. 9 Alkaloids, flavonoid, saponins, and tannins are known to possess medicinal and pesticidal properties. 10 These phytochemicals present in the Pinus merkusii extract. It has been reported that pinus plant components like phenolics, flavonoids, tannins and other constituents can be used to treat oxidative, inflammatory and microbial.11 The aim of this study was to investigate the potential of Pinus merkusii leaf extract against the larvae of Aedes aegypti through larvicidal bioassays.

MATERIALS AND METHODS: Mosquito Culture: Aedes aegypti colonies were maintained in our insectary in large enamel basins (45×45×40 cm) and rearing conditions were 28±2°C temperature, 65±5% relative humidity (RH) and photoperiod of 14:10 h light and darkperiod.12The egg strips were obtained from Institute of Tropical Disease Airlangga University Surabaya to start the colony. The strips were immersed in dechlorinated tap water for hatching. Larvae were fed with a diet of finely ground brewer yeast and dog biscuits (3:1). The emerged adults were fed with rabbit blood and with 10% glucose solution. Small porcelain dishes having 50 ml of tap water lined with filter paper was kept inside the cage for oviposition. Preparation of Ethanol Leaf Extract of Pinus merkusii: Plant material and extract preparation leaf of Pinus merkusii were collected from Pacet, Mojakerto, Indonesia. Leaves were cleaned with running tap water and were chopped into pieces. They were dried under shade at ambient temperature for 5 days and the air-dried leaf were then ground to powder for extraction. The powdered leaf (1 kg) was macerated with ethanol 96 % (5 L) for a week at 37°C. The supernatant was then collected and filtered through Whatman No. 1 filter paper in a Buchner funnel under vacuum. The filtrate was concentrated by evaporation with a vacuum rotary evaporator at 45°C. The extract was dried at reduced pressure, stored at 0-4oC and used for the experimentation. Phytochemical Analysis: The filtrate was tested for the presence of phytochemicals such as alkaloids, flavanoids, saponins, tannins and terpenoids using standard procedures.13 Larvicidal Bioassay Ethanol Leaf Extract of Pinus merkusii: The tests were conducted at room temperature. The Pinus merkusii leaf extracts as larvicide were tested against the third instar larvae of A. aegypti mosquitoes.14 Five replicates of Pinus merkusii extracts dilution with 0, 10, 20, 40, 80, 160, 320 and 640 ppm concentrations were prepared. Each replicate containing 200 ml of the described Pinus merkusii leaf extract was placed in a 500 ml glass beaker. 20 third-instar larvae of A. aegypti were transferred into each beaker14. After that, the number of dead larvae in each beaker was counted after 1, 5; 3; 6; 12 and 24 h. Identification of the Aedes aegypti larvae were done by tapping it with a needle in the siphon or cervical area. Each treatment was conducted in five replicates. The larvae were considered dead if, at the end of 24 hrs, they showed no sign of swimming movements even after gentle touching with a glass rod, as described in the World Health Organization's technical report series. 15The effects of the Pinus merkusii extracts were monitored through carefully counting the number of dead larvae after 24 hours of treatment, and the percentage mortality was computed. Number of dead larvae Percentage mortality=---------- x 100 Number of larvae introduced Lethal Concentration of Ethanol Leaf Extract of Pinus merkusii: The LC50 and LC90 of the plant extract that showed 100% mortality was determined by a similar procedure as mentioned above. 0, 10, 20, 40, 80, 160, 320 and 640 ppm concentration were tested and the observation was recorded after 1.5 ; 3; 6; 12 and 24 hrs of incubation. Statistical Analysis: The statistical tools that were used in this study are the following: the Arithmetic Mean to get the average number of dead mosquito larvae, and Probit Analysis to calculate LC50 and LC90 values to determine Lethal Concentrations of the plant extracts on Aedes aegypti mosquito larvae after 12 and 24 hours of treatment

RESULTS: The preliminary phytochemical analysis of ethanol leaf extract of Pinus merkusii (Table 1) showed the presence of alkaloids, saponins, flavonoids, triterpenoids and tannins of phytochemicals. Any of these phytochemicals, either singly or in a combination with each other could be responsible for the larvacidal activity of the Pinus merkusii leaf extract.

