Korespondensi Jurnal

Judul Artikel : Morphological and Histological Effect of Bruceine a on the Larvae of Aedes aegypti Linnaeus (Diptera : Culicidae)

Nama Jurnal : Asian Journal of Pharmaceutichal and Clinical Research

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No	Kegiatan	Tanggal	Keterangan	Halaman
1	Submission	15 Mei 2018	Editor (Journal System Dashboard) https://innovareacademics.in/ journals/index.php/ajpcr/submissions	1
2	Request for revised article	16 Juni 2018	Email dari Editor	1
3	Request for payment	16 Juni 2018	Email dari Editor	4
4	Balasan penulis ke Editor (Re-request for revised article & payamet)	28 Juni 2018	Email Penulis ke editor	5
5	Evaluation Report dari Reviewer	16 Juni 2018	Email dari Editor	7
6	Revised article	28 Juni 2018	Email Penulis ke editor	25
7	Bukti Pembayaran via transfer BNI 46	26 Juni 2018	Email Penulis ke editor	42
8	Final Proof	13 Juli 2018	Journal System Dashboard	43
9	Published	10 Oktober 2018	Journal System Dashboard https://innovareacademics.in/journals /index.php/ajpcr/article/view/27315	49

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MORPHOLOGICAL AND HISTOLOGICAL EFFECTS OF BRUCEINE A ON THE LARVAE OF *Aedes aegypti* Linnaeus (DIPTERA : CULICIDAE)

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ABSTRACT

Objective: This study aimed to determine the target of action of bruceine A on the basis of its morphological and histological effects on the larvae of *A. aegypti* (L.).

Methods: Bruceine A was isolated from *B. javanica* (L.) Merr seeds in accordance with the Mangungsong method. Larvae of *A. aegypti* (L.) in instar III to the beginning of instar IV were treated with various concentrations of bruceine A. The negative control group did not receive any treatment, whereas the positive control group received 1 ppm temephos. Dead larvae were collected after 24 h of treatment for the examination of morphological and histological changes.

Results: The negative control group did not exhibit any morphological and histological changes. Larvae treated with bruceine A, however, had visibly damaged heads, cuticles, digestive and respiration tracts, respiratory siphons, and setae, and were smaller than normal larvae. Larvae treated with temephos exhibited gastrointestinal damage, narrowed breathing tubes, cuticle damage, and detached/damaged setae feathers. The necrosis of gastrointestinal epithelial cells was the major histological change exhibited by larvae treated with various concentrations of bruceine A or 1 ppm temephos.

Conclusion: The targets of action of bruceine A in *A. aegypti* (L.) larvae are the head/caput, cuticle, setae, siphon, and gastrointestinal and respiratory tracts.

Key words: Bruceine A, *Brucea javanica* (L.) Merr, action target, morphology, histology, *Aedes aegypti* Linnaeus

INTRODUCTION

Vector control is a method for suppressing the incidence of vector-borne diseases. It is widely conducted as a public health intervention. *Aedes aegypti* Linnaeus is a mosquito species that is an important disease vector in tropical and subtropical regions [1]. *A. aegypti* (L.) is a vector of dengue fever, dengue hemorrhagic fever [2], chikungunya fever, yellow fever, and Zika viral disease [3]. The wide use of synthetic organic insecticides for vector control harms the environment and causes the emergence of insecticide-resistant vectors, as well as the deaths of nontarget animals. Earlier intervention studies have shown that although the use of synthetic insecticides, such as temephos, in risky or potential places can decrease disease transmission by mosquitoes, prolonged exposure to these chemicals will promote the adaptation, evolution and selection of mosquitoes [4]. Thus, plant-derived insecticides/larvicides should be developed as another option for controlling vector-borne diseases. The two essential oils of *Thymus vulgaris* and *Origanum majorana* (Lamiaceae) demonstrate an interesting larvicidal activity. The *O. majorana* essential oil is the most effective compared to the essential oil of *T. vulgaris* with an LC_{50} of 107.13 μg/mL and LC_{90} of 365.90 μg/mL on the malaria vector *Anopheles labranchiae* [5]. The crude ethanolic extract of *Smilax larvata* (Sarsaparilla) is a potential source of an eco-friendly larvicide against *Aedes aegypti* larvae with LC_{50} 225μg/mL^{-1} and LC_{90} 350μg/mL^{-1} [6]. Compounds from *Brucea javanica* (L.) Merr have potential applications as agricultural insecticides. Zhang et al. [7] proved that brusatol isolated from *B. javanica* (L.) Merr has insecticidal and antifeeding effects against the third-instar larvae of *Spodoptera exigua*. Brusatol can also induce apoptosis in the insect cell lines IOZCAS-Spec-II and Sf21. In these cell lines, apoptosis is characterized by DNA fragmentation, caspase-3 activation, and cytochrome-c release from mitochondria. Sutiningsih & Nurjazuli [8] proved that brusatol isolated from the seeds of *B. javanica* (L) Merr has larvicidal activity against *A. aegypti* at the Lethal Concentrations of 50 and 90 (LC_{50}, LC_{90}) of 0.669 and 8.331 ppm, respectively.

Bruceine A ([15]-3-methyl-2-butanoil-bruseolid) is a quassinoid derived from *B. javanica* (L.) Merr [9]. Its molecular formula is $C_{26}H_{34}O_{11}$, and its mass is 522.54 g/mol. Physically, it is an amorphous powder with bitter taste. Bruceine A has extensive broad

biological activity as an antibabesiosis, antitrypanosomal, and anti-malarial, as well as cytotoxic properties against cancer cell lines [10–12]. It also has insecticidal, antifeeding, and growth-inhibiting activities against tobacco budworm (*Heliothis virescens* F.) and *Spodoptera frugiperda* armyworm [13] and Mexican bean beetle larvae in the fourth instar (*Epilachna varivestis* Mulsant) [14]. Bruceine A can also act as a neurotoxin [15] and inhibitor of growth [16] against the larvae of *A. aegypti* (L.) The biolarvicidal mechanism of action of bruceine A occurs through the inhibition of acetylcholinesterase and VGSC gene. The behavioral responses of larvae treated with bruceine A include hyperexcitation, convulsions, paralysis, and aggressive biting of the anal gills; these behaviors indicate that bruceine A affects the larval neuromuscular systems [15]. Therefore, this study aimed to determine the targets of action of bruceine A and to identify its effects on the morphology and histology of *A. aegypti* (L.) larvae.

MATERIALS AND METHODS

Materials

Makasar Fruit (*B. javanica* L. Merr) was purchased from a wholesaler of medicinal plants (Aneka Herbal Yogyakarta, Indonesia). The specimen was further identified at the Laboratory of Pharmaceutical Biology, Faculty of Pharmacy, Gadjah Mada University, Yogyakarta, Indonesia, to confirm its identity and to obtain its relevant scientific information. *A. aegypti* (L.) larvae in instar III to the beginning of instar IV were obtained from colonies maintained at the Laboratory of Parasitology, Faculty of Medicine, University of Gadjah Mada, Yogyakarta. All commercial reagents and other chemicals used in this study were of analytical quality with the highest purity available and purchased from commercial suppliers. The selection of temephos dosage (1 ppm) is based on lethal damage consideration used in the field.

Extraction and isolation of bruceine A

Bruceine A was isolated from *B. javanica* (L.) Merr seeds in accordance with the method described by Mangungsong [17]. Dried seeds of *B. javanica* (L.) Merr (5 kg) were ground into powder and shaken with a hexane solution (15 L). The solution was then filtered and extracted with methanol (15 L). Methanol was evaporated to obtain a thick extract, which was then mixed with an equal volume of distilled water to form a suspension. The suspension was partitioned with hexane (3 L). The hexane fraction was separated from the suspension, and methanol–water fractions were collected for repeated extraction with

dichloromethane (1 L). The organic layer was collected and evaporated to obtain a concentrate, which was then diluted with methanol (100–250 mL) at 60°C and stored at room temperature. The methanol solution was maintained at room temperature to allow the crystallization of bruceine A. Further separation was conducted through filtration. The remainder of the filtrate / residue was separated through Thin Layer Chromatography (TLC) and evaporated. The purity levels of the amorphous powder were measured using High Performance Liquid Chromatography (HPLC).

Morphology test

Morphological tests were conducted in accordance with the method reported by Sharma et al. [18] with slight modifications. *A. aegypti* (L.) larvae in instar III to the beginning of instar IV were placed in glass jars, each containing 199 mL of water and 1mL of bruceine A at various lethal concentrations or 1 ppm of the positive control temephos. Negative controls were treated with distilled water. The larvae found dead after 24 h were separated and studied underlight microscopy for the examination of morphological. Larvae were scrutinized after mounting with Hoyer's medium and morphological changes in body segments including the head, setae, cuticle, abdomen, and anals gills were observed, photographed and compared with those of the controls.

Histology test

Histological tests were performed in accordance with the method of Narciso et al.[19] with slight modifications. Larvae treated with different concentrations of bruceine A, 1 ppm of temephos, or distilled water were fixed in 2.5% glutaraldehyde in sodium cacodylate buffer (0.1 M, pH 7.4) for 4 h. Samples were then dehydrated in a gradient ethanol series (70%, 80%, 90% 96%, and 100%). Samples were immersed in each ethanol solution for 15 min. Samples were embedded in Historesin JB4 and the resulting blocks were sliced using a microtome to obtain a series of 3µm thick sections. These sections were stained with hematoxylin–eosin, and then examined and photographed using a light microscope. Morphological and histological changes in larvae were analyzed descriptively.

RESULTS AND DISCUSSION

Isolation of bruceine A from *B. javanica* L. Merr

Based on the extraction and isolation method by Mangungsong [17] as much as 150 mg of isolate compounds of bruceine A was obtained from each of 5 kg of Makasar Fruit (*B. javanica* L. Merr). The purity levels of the amorphous powder was measured using two-dimensional chromatography with stationary phase silica gel 60 F254 on TLC plate and mobile phase of mixed solvent of chloroform and ethyl acetate with ratio of 1: 2 to produce a single purple spot seen in UV 366nm with Rf of 0.88. The results of this research is in line with the results from Mangungsong [17] which suggested that there was a single purple spot on bruceine A isolate under UV ray 366nm observation. The purple spot indicated that bruceine A isolate is single/pure apart from other chemical components [20]. Rf value of bruceine A isolate of 0.8 is still considered as ideal average value that is between 0.2-0.8. Rf value is the distance traveled by compound divided by distance traveled by eluen. Higher Rf value showed that isolate / chemical compound has low polarity and otherwise [21]. The result of calculation based on area under the graph of the High Performance Liquid Chromatography (HPLC) of bruceine A isolates showed single dominant peak with area width percentage of 92.796% and retention time (Rt) of 4.633 minutes. Although bruceine A compound has not reached 99-100%, bruceine A isolate compound inside the isolate is shown with single dominant peak on the produced chromatograph. The result of this research is not very different from Mangungsong [17], which showed that the pureness of bruceine A isolate of 94.88% with retention time (Rt) of 4.83 minutes.

Morphological changes of *A. aegypti* (L.) larvae

Observation on morphological changes on *A. aegypti* (L.) larvae is meant to decide the damaged the target body part after treatment with bruceine A at various concentrations compared to treatment with control. An overview of the morphological changes is presented in Figs. 1–2.

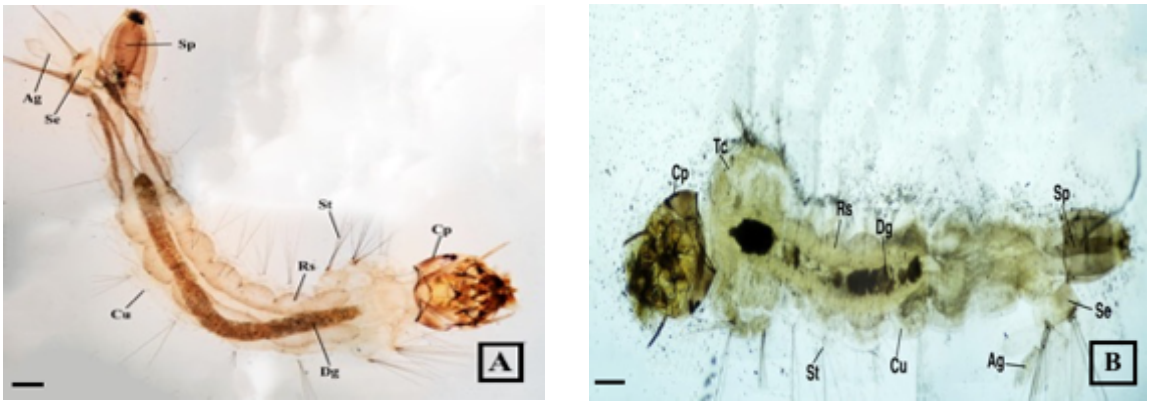

Fig.1: Microscopy images of control and temephos-treated *A. aegypti* (L.) larvae (A). Control larva (untreated), 40x. The heads, thoraxes, and abdomens of larvae are still complete. **(B). Temephos-treated (1 ppm) larva, 100x.** Respiratory and digestive tracts are severely damaged. Cuticle and setae are damaged/detached. **Cp: caput, Dg: digestive tract, Rs: respiratory tract, St: setae, Cu: cuticle, Sp: siphon, Se: saddle, Ag: anal gills.**

Bodies of control larvae did not show any damages (Fig. 1A). Larvae treated with 1 ppm temephos exhibited damaged cuticles and digestive tracts with some dark spots, narrowed breathing tubes, and some detached/damaged setae feathers (Fig. 1B). By contrast, larvae treated with lowest concentrations of bruceine A (1 ppm) exhibited morphological damage to head, which appeared dark, and some parts of the cuticle layer, as well as narrowed breathing tubes (Fig. 2A). At the higher concentration of bruceine A (2 ppm) damaged or caused the detachment of anal papillae / anal gills, as well as decreased body size and caused discoloration (Fig. 2B).

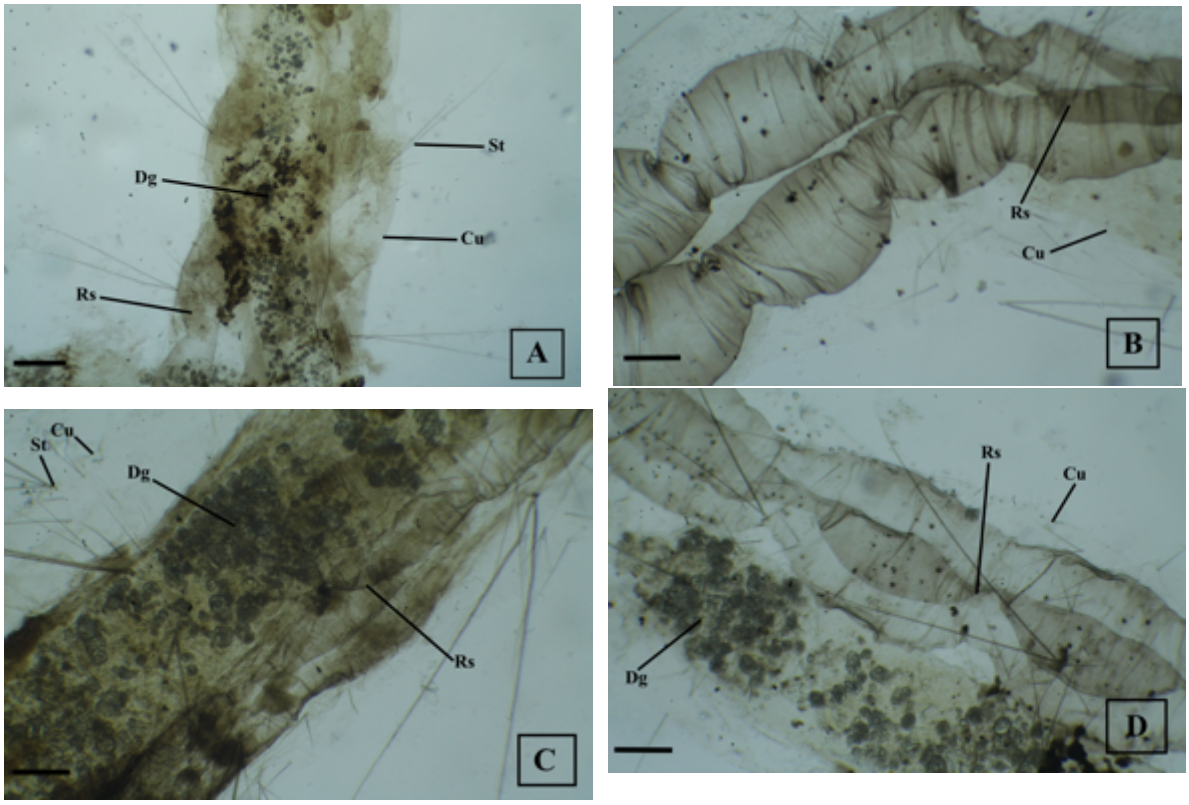

Fig. 2: Microscopy images of *A. aegypti* (L.) larvae treated with (A). 1 ppm (B). 2 ppm, (C). 4 ppm, and (D). 8 ppm bruceine A, 100x. Rs: respiratory tract, Cu: cuticle, Dg: digestive tract, St: setae. Larvae of *A. aegypti* (L.) treated with various concentrations of bruceine A exhibited damaged digestive and respiratory tracts, numerous loose setae and cuticle, and damaged siphons.

These results are consistent with previous studies, proved that larvae of *A. aegypti* (L.) treated with bruceine A at sub lethal concentration (0.2 ppm) causes damage to the digestive with the exitence of of black spots, folded respiratory tubes, setae and cuticles are detached [16]. The research of Warikoo and Kumar [22], who reported that treatment with excess *Argemone mexicana* damaged the anal papillae of *A. aegypti* larvae. Sharma et al. [18] showed that treatment with extracts of the stems and leaves of *Achyranthes aspera* caused structural damage to the anal papillae of larvae of *A. aegypti* in the early fourth instar. In the present study, microscopy observations showed that the internal membranes of anal papillae shrank, whereas external membranes remained normal. The larvae of *C. quinquefasciatus* treated with ethanol extracts of *Kaempferia galanga* exhibited damaged anal papillae and cuticle shrinkage, as reported by Insun et al. [23]. According to Chaithong et al.[24] the structural deformity of the anal papillae may result from osmotic and ionic dysregulation. Thus, osmotic and ionic dysregulation are possible

causes of death of the larvae of *A. aegypti* (L.).

Observation of morphology of *A. aegypti* (L.) larvae after the treatment with 4 ppm of bruceine A showed swollen digestive tracts, narrowed and folded respiratory tubes, damaged cuticle, and detached setae feathers (Fig. 2C). Larvae treated with the highest concentration of bruceine A (8 ppm) exhibited darkened heads with black spots, swollen or lysed digestive tracts with some blackened areas, small and highly folded respiratory tubes, enlarged siphons, and damaged cuticle and setae feathers (Fig. 2D). The higher the concentration of bruceine A, the morphological damage of *A. aegypti* (L.) larvae was getting worse and widespread to cause damage to the digestive tract and cuticle. In addition, respiratory tubes, siphon and anal gills were having more severe damage. These results are similar to those observed by Sharma et al. [18], who reported that the larvae of *A. aegypti* exhibited distorted midguts, pigmentation loss, and partial or total cell damage after treatment with extracts from the stems and leaves of *A. aspera*. Digestive tract damage was more visible in larvae treated with the hexane extract of *A. aspera* leaves than those treated with extracts from *A. aspera* stems. Light/electron microscopy observations at 6, 12, 24, and 48 h after *A. aspera* treatment showed that midgut epithelial damage intensified over time. Chaithong et al.[24] reported that pepper extract had similar effects on the midguts of *A. aegypti* larvae.

Based on the results of this study, it proves that toxic substances in bruceine A cause morphological damage in the body of *A. aegypti* (L.) larvae. Bruceine A acts as a contact poison to the gastrointestinal and respiratory systems and likely enters the larval body through the pores of the skin / cuticle, digestive tract, and siphon. Bruceine A is a nonpolar compound that is soluble in the lipids of the insect cuticle. Its solubility in lipids accelerates its rate of penetration into the insect hemocoel (body cavity). The rate of penetration of bruceine A through the cuticle depends on cuticle structure and thickness [25]. Toxic substances generally tend to enter through larval body parts that are thinly coated with cuticle; examples of such body parts include intersegmental membranes, membrane joints, and chemoreceptors on the tarsus [26]. Bruceine A is absorbed by the body wall of insects and taken by body fluids to the active target area. It causes the dysfunction of the digestive, respiratory, and nervous systems of larvae [27]. Toxic substances enter the skin membrane of larvae through simple diffusion [28]. These compounds then damage skin cells, causing the skin membrane to lose its impermeability and thus allowing other free toxic compounds to enter the larval body. Toxic compounds also damage proteins in the skin membrane, thus disturbing the function of the skin as the

protector of the body [29]. In addition to diffusion through the skin, toxic substances enter through the digestive tract [30]. The digestive tract of the mosquito larva consists of the anterior, mid, and posterior parts [31]. Food digestion and nutrient absorption occurs in the midgut [29]. The insect midgut is covered with epithelial tissue. Toxic substances enter through the mouth of the larva and continue to the midgut while lysing epithelial cells. Cell lysis decreases the surface tension of mucous membranes, ultimately inhibiting digestion and nutrient absorption [26,31]. Toxic substances may also enter the larval body through the respiratory tract. Air enters through a siphon attached to the water surface. Toxic substances cover the surface of the water medium and thus prevent the siphon from obtaining oxygen. Wulandari et al.[32] stated that secondary metabolites can interfere with oxygen collection. Given that the neural networks of larvae are highly sensitive tooxygen balance, neural atrophy and siphon damage may hinder breathing and eventually cause larvae to die.

Meanwhile, *A. aegypti* (L.) larvae treated with temephos 1 ppm caused damage on the entire body (Fig.1B). The body size of the larvae shrinks compared to its body size after treatment with bruceine A and control (untreated). The result of this research is not very different from Yulidar and Hadifah [33] which showed the morphological damages of *A.aegypti* larvae on the head, thorax, abdomen, and detached setae feathers, and shrinking body size after treatment with temephos at lethal concentration. This is thought to happen because the diferences in water content inside larvae's body and the environment so the water from the body is released through abdominal sockets and moved out to the environment. According to Badvaev [34], the water movement from larvae's body to the environment is caused by high temephos inside the media causing the osmotic pressure of the environment is higher. The higher temephos concentration on water media, causing water content in the body of larvae getting higher and the differences in osmotic pressure happens. The balance of osmosis chemical solution can happen by diffusion [35]. On the dead larvae, there is water movement from higher water molecules in the environment to the inside of *A. aegypti* (L.) larvae that has lower osmotic pressure [36]. Allegedly, this is what causes the outer layer of abdomen is seen shrinking bacause the water from inside of larvae's body is leaked outside.

Temephos likely enters the bodies of larvae through cuticle contact, inhalation, and or ingestion [37]. Temephos contains phosphorothionate, a lipophilic group. Thus, it easily penetrates the hydrophilic epicuticular parts of *A. aegypti* (L.) larvae and causes the cuticle and setae feathers to detach from the bodies of larvae [38]. After penetrating the

cuticle / skin, temephos then enters nerve cells in the gastrointestinal and respiratory tracts of larvae. Temephos poisoning is characterized by restlessness, hyperexcitability, tremors, convulsions, and eventually muscle paralysis [38]. In addition to the cuticle, temephos enters the larval body through the respiratory tract, thus causing the breathing tube to shrink. Temephos also enters the larval body when consumed with food in breeding media [37].

Histological changes of *A. aegypti* (L.) larvae

Histomorphological analysis was conducted to gain further insight on the targets of action of bruceine A in the larvae of *A. aegypti* (L.). Figs. 3–4 show the differences between the histology of control larvae and temephos with that of larvae treated with lethal concentrations of bruceine A.