Table 1: Phytochemical analysis of extracts of Pinus merkusii leaf Pinus merkusii tree bark extract Phytochemicals Level Alkaloids Flavanoids Saponins Tannins Terpenoids ++ +++ + ++ +++ +: low, ++: immediate, +++: high

Different concentrations (0, 10, 20, 40, 80, 160, 320 and 640 ppm) of Pinus merkusii leaf extract solutions were bioassayed against the third instar larvae of Aedes aegypti. The results were shown after 1.5, 3, 6, 12 and 24 h of treatment (Table 2). In control treatments, no larvicidal effect was observed; the larvae remained alive, and they moulted into fourth instar larvae. Whereas, when the different Pinus merkusii leaf extract concentrations were tested, different mortality rates were recorded with respect to exposure time. At 40 ppm Pinus merkusii leaf extract, the larvae

remained immobile after 3 h of treatment. When 80 ppm Pinus merkusii leaf extract was tested, 1.4 % and 46.3 % larval mortality was recorded after 1.5 h and 24 h of treatment respectively. When 160 ppm Pinus merkusii leaf extractsolution was tested, 3.4 % and 87.2 % mortality was recorded after 1.5 h and 24 hoftreatment respectively. At 320 ppm Pinus merkusii leaf extract, complete mortality (100 %) was recorded after 24 h of exposure, while at 640 ppm, 100 % mortality was recorded after 12 h. The maximum result (100 %) was recorded with 320 ppm Pinus merkusii leaf extract concentration after 24 h exposure (Table 2). Table 2:Effect of different concentrations of Pinus merkusii leaf extract solution and exposure time on larvicidal bioassay of third instar larvae of Aedes aegypti Pinus Merkusii Extract(ppm) Larval Mortality Rate (%) After 1.5 h 3 h 6 h 12 h 24 h 0 (Control) 10 20 40 80 160 320 640 0 0 0 0 1.4 ± 0.3 3.4 ± 0.6 5.9 ± 0.4 8.2± 0.7 0 0 0 2.4 ± 0.3 3.8 ± 0.6 9.3 ± 0.8 14.1 ± 1.8 24.3 ± 2.9 0 0 0 4.1 ± 0.6 11.3 ± 2.1 20.3 ± 4.2 32.3 ± 3.9 40.3 ± 6.1 0 0 1.2 ± 0.3 7.6 ± 1.3 22.9 ± 2.3 58.3 ± 5.6 85.4 ± 4.9 100 ± 0 0 0 4.5 ± 0.9 21.6 ± 2.7 46.3 ± 3.9 87.2 ± 6.1 100 ± 0 100 ± 0 *The values are mean ± SEM of five replicate After 12 h, lethal concentration 50 (50 % larvicidal activity) of Pinus merkusii leaf extract was 145.7 ppm while lethal concentration 90 (90 % larvicidal activity) was 379.4 ppm. However after 24 h,lethal concentration (50 % larvicidal activity) of Pinus merkusii leaf extract was 86.3 ppm while lethal concentration (90 % larvicidal activity) was 173.6 ppm (Table 3). The exposure time is very important for larvicidal activity of the Pinus merkusii tree bark extract solution. Table 3: LC50 and LC90 of extracts of Pinus merkusii leaf Time Pinus merkusii tree bark Extract LC50 (ppm) LC90 (ppm) After 12 h After 24 h 145.7 86.3 379.4 173.6 DISCUSSION: Mosquitoes are responsible for the spread of more diseases than any other group of arthropods. The medical importance of mosquitoes as vectors for the transmission of serious diseases that cause morbidity, mortality, economical loss, and social disruption such asmalaria, lymphatic filariasis, and viral diseases is well documented. Aedes aegypti, the primary carrier for viruses that cause dengue and dengue hemorrhagic fever and yellow fever, are widespread over large areas of the tropics and subtropics. 16 There is no vaccine to prevent dengue infection, nor are there drugs to combat the disease in infected persons, so vector control is the most opted solution available so far for reducing the morbidity. The prevention of mosquito breeding through the use of larvicides is the most effective way to fight with this mosquito importunation. Synthetic insecticides have been used as larvicide in several countries for the last 30 years.17 However, the non-selectiveness of insecticides and harmful effects on other organisms is the major hindrance with the use of these chemical insecticides.18 The need for development of effective insecticides should be taken into consideration