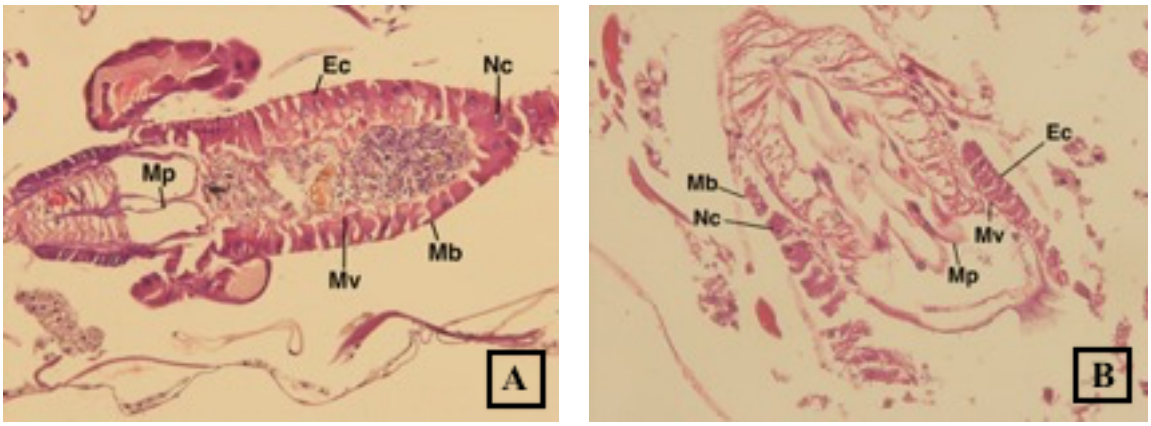

Fig.3: Longitudinal sections of gastrointestinal tracts from from *A. aegypti* (L.) larvae, (A). Control larvae, 400x. Gastrointestinal epithelial cells are normal with compactly stained cytoplasm; **(B). Temephos-treated larva (1 ppm), 400x.** Gastrointestinal epithelial cells are necrotic and have detached from the basement membrane. **Mb: basement membrane, Ec: epithelial cells, Mv: microvilli, Mp: periotropic membrane, Nc: nucleus.**

Gastrointestinal epithelial cells from the control *A. aegypti* (L.) larvae were normal with compactly stained cytoplasms, spherical nuclei, clearly defined chromatin, and visible periotropic membranes. Moreover, the majority of microvilli appeared normal. Epithelial cells remained attached to the basement membrane (Fig. 3A). Larvae treated with 1 ppm temephos exhibited necrotic, shrunken, diffuse gastrointestinal epithelial cells with karyopyknotic nuclei. Necrotic epithelial cells remained attached to the basement membrane. Necrotic microvilli and periotropic membranes appeared diffuse, and

epithelial cells appeared disorganized (Fig. 3B).

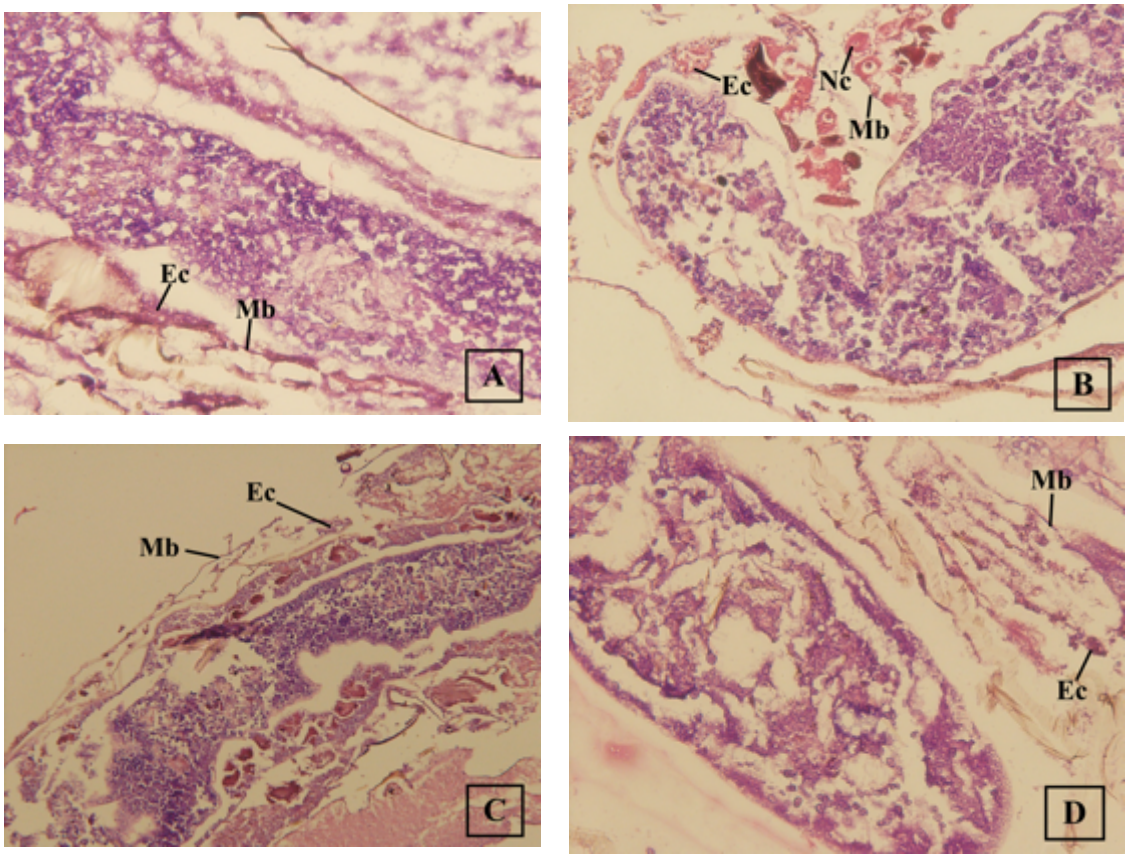

Fig.4: Longitudinal sections of gastrointestinal tracts from *A. aegypti* (L.) larvae treated with: (A). 1 ppm bruceine A; (B). 2 ppm bruceine A; (C). 4 ppm bruceine A; (D). 8 ppm bruceine A, 400x. *A. aegypti* (L.) larvae treated with various concentrations of bruceine A exhibited diffuse necrotic epithelial cells (lysis). Periotropic membranes are absent or are detached from the basement membrane. **Mb: basement membrane, Ec: epithelial cells, Mv: microvilli, Mp: periotropic membrane.**

A. aegypti (L.) larvae treated with the low concentration of 1 ppm bruceine A exhibited necrotic gastrointestinal epithelial cells that remained attached to the basement membrane. Microvilli and periotropic membranes became diffuse and necrotic, and epithelial cells became structurally disorganized (Fig. 4A). The same comprehensive histological changes were also observed in *A. aegypti* (L.) larvae treated with bruceine A at concentrations of 2 and 4 ppm (Fig. 4B–4C). Damage intensified with increasing bruceine A concentration. Larvae treated with 8 ppm bruceine A exhibited completely diffuse necrotic gastrointestinal epithelial cells, which completely detached from the

basement membrane and localized in the lumen. These results are consistent with that reported by Sharma et al. [18], who stated that midgut epithelial cells exhibited intense damage at 6, 12, 24, and 48 h after treatment with *A. aspera* extract. Highly typical changes include the vacuolization of midgut columnar cells, damage of microvilli, release of epithelial cell content into the midgut lumen, and eventual cell death. Sutiningsih et al. [16] reports that there was necrosis on gastrointestinal epithel cells indiacted by shrunken cells and diminished core (karyolysis) on *A. aegypti* (L.) larvae after treatment with bruceine A at sub lethal dosage (0.2 ppm). The results from Patil et al. [39] showing that there were extruded periotic membrane on posterior peak between anal papilla on the dead *A. aegypti* (L.) larvae after treated with *Clerodendron inerme* extract at lethal concentration. The extruded periotic membrane indicated that *Clerodendron inerme* extract affected the intestinal area, that can cause substancial effect on nutrition absorbing and inhibition of larvae's development process. Peritropic membrane is a sheath containing acellular chitin that separates the content of intestines from secretory epithel / intestinal absorption, it also acts as barrier for pathogens which protect the are of midgut [40–42].

Narciso et al.[19] reported that histomorphological changes resulting from treatment with burchelin from *Ocotea cymbarium* caused the death of L3–L4 larvae of *A. aegypti*. The midgut epithelial cells of the larvae exhibited disorganization, damage, and vacuolization. The histological analysis of larvae of *Culex nigripalpus* infected by *Bacillus thuringiensis* Medelin (Cry 11Bb) [42] revealed similar damages as that observed in the intestinal cells of *Aedes albopictus* infected by *B. thuringiensis* var Israelensis (Bti) [43]. Infection is characterized by the presence of rounded mesenteric cells with granular cytoplasm, absent or clear nucleus, and cytoplasmic vacuolization. The mesentery actively participates in secretion and absorption. The disintegration of mesentery cells occurs through the accumulation of granular material in the apical part followed by the release of material into the gut lumen. Mosquito larvae treated with the tested substance exhibited gastric vacuolization, cellular disorganization within intersegmental cells, and clear or absent nucleus. These comprehensive changes are not limited to chemical damage; infection with *Baculovirus* resulted in the same changes to the gastric and Malpighian tubules of *C. nigripalpus* Theobald larvae [42].

Al-Mehmadi & Al-Khalaf [44] stated that histopathological changes are qualitatively different in terms of localization and quantitatively correspond to the duration or length of observation time. Histopathological effects on the midgut and gastric caeca confirmed

that these areas are in direct contact with toxic substances. *C. quinquefasciatus* larvae treated with *Melia azedarach* extract exhibited serious damage and necrotic columnar epithelial cells of the gastric caeca. At 24 h after treatment, the epithelial cells of the gastric caeca exploded or shrunk and underwent lysis. Changes were also observed in the anterior and posterior of the midgut, and epithelial cells detached from the basal membrane with periotrophic membrane damage and the cell wall rupture [44]. The mixing of intestinal contents with hemolymph causes larvae to die. Assar and El-Sobky [45] also observed that aqueous hyacinth extracts can severely damage the larval midgut. Damage after 48 and 72 h of observation is characterized by vacuolization and shrinkage of epithelial cells.

Bruceine A is a toxic substance may enter the digestive tract through the skin or buccal membrane. This toxic substance first causes midgut epithelial cells to undergo lysis or necrosis. Cell death or lysis, in turn, decreases the surface tension of the mucous membranes of the midgut to inhibit the digestion and absorption of food, ultimately resulting in larval death [29]. The results of this study prove that bruceine A is a potential natural larvicide that can be used to control the population of *A. aegypti* (L.) larvae as disease vectors. Its targets of action for morphological damage include the head, cuticle, setae, siphons, and gastrointestinal and respiratory tracts, whereas those of histological damage are the midgut or gastrointestinal epithelial cells. It is necessary to conduct further research on larvicidal action target of bruceine A on different species of mosquitoes as well as microscopic examination on body parts of larvae in details using transmission electron microscope.

CONCLUSION

Larvicidal action targets of bruceine A are as follows: (a) Morphologically damage the head, cuticles, setae, digestive and respiratory tracts and siphon, (b) Histologically damage by causing necrosis on gastrointestinal epithelial cells, periotropic membrane, microvilli and disorganized epithel cells, detached from basalis membrane.

ACKNOWLEDGMENTS

The authors of this research would like to thank the Directorate for Research and Community Service, Ministry of Research, Technology and Higher Education of the Republic of Indonesia, which has funded this research on Doctoral Dissertation Research year 2017.

CONFLICTS OF INTERESTS

The authors have no conflict of interest or financial interest in regard to the result of the research.

AUTHORS CONTRIBUTION

Dwi Sutiningsih: Conceived and designed the experiments, review literature and write the manuscript.

Mustofa: Performed the experiments and contributed to result analysis and the writing manuscript.

Tri Baskoro Tunggul Satoto: Perform morphological and histological analysis.

Edhi Martono: Designed the research plan and contributed to the writing manuscript.

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MORPHOLOGICAL AND HISTOLOGICAL EFFECTS OF BRUCEINE A ON THE LARVAE OF *Aedes aegypti* Linnaeus (DIPTERA: CULICIDAE)

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ABSTRACT

Objective: This study aimed to determine a target of action of bruceine A on the basis of its morphological and histological effects on the larvae of *Aedes aegypti* Linnaeus.

Methods: Bruceine A was isolated from *Brucea javanica* (L.) Merr seeds in accordance with the Mangungsong method. Larvae of *A. aegypti* (L.) in instar III to the beginning of instar IV were treated with various concentrations of bruceine A. The negative control group did not receive any treatment, whereas the positive control group received 1 ppm temephos. Dead larvae were collected after 24 hours of treatment for the examination of morphological and histological changes.

Results: The negative control group did not exhibit any morphological and histological changes. Larvae treated with bruceine A, however, had visible damaged heads, cuticles, digestive and respiration tracts, respiratory siphons, and setae, and they were smaller than normal larvae. Larvae treated with temephos exhibited gastrointestinal damage, narrowed breathing tubes, cuticle damage, and deta d/damaged setae feathers. The necrosis of gastrointestinal epithelial cells was the major histological change exhibited by larvae treated with various concentrations of bruceine A or 1 ppm temephos.

Conclusion: The targets of action of bruceine A in *A. aegypti* (L.) larvae are the head/caput, cuticle, setae, siphon, and gastrointestinal and respiratory tracts.

Key words: Bruceine A, *Brucea javanica* (L.) Merr, action target, morphology, histology, *Aedes aegypti* Linnaeus

INTRODUCTION

Vector control is a method to suppress the incidence of vector-borne diseases. It is widely conducted as a public health intervention. *Aedes aegypti* Linnaeus is a mosquito species that is proved to be an important disease vector in tropical and subtropical regions [1]. *A. aegypti* (L.) is a vector of dengue fever, dengue hemorrhagic fever [2], chikungunya fever, yellow fever, and Zika viral disease [3]. The wide use of synthetic organic insecticides for vector control harms the environment and causes the emergence of insecticide-resistant vectors, as well as the deaths of non-target animals. Earlier intervention studies have shown that although the use of synthetic insecticides such as temephos, especially in risky or potential places can decrease disease transmission by mosquitoes, prolonged exposure to these chemicals will promote the adaptation, evolution, and selection of mosquitoes [4]. Thus, plant-derived insecticides/larvicides should be developed as another option for controlling vector-borne diseases. The two essential oils of *Thymus vulgaris* and *Origanum majorana* (Lamiaceae) demonstrate an interesting larvicidal activity. The *O. majorana* essential oil is more effective compared to the essential oil of *T. vulgaris* with an LC_{50} of 107.13 µg/mL and LC_{90} of 365.90 µg/mL on the malaria vector *Anopheles labranchiae* [5]. The crude ethanolic extract of *Smilax larvata* (Sarsaparilla) is a potential source of an eco-friendly larvicide against *Aedes aegypti* larvae with LC_{50} 225 $\mu g/mL^{-1}$ and LC_{90} 350 $\mu g/mL^{-1}$ [6]. Compounds from *Brucea javanica* (L.) Merr has potential applications as agricultural insecticides. Zhang et al. [7] proved that brusatol isolated from *B. javanica* (L.) Merr has insecticidal and antifeeding effects against the third-instar larvae of *Spodoptera exigua*. Brusatol can also induce apoptosis in the insect cell lines IOZCAS-Spec-II and Sf21. In these cell lines, apoptosis is characterized by DNA fragmentation, caspase-3 activation, and cytochrome-c release from mitochondria. Sutiningsih & Nurjazuli [8] proved that brusatol isolated from the seeds of *B. javanica* (L) Merr has larvicidal activity against *A. aegypti* at the Lethal Concentrations of 50 and 90 (LC_{50}, LC_{90}) of 0.669 and 8.331 ppm, respectively.

Bruceine A ([15]-3-methyl-2-butanoil-bruseolid) is a quassinoid derived from *B. javanica* (L.) Merr [9]. Its molecular formula of $C_{26}H_{34}O_{11}$, and has mass of 522.54 g/mol. Physically, it is an amorphous powder with a bitter taste. Bruceine A has extensive broad biological activity as an antibabesiosis, antitrypanosomal, and anti-malarial as well as

cytotoxic properties against cancer cell lines [10–12]. It also has insecticidal, antifeeding, and growth-inhibiting activities against tobacco budworm (*Heliothis virescens* F.), *Spodoptera frugiperda* armyworm [13] and Mexican bean beetle larvae in the fourth instar (*Epilachna varivestis* Mulsant) [14]. Bruceine A can also act as a neurotoxin [15] and inhibitor of growth [16] against the larvae of *A. aegypti* (L.) The biolarvicidal mechanism of action of bruceine A occurs through the inhibition of acetylcholinesterase and VGSC gene. The behavioral responses of larvae treated with bruceine A include hyperexcitation, convulsions, paralysis, and aggressive biting of the anal gills; these behaviors indicate that bruceine A affects the larval neuromuscular systems [15]. Therefore, this study aimed to determine the targets of action of bruceine A and to identify its effects on the morphology and histology of *A. aegypti* (L.) larvae.

MATERIALS AND METHODS

Materials

Makassar Fruit (*B. javanica* L. Merr) was purchased from a wholesaler of medicinal plants (Aneka Herbal Yogyakarta, Indonesia). Confirming its identity as well as obtaining its relevant scientific information, the specimen was further identified at the Laboratory of Pharmaceutical Biology, Faculty of Pharmacy, Gadjah Mada University, Yogyakarta, Indonesia. *A. aegypti* (L.) larvae in instar III to the beginning of instar IV were obtained from colonies maintained at the Laboratory of Parasitology, Faculty of Medicine, University of Gadjah Mada, Yogyakarta. All commercial reagents and other chemicals used in this study purchased from commercial suppliers and were of analytical quality with the highest purity available. The selection of temephos dosage (1 ppm) was based on lethal damage consideration used in the field.

Extraction and isolation of bruceine A

Bruceine A was isolated from *B. javanica* (L.) Merr seeds in accordance with the method described by Mangungsong [17]. Dried seeds of *B. javanica* (L.) Merr (5 kg) were ground into powder and shaken with a hexane solution (15 L). The solution was then filtered and extracted with methanol (15 L). Methanol was evaporated to obtain a thick extract, which was then mixed with an equal volume of distilled water to form a suspension. Then, the suspension was partitioned with hexane (3 L). After, the hexane fraction was separated from the suspension, and methanol-water fractions were collected for repeated extraction with dichloromethane (1 L). Later, the organic layer was collected and evaporated to obtain a concentrate, which was then diluted with methanol (100–250 mL) at 60 °C and

stored at room temperature. The methanol solution was maintained at room temperature to allow the crystallization of bruceine A. Further separation was conducted through filtration. The remainder of the filtrate/residue was separated through Thin Layer Chromatography (TLC) and evaporated. Finally, purity levels of the amorphous powder were measured using High-Performance Liquid Chromatography (HPLC).

Morphological test

Morphological tests were conducted in accordance with the method reported by Sharma et al. [18] with slight modifications. *A. aegypti* (L.) larvae in instar III to the beginning of instar IV were placed in glass jars, each containing 199 mL of water and 1 mL of bruceine A at various lethal concentrations or 1 ppm of the positive control temephos. Negative controls were treated with distilled water. The larvae found dead after 24 h were separated and studied under light microscopy to examine its morphology. Larvae were scrutinized after mounting with Hoyer's medium and morphological changes in body segments including the head, setae, cuticle, abdomen, and anal gills. They were observed, photographed and compared with those of the controls.

Histological test

Histological tests were performed in accordance with the method of Narciso et al.[19] with slight modifications. Larvae treated with different concentrations of bruceine A, 1 ppm of temephos, or distilled water were fixed in 2.5% glutaraldehyde in sodium cacodylate buffer (0.1 M, pH 7.4) for 4 h. Samples were then dehydrated in a gradient ethanol series (70%, 80%, 90% 96%, and 100%). Samples were immersed in each ethanol solution for 15 min. Samples were embedded in Historesin JB4 and the resulting blocks were sliced using a microtome to obtain a series of 3 μm thick sections. These sections were stained with hematoxylin-eosin and then examined and photographed using a light microscope. Morphological and histological changes in larvae were analyzed descriptively.

RESULTS AND DISCUSSION

Isolation of bruceine A from *B. javanica* L. Merr

Based on the extraction and isolation method by Mangungsong [17], as much as 150 mg of isolate compounds of bruceine A was obtained from each of 5 kg of Makassar Fruit (*B. javanica* L. Merr). The purity levels of the amorphous powder were measured using two-dimensional chromatography with stationary phase silica gel 60 F254 on TLC plate

and mobile phase of mixed solvent of chloroform and ethyl acetate with ratio of 1: 2 to produce a single purple spot seen in UV 366 nm with retardation factor (Rf) of 0.88. The results of this research are in line with the results from Mangungsong [17] which suggested that there was a single purple spot on bruceine A isolate under UV ray 366 nm observation. The purple spot indicated that bruceine A isolate is single/pure apart from other chemical components [20]. Rf value of bruceine A isolate of 0.8 is still considered as an ideal average value that is between 0.2-0.8. Rf value is the distance traveled by compound divided by distance traveled by eluent. Higher Rf value showed that isolate/chemical compound has low polarity and otherwise [21]. The result of a calculation based on area under the graph of the High-Performance Liquid Chromatography (HPLC) of bruceine A isolate showed a single dominant peak with area width percentage of 92.796% and retention time (Rt) of 4.633 minutes. Although bruceine A compound has not reached 99-100%, bruceine A isolate compound inside the isolate is shown with a single dominant peak on the produced chromatograph. The result of this research is not very different from Mangungsong [17] which showed that the pureness of bruceine A isolate was of 94.88% with a retention time (Rt) of 4.83 minutes.

Morphological changes of *A. aegypti* (L.) larvae

Observation on morphological changes in *A. aegypti* (L.) larvae was meant to decide damaged target body part after the treatment with bruceine A at various concentrations comparing the treatment with a control larvae. An overview of the morphological changes is presented in Figs. 1–2.

Bodies of control larvae did not show any damages (Fig. 1A). Those larvae treated with 1 ppm temephos exhibited damaged cuticles and digestive tracts with some dark spots narrowed breathing tubes, and some detached/damaged setae feathers (Fig. 1B). By contrast, larvae treated with lowest concentrations of bruceine A (1 ppm) exhibited morphological damage to the head, which appeared dark, and some parts of the cuticle layer, as well as narrowed breathing tubes (Fig. 2A). At the higher concentration, bruceine A (2 ppm) damaged or caused the detachment of anal papillae/anal gills, as well as decreased body size and caused discoloration (Fig. 2B).

These results are consistent with previous studies confirming that larvae of *A. aegypti* (L.) treated with bruceine A at sub-lethal concentration (0.2 ppm) causes damage to their digestive with the existence of black spots, folded respiratory tubes, and detached setae and cuticles [16]. The research of Warikoo and Kumar [22], who reported that treatment

with excess *Argemone mexicana* damaged the anal papillae of *A. aegypti* larvae. Sharma et al. [18] showed that treatment with extracts of the stems and leaves of *Achyranthes aspera* caused structural damage to the anal papillae of larvae of *A. aegypti* in the early fourth instar. In the present study, microscopic observations showed that the internal membranes of anal papillae were shrank, whereas external membranes were remained normal. As reported by Insun et al. [23], the larvae of *Culex quinquefasciatus* treated with ethanol extracts of *Kaempferia galanga* exhibited anal papillae damage and cuticle shrinkage. According to Chaithong et al.[24], the structural deformity of the anal papillae may result from osmotic and ionic dysregulation. Thus, osmotic and ionic dysregulation are possible causes of death of the larvae of *A. aegypti* (L.).

Observation of morphology of *A. aegypti* (L.) larvae after the treatment with 4 ppm of bruceine A showed swollen digestive tracts, narrowed and folded respiratory tubes, damaged cuticle, and detached setae feathers (Fig. 2C). Larvae treated with the highest concentration of bruceine A (8 ppm) exhibited darkened heads with black spots, swollen or lysed digestive tracts with some blackened areas, small and highly folded respiratory tubes, enlarged siphons, and damaged cuticle and setae feathers (Fig. 2D). The higher the concentration of bruceine A, the worse and more widespread of *A. aegypti* (L.) larvae morphological damages are to cause damage to the digestive tract and cuticle. In addition, respiratory tubes, siphon and anal gills were having more severe damage. These results are similar to those observed by Sharma et al. [18], who reported that the larvae of *A. aegypti* exhibited distorted midguts, pigmentation loss, and partial or total cell damage after treatment with extracts from the stems and leaves of *A. aspera*. Digestive tract damage was more visible in larvae treated with the hexane extract of *A. aspera* leaves than those treated with extracts from *A. aspera* stems. Light/electron microscopic observations at 6, 12, 24, and 48 h after *A. aspera* treatment showed that midgut epithelial damage intensified over time. Chaithong et al.[24] reported that pepper extract had similar effects on the midguts of *A. aegypti* larvae.

Based on the results of this study, it proves that toxic substances in bruceine A cause morphological damage in the body of *A. aegypti* (L.) larvae. Bruceine A acts as a contact poison to the gastrointestinal and respiratory systems and likely enters the larval body through the pores of the skin/cuticle, digestive tract, and siphon. Bruceine A is a nonpolar compound that is soluble in the lipids of the insect cuticle. Being soluble in lipids accelerates its rate of penetration into the insect hemocoel (body cavity). The penetration rate of bruceine A through the cuticle depends on cuticle structure and thickness [25].