due to the toxicity problems, together with the increased incidence of insect resistance. In this study was undertaken to access the toxicant potential of the Pinus merkusii against mosquito larvae of A. aegypti. The Pinus merkusii extracts exhibited a concentration dependent activities against Aedes aegypti larvae since the percentage mortality were observed to increase with increasing concentrations of the Pinus merkusii leaf extracts. The increase of percentage mortality of the treated Aedes aegypti larvae is supported by the presence of phytochemicals in the Pinus merkusii extracts which have insecticidal activities. The least percentage mortality was noted in the control group (0 ppm concentration) which is extremely lower compared to those in the experimental groups. It reveals that all tested concentrations of the Pinus merkusii tree bark extracts (20, 40, 80, 160, 320 and 640) caused mortality of Aedes aegypti larvae in comparison to those in the control group. Result also indicates that 160 ppm concentrations of Pinus merkusii leaf extract have the high percentage of mortality after 24 h. The Pinus merkusii leaf extract high larvicidal activity is supported by the presence of phytochemicals such as alkaloids, saponins, flavonoids, triterpenoids and tannins which are known to have insecticidal and pesticidal properties.11 The phytochemicals of the plants serve as huge storage of compounds that have biological action. 19 This is supported by the presence of phytochemicals such as alkaloids, saponins, and tannins are known to possess medicinal and pesticidal properties. 10 It is further noted that the percentage mortality increased with increasing concentrations of the plant extracts. Moreover, the mortality of mosquito larvae was also increased in relation to the time of exposure. The larvicidal activity of the highest concentration (320 ppm) of the Pinus merkusii leaf extracts on A. aegypti mosquito larvae within 24 hours of exposure. Moreover, the results also exhibited that there is a significant difference on the mortality of A. aegypti mosquito larvae between the control group andvarious concentration of the Pinus merkusii leaf extracts. This result denotes that higher concentration of the Pinus merkusii extracts would lead to greater number of mortality in the A. aegypti mosquito larvae. The lethal concentrations (LC50 and LC90) values of these Pinus merkusii extracts on Aedes aegypti mosquito larvae after 24 hours of exposure are 86.3 ppm and 173.6 ppm. It shows that Pinus merkusii tree extract is the most effective in terms of insecticidal activity. Results show that Pinus merkusii is highly lethal to A. aegypti larvae. CONCLUSION: This study indicates that the ethanolic leaf extract of Pinus merkusii has larvicidal properties and its use as a larvicide against the dengue-vector, Aedes aegypti mosquito should be explored. The percentage mortality increased with increasing concentrations of the Pinus merkusii leaf extracts and also increased in relation to the time of exposure. It is worthwhile to study extensively the larvicidal properties of the Pinus merkusii by isolating and identifying the active components responsible for larval mortality, and then test them in field trials in order to assess their potential as an alternative to synthetic chemical larvicides. CONFLICT Of INTEREST: All authors declare no conflict of interest ACKNOWLEDGEMENT: The authors would like to acknowledge the support of The Ministry of Health Republic of Indonesia through the Health Polytecnic Surabaya Indonesia in conducting this research work REFERENCES: 1. Hales S, Wet ND, Maindonald J, Woodward A. Potential effect of population and climate changes on global distribution of dengue fever: an empirical model. The Lancet. 2002; 360:830-834. 2. Kovendan K, Murugan K. 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