Toxic substances generally tend to penetrate through larval body parts that are thinly coated with cuticle; examples of such body parts include intersegmental membranes, membrane joints, and chemoreceptors on the tarsus [26]. Bruceine A is absorbed by the body wall of insects and taken by body fluids to the active target area. It causes the dysfunction of the digestive, respiratory, and nervous systems of larvae [27]. Toxic substances enter the skin membrane of larva through simple diffusion [28]. These compounds then damage skin cells, causing the skin membrane to lose its impermeability and thus allowing other free toxic compounds to penetrate into the larval body. Toxic compounds also damage proteins in the skin membrane, thus disturbing the function of the skin as the protector of the body [29]. In addition to diffusion through the skin, toxic substances enter through the digestive tract [30]. The digestive tract of the mosquito larva consists of the anterior, mid, and posterior parts [31]. Food digestion and nutrient absorption occur in the midgut [29]. The insect midgut is covered with epithelial tissue. Toxic substances enter through the mouth of the larva and continue to the midgut while lysing epithelial cells. Cell lysis decreases the surface tension of mucous membranes ultimately inhibiting digestion and nutrient absorption [26,31]. Toxic substances may also penetrate the larval body through respiratory tracts. Air enters through a siphon attached to the water surface. Thus, toxic substances covering the surface of the water medium prevent the siphon from obtaining oxygen. Wulandari et al.[32] stated that secondary metabolites can interfere with oxygen collection. Given that the neural networks of larvae are highly sensitive to oxygen balance, neural atrophy and siphon damage may hinder breathing and eventually cause larvae to die.

Meanwhile, *A. aegypti* (L.) larvae treated with temephos 1 ppm caused damage on the entire body (Fig.1B). The body size of the larvae shrinks compared to its body size after treatment with bruceine A and control (untreated). The result of this research is not very different from Yulidar and Hadifah [33] which showed the morphological damages of *A.aegypti* larvae on the head, thorax, abdomen, and detached setae feathers, and shrinking body size after treatment with temephos at lethal concentration. This is thought to happen because the differences in water content inside larvae's body and the environment so the water from the body is released through abdominal sockets and moved out to the environment. According to Badvaev [34], the water movement from larvae's body to the environment is caused by high temephos inside the media leading to the osmotic pressure of the environment is higher. The higher temephos concentration on water media bring about water content in the body of larvae getting higher and the differences in osmotic pressure happen. The balance of osmosis chemical solution can transpires through

diffusion [35]. On the dead larvae, there is water movement from higher water molecules in the environment to the inner part of *A. aegypti* (L.) larvae that has lower osmotic pressure [36]. Allegedly, this is what causes the outer layer of the abdomen is seen shrinking because the water from inside of larvae's body is leaked outside.

Temephos likely gets into the bodies of larvae through cuticle contact, inhalation, and or ingestion [37]. Temephos contains phosphorothioate, a lipophilic group. Thus, it easily penetrates the hydrophilic epicuticular parts of *A. aegypti* (L.) larvae and causes the cuticle and setae feathers to detach from the bodies of larvae [38]. After penetrating the cuticle/skin, temephos then enters nerve cells in the gastrointestinal and respiratory tracts of larvae. Temephos poisoning is characterized by restlessness, hyperexcitability, tremors, convulsions, and eventually muscle paralysis [38]. In addition to the cuticle, temephos enters the larval body through the respiratory tract, thus causing the breathing tube to shrink. Temephos also enters the larval body when consumed with food in breeding media [37].

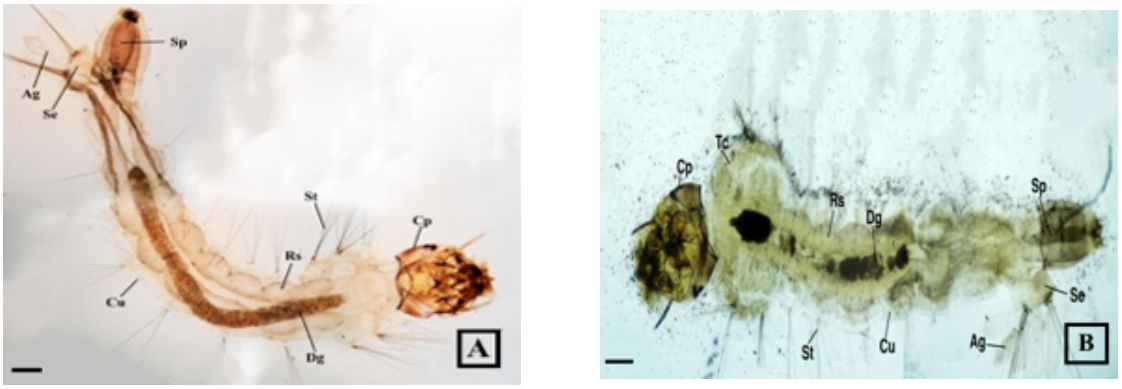

Fig.1: Microscopic images of control and temephos-treated *A. aegypti* (L.) larvae. (a) control larva (untreated), 40x. The heads, thoraxes, and abdomens of larvae are still complete, (b) temephos-treated (1 ppm) larva, 100x. Respiratory and digestive tracts are severely damaged, cuticle and setae are damaged/detached. Cp: caput, Dg: digestive tract, Rs: respiratory tract, St: setae, Cu: cuticle, Sp: siphon, Se: saddle, Ag: anal gills

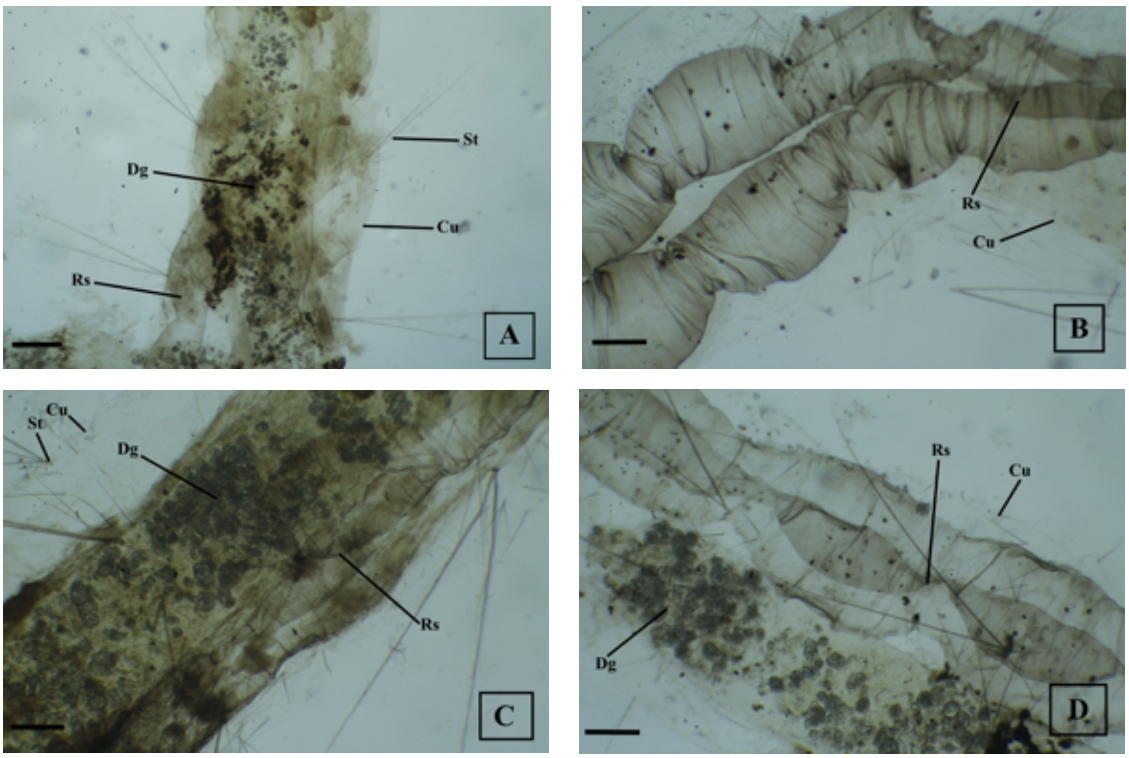

Fig. 2: Microscopic images of *A. aegypti* (L.) larvae treated with (a) 1 ppm, (b) 2 ppm, (c) 4 ppm, and (d) 8 ppm bruceine A, 100x. Larvae of *A. aegypti* (L.) treated with various concentrations of bruceine A exhibited damaged digestive and respiratory tracts, numerous loose setae and cuticle, and damaged siphons. Rs: respiratory tract, Cu: cuticle, Dg: digestive tract, St: setae

Histological changes of *A. aegypti* (L.) larvae

The histomorphological analysis was conducted to gain further insight into the targets of action of bruceine A in the larvae of *A. aegypti* (L.). Figs. 3-4 show the differences between the histology of control larvae and temephos with that of larvae treated with lethal concentrations of bruceine A.

Gastrointestinal epithelial cells from the control *A. aegypti* (L.) larvae were normal with compactly stained cytoplasm, spherical nuclei, clearly defined chromatin, and visible peritrophic membranes. Moreover, the majority of microvilli appeared normal where epithelial cells remained attached to the basement membrane (Fig. 3A). Larvae treated with 1 ppm temephos exhibited necrotic, shrunken, and diffuse gastrointestinal epithelial cells with karyopyknotic nuclei. Necrotic epithelial cells remained attached to the

basement membrane. Necrotic microvilli and peritrophic membranes appeared diffuse, and epithelial cells appeared disorganized (Fig. 3B).

A. aegypti (L.) larvae treated with the low concentration of 1 ppm bruceine A exhibited necrotic gastrointestinal epithelial cells that remained attached to the basement membrane. Microvilli and peritrophic membranes became diffuse and necrotic, and epithelial cells became structurally disorganized (Fig. 4A). The same comprehensive histological changes were also observed in *A. aegypti* (L.) larvae treated with bruceine A at concentrations of 2 and 4 ppm (Fig. 4B-C). It showed that damage intensified with increasing bruceine A concentration. Larvae treated with 8 ppm bruceine A exhibited completely diffuse necrotic gastrointestinal epithelial cells which completely detached from the basement membrane and localized in the lumen. These results are consistent with that reported by Sharma et al. [18] who stated that midgut epithelial cells exhibited intense damage at 6, 12, 24, and 48 h after treatment with *A. aspera* extract. Highly typical changes include the vacuolization of midgut columnar cells, damage to microvilli, the release of epithelial cell content into the midgut lumen, and eventual cell death. Sutiningsih et al. [16] report that there was necrosis on gastrointestinal epithelial cells indicated by shrunken cells and diminished core (karyolysis) on *A. aegypti* (L.) larvae after treatment with bruceine A at the sub-lethal dosage (0.2 ppm). The results from Patil et al. [39] showing that there were extruded peritrophic membrane on posterior peak between anal papilla on the dead *A. aegypti* (L.) larvae after treated with *Clerodendron inerme* extract at lethal concentration. The extruded peritrophic membrane indicated that *Clerodendron inerme* extract affected the intestinal area, that can cause a substantial effect on nutrition absorbing and inhibition of larvae's development process. The peritrophic membrane is a sheath containing acellular chitin that separates the content of intestines from secretory epithelial/intestinal absorption that also acts as a barrier for pathogens which protect the area of midgut [40–42].

Narciso et al.[19] reported that histomorphological changes resulted from treatment with burchelin from *Ocotea cymbarium* caused the death of L3–L4 larvae of *A. aegypti*. The midgut epithelial cells of the larvae exhibited disorganization, damage, and vacuolization. The histological analysis of larvae of *Culex nigripalpus* infected by *Bacillus thuringiensis* Medelin (Cry 11Bb) [42] revealed similar damages as that observed in the intestinal cells of *Aedes albopictus* infected by *B. thuringiensis* var Israelensis (Bti) [43]. Infection is characterized by the presence of rounded mesenteric cells with granular cytoplasm, absent or clear nucleus, and cytoplasmic vacuolization. The mesentery actively

participates in secretion and absorption. The disintegration of mesentery cells occurs through the accumulation of granular material in the apical part followed by the release of material into the gut lumen. Mosquito larvae treated with the tested substance exhibited gastric vacuolization, cellular disorganization within intersegmental cells, and clear or absent nucleus. These comprehensive changes are not limited to chemical damage; infection with *Baculovirus* resulted in the same changes to the gastric and Malpighian tubules of *C. nigripalpus* Theobald larvae [42].

Al-Mehmadi & Al-Khalaf [44] stated that histopathological changes are qualitatively different in terms of localization and quantitatively correspond to the duration or length of observation time. Histopathological effects on the midgut and gastric caeca confirmed that these areas are in direct contact with toxic substances. *C. quinquefasciatus* larvae treated with *Melia azedarach* extract exhibited serious damage and necrotic columnar epithelial cells of the gastric caeca. At 24 h after treatment, the epithelial cells of the gastric caeca exploded or shrunk and underwent lysis. Changes were also observed in the anterior and posterior of the midgut which showed epithelial cells detached from the basal membrane with peritrophic membrane damage and cell wall rupture [44]. The mixing of intestinal contents with hemolymph causes larvae to die. Assar and El-Sobky [45] also observed that aqueous hyacinth extracts can severely damage the larval midgut. They reported that damage after 48 and 72 h of observation is characterized by vacuolization and shrinkage of epithelial cells.

Bruceine A is a toxic substance may enter the digestive tract through the skin or buccal membrane. This toxic substance first causes midgut epithelial cells to undergo lysis or necrosis. Cell death or lysis, in turn, decreases the surface tension of the mucous membranes of the midgut to inhibit the digestion and absorption of food, ultimately resulting in larval death [29]. The results of this study prove that bruceine A is a potential natural larvicide that can be used to control the population of *A. aegypti* (L.) larvae as disease vectors. Its targets of action for morphological damage include the head, cuticle, setae, siphons, and gastrointestinal and respiratory tracts, whereas those of histological damage is the midgut or gastrointestinal epithelial cells. It is necessary to conduct further research on larvicidal action target of bruceine A on different species of mosquitoes as well as a detailed microscopic examination on body parts of larvae using transmission electron microscope.

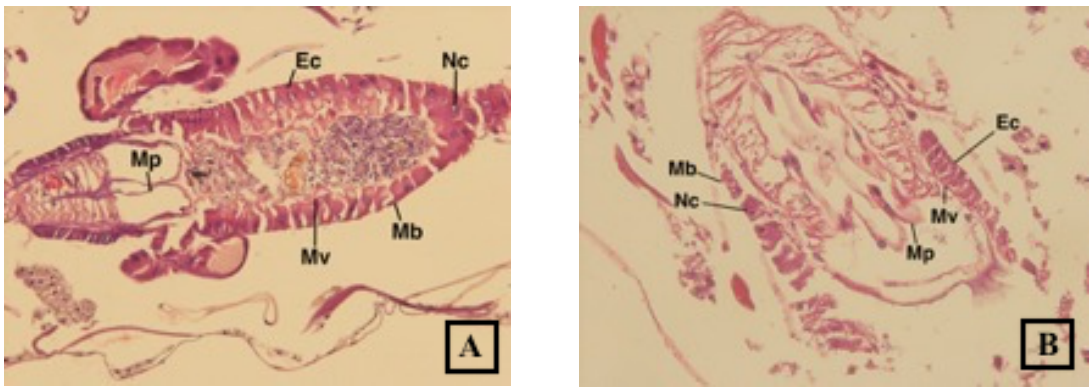

Fig. 3: Longitudinal sections of gastrointestinal tracts from *A. aegypti* (L.) larvae. (a) control larvae, 400x. Gastrointestinal epithelial cells are normal with compactly stained cytoplasm, (b) temephos-treated larva (1 ppm), 400x. Gastrointestinal epithelial cells are necrotic. Mb: basement membrane, Ec: epithelial cells, Mv: microvilli, Mp: peritrophic membrane, Nc: nucleus

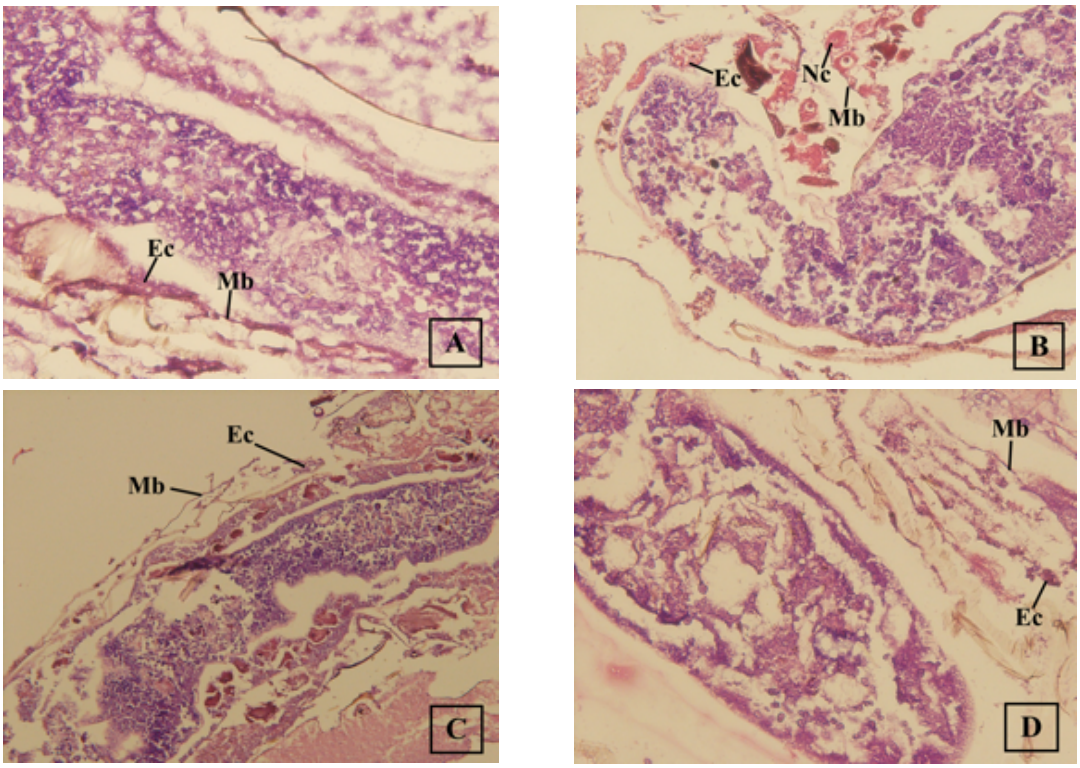

Fig. 4: Longitudinal sections of gastrointestinal tracts from *A. aegypti* (L.) larvae treated with (a) 1 ppm bruceine A, (b) 2 ppm bruceine A, (c) 4 ppm bruceine A, (d) 8 ppm bruceine A, 400x. *A. aegypti* (L.) larvae treated with various concentrations of bruceine A exhibited diffuse necrotic epithelial cells. Mb: basement membrane, Ec: epithelial cells, Mv: microvilli, Mp: peritrophic membrane

CONCLUSION

Larvicidal action targets of bruceine A are as follows: (a) Morphologically damage the head, cuticles, setae, digestive and respiratory tracts and siphon, (b) histologically damage by causing necrosis on gastrointestinal epithelial cells, peritrophic membrane, microvilli and disorganized epithelial cells, detached from basalis membrane.

ACKNOWLEDGMENTS

The authors of this research would like to thank the Directorate for Research and Community Service, Ministry of Research, Technology and Higher Education of the Republic of Indonesia which has funded this research on Doctoral Dissertation Research year 2017.

CONFLICTS OF INTERESTS

The authors have no conflict of interest or financial interest in regard to the result of the research.

AUTHORS CONTRIBUTION

Dwi Sutiningsih: Conceived and designed the experiments, reviewed literatures and wrote the manuscript.

Mustofa: Performed the experiments and contributed to analyzing result and writing manuscript.

Tri Baskoro Tunggul Satoto: Performed morphological and histological analysis.

Edhi Martono: Designed the research plan and contributed to writing manuscript.

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Vol 11, Issue 10, 2018

Research Article

MORPHOLOGICAL AND HISTOLOGICAL EFFECTS OF BRUCEINE A ON THE LARVAE OF *AEDES AEGYPTI* LINNAEUS (DIPTERA: CULICIDAE)

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Received: 15 May 2018, Revised and Accepted: 28 June 2018

ABSTRACT

Objective: This study aimed to determine a target of action of bruceine A on the basis of its morphological and histological effects on the larvae of *Aedes aegypti* Linnaeus.

Methods: Bruceine A was isolated from *Brucea javanica* (L.) Merr. seeds in accordance with the Mangungsong method. Larvae of *A. Aegypti* (L.) in instar III to the beginning of instar IV were treated with various concentrations of bruceine A. The negative control group did not receive any treatment, whereas the positive control group received 1 ppm temefos. Dead larvae were collected after 24 h of treatment for the examination of morphological and histological changes.

Results: The negative control group did not exhibit any morphological and histological changes. Larvae treated with bruceine A, however, had visible damaged heads, cuticles, digestive and respiration tracts, respiratory siphons, and setae, and they were smaller than normal larvae. Larvae treated with temefos exhibited gastrointestinal damage, narrowed breathing tubes, cuticle damage, and detached/damaged seta feathers. The necrosis of gastrointestinal epithelial cells was the major histological change exhibited by larvae treated with various concentrations of bruceine A or 1 ppm temefos.

Conclusion: The targets of action of bruceine A in *A. aegypti* (L.) larvae are the head/caput, cuticle, setae, siphon, and gastrointestinal and respiratory tracts.

Keywords: Bruceine A, *Brucea javanica* (L.) Merr., Action target, Morphology, Histology, *Aedes aegypti* Linnaeus.

 DOI: http://dx.doi.org/10.22159/ajpcr.2018.v11i10.27315

INTRODUCTION

Vector control is a method to suppress the incidence of vector-borne diseases. It is widely conducted as a public health intervention. *Aedes aegypti* Linnaeus is a mosquito species that is proved to be an important disease vector in tropical and subtropical regions [1]. *A. aegypti* (L.) is a vector of dengue fever, dengue hemorrhagic fever [2], chikungunya fever, yellow fever, and Zika viral disease [3]. The wide use of synthetic organic insecticides for vector control harms the environment and causes the emergence of insecticide-resistant vectors, as well as the deaths of non-target animals. Earlier intervention studies have shown that although the use of synthetic insecticides such as temefos, especially in risky or potential places, can decrease disease transmission by mosquitoes, prolonged exposure to these chemicals will promote the adaptation, evolution, and selection of mosquitoes [4]. Thus, plant-derived insecticides/larvicides should be developed as another option for controlling vector-borne diseases. The two essential oils of *Thymus vulgaris* and *Origanum majorana* (Lamiaceae) demonstrate an interesting larvicidal activity. The *O. majorana* essential oil is more effective compared to the essential oil of *T. vulgaris* with an lethal concentration 50 (LC_{50}) of 107.13 µg/mL and LC_{90} of 365.90 µg/mL on the malaria vector *Anopheles labranchiae* [5]. The crude ethanolic extract of *Smilax larvata* (Sarsaparilla) is a potential source of an eco-friendly larvicide against *A. aegypti* larvae with LC_{50} 225 µg/mL^{-1} and LC_{90} 350 µg/mL^{-1} [6]. Compounds from *Brucea javanica* (L.) Merr., have potential applications as agricultural insecticides. Zhang *et al*. [7] proved that brusatol isolated from *B. javanica* (L.) Merr. has insecticidal and antifeeding effects against the third-instar larvae of *Spodoptera exigua*. Brusatol can also induce apoptosis in the insect cell lines IOZCAS-Spec-II and Sf21. In these cell lines, apoptosis is characterized by DNA fragmentation, caspase-3 activation, and cytochrome-c release from mitochondria. Sutiningsih and Nurjazuli [8] proved that brusatol isolated from the seeds of *B. javanica* (L) Merr has larvicidal activity against *A. aegypti* at the LC_{50} and LC_{90} of 0.669 and 8.331 ppm, respectively.

Bruceine A ([15]-3-methyl-2-butanoil-bruseolid) is a quassinoid derived from *B. javanica* (L.) Merr [9]. It has a molecular formula of $C_{26}H_{34}O_{11}$ and has a mass of 522.54 g/mol. Physically, it is an amorphous powder with a bitter taste. Bruceine A has extensive broad biological activity as an antibabesiosis, antitrypanosomal, and anti-malarial as well as cytotoxic properties against cancer cell lines [10-12]. It also has insecticidal, antifeeding, and growth-inhibiting activities against tobacco budworm (*Heliothis virescens* F.), *Spodoptera frugiperda* armyworm [13], and Mexican bean beetle larvae in the fourth instar (*Epilachna varivestis* Mulsant) [14]. Bruceine A can also act as a neurotoxin [15] and an inhibitor of growth [16] against the larvae of *A. aegypti* (L.) The biolarvicidal mechanism of the action of bruceine A occurs through the inhibition of acetylcholinesterase and VGSC gene. The behavioral responses of larvae treated with bruceine A include hyperexcitation, convulsions, paralysis, and aggressive biting of the anal gills; these behaviors indicate that bruceine A affects the larval neuromuscular systems [15]. Therefore, this study aimed to determine the targets of action of bruceine A and to identify its effects on the morphology and histology of *A. aegypti* (L.) larvae.

METHODS

Materials

Makassar fruit (*B. javanica* L. Merr) was purchased from a wholesaler of medicinal plants (Aneka Herbal Yogyakarta, Indonesia). Confirming its identity as well as obtaining its relevant scientific information, the specimen was further identified at the Laboratory of Pharmaceutical Biology, Faculty of Pharmacy, Gadjah Mada University, Yogyakarta, Indonesia. *A. aegypti* (L.) larvae in instar III to the beginning of instar IV were obtained from colonies maintained at the Laboratory of Parasitology, Faculty of Medicine, University of Gadjah Mada, Yogyakarta. All commercial reagents and other chemicals used in this study purchased from commercial suppliers were of analytical quality with the highest purity available. The selection of temefos dosage (1 ppm) was based on lethal damage consideration used in the field.

Extraction and isolation of bruceine A

Bruceine A was isolated from *B. javanica* (L.) Merr seeds in accordance with the method described by Mangungsong [17]. Dried seeds of *B. javanica* (L.) Merr (5 kg) were ground into powder and shaken with a hexane solution (15 L). The solution was then filtered and extracted with methanol (15 L). Methanol was evaporated to obtain a thick extract, which was then mixed with an equal volume of distilled water to form a suspension. Then, the suspension was partitioned with hexane (3 L). After, the hexane fraction was separated from the suspension, and methanol-water fractions were collected for repeated extraction with dichloromethane (1 L). Later, the organic layer was collected and evaporated to obtain a concentrate, which was then diluted with methanol (100–250 mL) at 60°C and stored at room temperature. The methanol solution was maintained at room temperature to allow the crystallization of bruceine A. Further separation was conducted through filtration. The remainder of the filtrate/residue was separated through thin-layer chromatography (TLC) and evaporated. Finally, purity levels of the amorphous powder were measured using high-performance liquid chromatography (HPLC).

Morphological test

Morphological tests were conducted in accordance with the method reported by Sharma *et al*. [18] with slight modifications. *A. aegypti* (L.) larvae in instar III to the beginning of instar IV were placed in glass jars, each containing 199 mL of water and 1 mL of bruceine A at various LC or 1 ppm of the positive control temefos. Negative controls were treated with distilled water. The larvae found dead after 24 h were separated and studied under light microscopy to examine its morphology. Larvae were scrutinized after mounting with Hoyer's medium and morphological changes in body segments including the head, setae, cuticle, abdomen, and anal gills. They were observed, photographed, and compared with those of the controls.

Histological test

Histological tests were performed in accordance with the method of Narciso *et al*. [19] with slight modifications. Larvae treated with different concentrations of bruceine A, 1 ppm of temefos, or distilled water were fixed in 2.5% glutaraldehyde in sodium cacodylate buffer (0.1 M, pH 7.4) for 4 h. Samples were then dehydrated in a gradient ethanol series (70%, 80%, 90% 96%, and 100%). Samples were immersed in each ethanol solution for 15 min. Samples were embedded in Historesin JB4, and the resulting blocks were sliced using a microtome to obtain a series of 3 µm thick sections. These sections were stained with hematoxylin-eosin and then examined and photographed using a light microscope. Morphological and histological changes in larvae were analyzed descriptively.

RESULTS AND DISCUSSION

Isolation of bruceine A from *B. javanica* L. Merr

Based on the extraction and isolation method by Mangungsong [17], as much as 150 mg of isolate compounds of bruceine A was obtained from each of 5 kg of Makassar fruit (*B. javanica* L. Merr). The purity levels of the amorphous powder were measured using two-dimensional chromatography with stationary phase silica gel 60 F254 on TLC plate and mobile phase of mixed solvent of chloroform and ethyl acetate with ratio of 1: 2 to produce a single purple spot seen in ultraviolet (UV) 366 nm with retardation factor (Rf) of 0.88. The results of this research are in line with the results from Mangungsong [17] which suggested that there was a single purple spot on bruceine A isolate under UV ray 366 nm observation. The purple spot indicated that bruceine A isolate is single/pure apart from other chemical components [20]. Rf value of bruceine A isolate of 0.8 is still considered as an ideal average value that is between 0.2 and 0.8. Rf value is the distance traveled by compound divided by distance traveled by eluent. Higher Rf value showed that isolate/chemical compound has low polarity and otherwise [21]. The result of a calculation based on area under the graph of the HPLC of bruceine A isolate showed a single dominant peak with area width percentage of 92.796% and retention time (Rt) of 4.633 min. Although bruceine A compound has not reached 99–100%, bruceine A isolate compound inside the isolate is shown with a single dominant peak on the produced chromatograph. The result of this research is not very different from Mangungsong [17] which showed that the pureness of bruceine A isolate was of 94.88% with a Rt of 4.83 min.

Morphological changes of *A. aegypti* (L.) larvae

Observation on morphological changes in *A. aegypti* (L.) larvae was meant to decide damaged target body part after the treatment with bruceine A at various concentrations comparing the treatment with a control larvae. An overview of the morphological changes is presented in Figs. 1 and 2.

Bodies of control larvae did not show any damages (Fig. 1a). Those larvae treated with 1 ppm temefos exhibited damaged cuticles and digestive tracts with some dark spots narrowed breathing tubes and some detached/damaged setae feathers (Fig. 1b). By contrast, larvae treated with lowest concentrations of bruceine A (1 ppm) exhibited morphological damage to the head, which appeared dark, and some parts of the cuticle layer, as well as narrowed breathing tubes (Fig. 2a). At the higher concentration, bruceine A (2 ppm) damaged or caused the detachment of anal papillae/anal gills, as well as decreased body size and caused discoloration (Fig. 2b).

These results are consistent with previous studies confirming that larvae of *A. aegypti* (L.) treated with bruceine A at sub-LC (0.2 ppm) cause damage to their digestive with the existence of black spots, folded respiratory tubes, and detached setae and cuticles [16]. The research of Warikoo and Kumar [22] reported that treatment with excess *Argemone mexicana* damaged the anal papillae of *A. aegypti* larvae. Sharma *et al*. [18] showed that treatment with extracts of the stems and leaves of *Achyranthes aspera* caused a structural damage to the anal papillae of larvae of *A. aegypti* in the early fourth instar. In the present study, microscopic observations showed that the

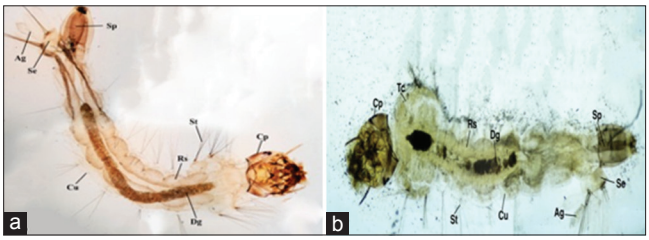

Fig. 1: Microscopic images of control and temefos-treated *Aedes aegypti* (L.) larvae. (a) control larva (untreated), ×40. The heads, thoraxes, and abdomens of larvae are still complete, (b) temefos-treated (1 ppm) larva, ×100. Respiratory and digestive tract are severely damaged, cuticle, and setae are damaged/detached. Cp: Caput, Dg: Digestive tract, Rs: Respiratory tract, St: Setae, Cu: Cuticle, Sp: Siphon, Se: Saddle, Ag: Anal gills

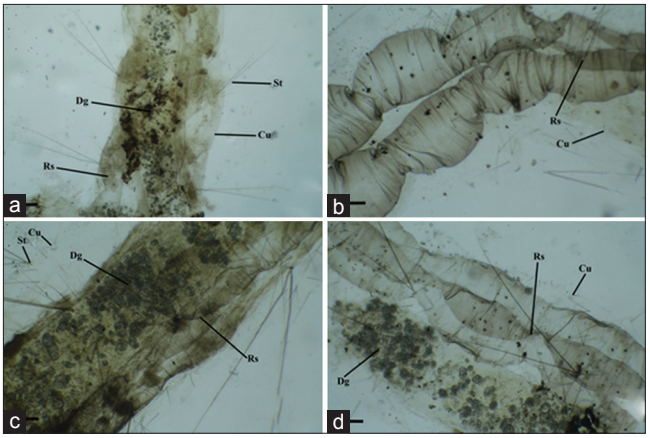

Fig. 2: Microscopic images of *Aedes aegypti* (L.) larvae treated with (a) 1 ppm, (b) 2 ppm, (c) 4 ppm, and (d) 8 ppm bruceine A, ×100. Larvae of *A. aegypti* (L.) treated with various concentrations of bruceine A exhibited damaged digestive and respiratory tract, numerous loose setae and cuticle, and damaged siphons. Rs: Respiratory tract, Cu: Cuticle, Dg: Digestive tract, St: Setae

internal membranes of anal papillae were shrank, whereas external membranes were remained normal. As reported by Insun *et al*. [23], the larvae of *Culex quinquefasciatus* treated with ethanol extracts of *Kaempferia galanga* exhibited anal papillae damage and cuticle shrinkage. According to Chaithong *et al*. [24], the structural deformity of the anal papillae may result from osmotic and ionic dysregulation. Thus, osmotic and ionic dysregulation is possible causes of death of the larvae of *A. aegypti* (L.).

Observation of morphology of *A. aegypti* (L.) larvae after the treatment with 4 ppm of bruceine A showed swollen digestive tracts, narrowed and folded respiratory tubes, damaged cuticle, and detached setae feathers (Fig. 2c). Larvae treated with the highest concentration of bruceine A (8 ppm) exhibited darkened heads with black spots, swollen or lysed digestive tracts with some blackened areas, small and highly folded respiratory tubes, enlarged siphons, and damaged cuticle and setae feathers (Fig. 2d). The higher the concentration of bruceine A, the worse and more widespread of *A. aegypti* (L.) larvae morphological damages is to cause damage to the digestive tract and cuticle. In addition, respiratory tubes, siphon, and anal gills were having more severe damage. These results are similar to those observed by Sharma *et al*. [18], who reported that the larvae of *A. aegypti* exhibited distorted midguts, pigmentation loss, and partial or total cell damage after treatment with extracts from the stems and leaves of *A. aspera*. Digestive tract damage was more visible in larvae treated with the hexane extract of *A. aspera* leaves than those treated with extracts from *A. aspera* stems. Light/electron microscopic observations at 6, 12, 24, and 48 h after *A. aspera* treatment showed that midgut epithelial damage intensified over time. Chaithong *et al*. [24] reported that pepper extract had similar effects on the midguts of *A. aegypti* larvae.

Based on the results of this study, it proves that toxic substances in bruceine A cause morphological damage in the body of *A. aegypti* (L.) larvae. Bruceine A acts as a contact poison to the gastrointestinal and respiratory systems and likely enters the larval body through the pores of the skin/cuticle, digestive tract, and siphon. Bruceine A is a nonpolar compound that is soluble in the lipids of the insect cuticle. Being soluble in lipids accelerates its rate of penetration into the insect hemocoel (body cavity). The penetration rate of bruceine A through the cuticle depends on cuticle structure and thickness [25]. Toxic substances generally tend to penetrate through larval body parts that are thinly coated with cuticle; examples of such body parts include intersegmental membranes, membrane joints, and chemoreceptors on the tarsus [26]. Bruceine A is absorbed by the body wall of insects and taken by body fluids to the active target area. It causes the dysfunction of the digestive, respiratory, and nervous systems of larvae [27]. Toxic substances enter the skin membrane of larva through simple diffusion [28]. These compounds then damage skin cells, causing the skin membrane to lose its impermeability and thus allowing other free toxic compounds to penetrate into the larval body. Toxic compounds also damage proteins in the skin membrane, thus disturbing the function of the skin as the protector of the body [29]. In addition to diffusion through the skin, toxic substances enter through the digestive tract [30]. The digestive tract of the mosquito larva consists of the anterior, mid, and posterior parts [31]. Food digestion and nutrient absorption occur in the midgut [29]. The insect midgut is covered with epithelial tissue. Toxic substances enter through the mouth of the larva and continue to the midgut while lysing epithelial cells. Cell lysis decreases the surface tension of mucous membranes ultimately inhibiting digestion and nutrient absorption [26,31]. Toxic substances may also penetrate the larval body through respiratory tracts. Air enters through a siphon attached to the water surface. Thus, toxic substances covering the surface of the water medium prevent the siphon from obtaining oxygen. Wulandari *et al*. [32] stated that secondary metabolites can interfere with oxygen collection. Given that the neural networks of larvae are highly sensitive to oxygen balance, neural atrophy and siphon damage may hinder breathing and eventually cause larvae to die.

Meanwhile, *A. aegypti* (L.) larvae treated with temefos 1 ppm caused damage on the entire body (Fig.1b). The body size of the larvae shrinks compared to its body size after treatment with bruceine A and control (untreated). The result of this research is not very different from Yulidar and Hadifah [33] which showed the morphological damages of *A. aegypti* larvae on the head, thorax, abdomen, and detached setae feathers, and shrinking body size after treatment with temefos at LC. This is thought to happen because of the differences in water content inside larvae's body and the environment, so the water from the body is released through abdominal sockets and moved out to the environment. According to Badyaev [34], the water movement from larvae's body to the environment is caused by high temefos inside the media leading to the osmotic pressure of the environment is higher. The higher temefos concentration on water media brings about water content in the body of larvae getting higher and the differences in osmotic pressure happen. The balance of osmosis chemical solution can transpire through diffusion [35]. On the dead larvae, there is water movement from higher water molecules in the environment to the inner part of *A. aegypti* (L.) larvae that have lower osmotic pressure [36]. Allegedly, this is what causes the outer layer of the abdomen is seen shrinking because the water from inside of larvae's body is leaked outside.

Temefos likely gets into the bodies of larvae through cuticle contact, inhalation, and/or ingestion [37]. Temefos contains phosphorothioate, a lipophilic group. Thus, it easily penetrates the hydrophilic epicuticular parts of *A. aegypti* (L.) larvae and causes the cuticle and setae feathers to detach from the bodies of larvae [38]. After penetrating the cuticle/skin, temefos then enters nerve cells in the gastrointestinal and respiratory tracts of larvae. Temefos poisoning is characterized by restlessness, hyperexcitability, tremors, convulsions, and eventually muscle paralysis [38]. In addition to the cuticle, temefos enters the larval body through the respiratory tract, thus causing the breathing tube to shrink. Temefos also enters the larval body when consumed with food in breeding media [37].

Histological changes of *A. aegypti* (L.) larvae

The histomorphological analysis was conducted to gain further insight into the targets of action of bruceine A in the larvae of *A. aegypti* (L.). Figs. 3 and 4 show the differences between the histology of control larvae and temefos with that of larvae treated with LC of bruceine A.

Gastrointestinal epithelial cells from the control *A. aegypti* (L.) larvae were normal with compactly stained cytoplasm, spherical nuclei, clearly defined chromatin, and visible peritrophic membranes. Moreover, the majority of microvilli appeared normal where epithelial cells remained attached to the basement membrane (Fig. 3a). Larvae treated with 1 ppm temefos exhibited necrotic, shrunken, and diffuse gastrointestinal epithelial cells with karyopyknotic nuclei. Necrotic epithelial cells remained attached to the basement membrane. Necrotic microvilli and peritrophic membranes appeared diffuse, and epithelial cells appeared disorganized (Fig. 3b).

A. aegypti (L.) larvae treated with the low concentration of 1 ppm bruceine A exhibited necrotic gastrointestinal epithelial cells that remained attached to the basement membrane. Microvilli and peritrophic membranes became diffuse and necrotic, and epithelial cells became structurally disorganized (Fig. 4a). The same comprehensive histological changes were also observed in *A. aegypti* (L.) larvae treated with bruceine A at concentrations of 2 and 4 ppm (Fig. 4b and c). It showed that damage intensified with increasing bruceine A concentration. Larvae treated with 8 ppm bruceine A exhibited completely diffuse necrotic gastrointestinal epithelial cells which completely detached from the basement membrane and localized in the lumen. These results are consistent with that reported by Sharma *et al*. [18] who stated that midgut epithelial cells exhibited intense damage at 6, 12, 24, and 48 h after treatment with *A. aspera* extract. Highly typical changes include the vacuolization of midgut columnar cells, damage to microvilli, the release of epithelial cell content into the midgut lumen, and eventual cell death. Sutiningsih *et al*. [16] report that there was necrosis on gastrointestinal epithelial cells indicated by shrunken cells and diminished core (karyolysis) on *A. aegypti* (L.) larvae after treatment with bruceine A at the sub-lethal dosage (0.2 ppm). The results from Patil *et al*. [39] showing that there were extruded peritrophic membrane on posterior peak between anal papilla on the dead *A. aegypti* (L.) larvae after treated with *Clerodendron inerme* extract at LC. The extruded peritrophic membrane indicated that *C. inerme* extract affected the intestinal area that can cause a substantial effect on nutrition absorbing and inhibition of larvae's development process. The peritrophic membrane is a sheath containing acellular chitin that separates the content of intestines from secretory epithelial/intestinal absorption that also acts as a barrier for pathogens which protect the area of midgut [40-42].

Narciso *et al*. [19] reported that histomorphological changes resulted from treatment with burchelin from *Ocotea cymbarium* caused the death of L3-L4 larvae of *A. aegypti*. The midgut epithelial cells of the larvae exhibited disorganization, damage, and vacuolization. The histological analysis of larvae of *Culex nigripalpus* infected by *Bacillus thuringiensis* Medelin (Cry 11Bb) [42] revealed similar damages as that observed in the intestinal cells of *Aedes albopictus* infected by *B. thuringiensis* var Israelensis (Bti) [43]. Infection is characterized by the presence of rounded mesenteric cells with granular cytoplasm, absent or clear nucleus, and cytoplasmic vacuolization. The mesentery actively participates in secretion and absorption. The disintegration of mesentery cells occurs through the accumulation of granular material in the apical part followed by the release of material into the gut lumen. Mosquito larvae treated with the tested substance exhibited gastric vacuolization, cellular disorganization within intersegmental cells, and clear or absent nucleus. These comprehensive changes are not limited to chemical damage; infection with *Baculovirus* resulted in the same changes to the gastric and Malpighian tubules of *C. nigripalpus* Theobald larvae [42].

Al-Mehmadi and Al-Khalaf [44] stated that histopathological changes are qualitatively different in terms of localization and quantitatively correspond to the duration or length of observation time. Histopathological effects on the midgut and gastric ceca

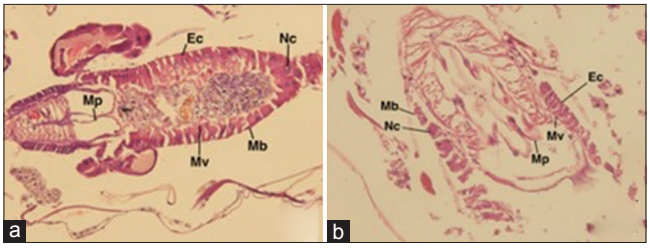

Fig. 3: Longitudinal sections of gastrointestinal tracts from *Aedes aegypti* (L.) larvae. (a) control larvae, ×400. Gastrointestinal epithelial cells are normal with compactly stained cytoplasm, (b) temefos-treated larva (1 ppm), ×400. Gastrointestinal epithelial cells are necrotic. Mb: Basement membrane, Ec: Epithelial cells, Mv: Microvilli, Mp: Peritrophic membrane, Nc: Nucleus

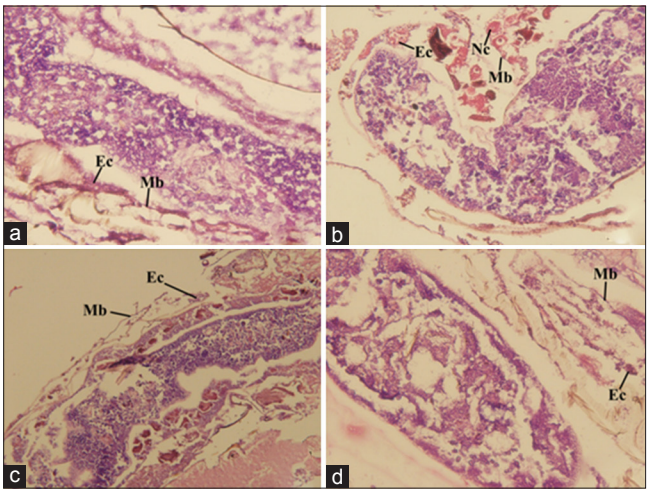

Fig. 4: Longitudinal sections of gastrointestinal tracts from *Aedes aegypti* (L.) larvae treated with (a) 1 ppm bruceine A, (b) 2 ppm bruceine A, (c) 4 ppm bruceine A, (d) 8 ppm bruceine A, ×400. *A. aegypti* (L.) larvae treated with various concentrations of bruceine A exhibited diffuse necrotic epithelial cells. Mb: Basement membrane, Ec: Epithelial cells, Mv: Microvilli, Mp: Peritrophic membrane

confirmed that these areas are in direct contact with toxic substances. *C. quinquefasciatus* larvae treated with *Melia azedarach* extract exhibited serious damage and necrotic columnar epithelial cells of the gastric ceca. At 24 h after treatment, the epithelial cells of the gastric ceca exploded or shrunk and underwent lysis. Changes were also observed in the anterior and posterior of the midgut which showed epithelial cells detached from the basal membrane with peritrophic membrane damage and cell wall rupture [44]. The mixing of intestinal contents with hemolymph causes larvae to die. Assar and El-Sobky [45] also observed that aqueous hyacinth extracts can severely damage the larval midgut. They reported that damage after 48 and 72 h of observation is characterized by vacuolization and shrinkage of epithelial cells.

Bruceine A is a toxic substance which may enter the digestive tract through the skin or buccal membrane. This toxic substance first causes midgut epithelial cells to undergo lysis or necrosis. Cell death or lysis, in turn, decreases the surface tension of the mucous membranes of the midgut to inhibit the digestion and absorption of food, ultimately resulting in larval death [29]. The results of this study prove that bruceine A is a potential natural larvicide that can be used to control the population of *A. aegypti* (L.) larvae as disease vectors. Its targets of action for morphological damage include the head, cuticle, setae, siphons, and gastrointestinal and respiratory tracts, whereas those of histological damage is the midgut or gastrointestinal epithelial cells.

It is necessary to conduct further research on larvicidal action target of bruceine A on different species of mosquitoes as well as a detailed microscopic examination on body parts of larvae using transmission electron microscope.

CONCLUSION

Larvicidal action targets of bruceine A are as follows: (a) Morphologically damage the head, cuticles, setae, digestive and respiratory tracts, and siphon and (b) histologically damage by causing necrosis on gastrointestinal epithelial cells, peritrophic membrane, microvilli, and disorganized epithelial cells, detached from basalis membrane.

ACKNOWLEDGMENTS

The authors of this research would like to thank the Directorate for Research and Community Service, Ministry of Research, Technology and Higher Education of the Republic of Indonesia which has funded this research on Doctoral Dissertation Research year 2017.

AUTHOR'S CONTRIBUTION

Dwi Sutiningsih: Conceived and designed the experiments, reviewed literatures, and wrote the manuscript. Mustofa: Performed the experiments and contributed to analyzing result and writing manuscript. Tri Baskoro Tunggul Satoto: Performed morphological and histological analysis. Edhi Martono: Designed the research plan and contributed to writing manuscript.

CONFLICTS OF INTEREST

The authors have no conflict of interest or financial interest in regard to the result of the research.

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9. Published

Asian Journal of Pharmaceutical and Clinical Research
E-ISSN: 2455-3891
P-ISSN: 0974-2441
EMBASE

MORPHOLOGICAL AND HISTOLOGICAL EFFECTS OF BRUCEINE A ON THE LARVAE OF AEDES AEGYPTI LINNAEUS (DIPTERA: CULICIDAE)

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DOI
https://doi.org/10.22159/ajpcr.2018.v11i10.27315

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Objective: This study aimed to determine a target of action of bruceine A on the basis of its morphological and histological effects on the larvae of *Aedes aegypti* Linnaeus.

Methods: Bruceine A was isolated from *Brucea javanica* (L.) Merr. seeds in accordance with the Mangungsong method. Larvae of *A. Aegypti* (L.) in instar III to the beginning of instar IV were treated with various concentrations of bruceine A. The negative control group did not receive any treatment, whereas the positive control group received 1 ppm temefos. Dead larvae were collected after 24 h of treatment for the

Online ISSN: 2455-3891
Print ISSN: 0974-2441
ICV 2019: 133.59

Journal Metrics 2018
Source Normalized Impact per Paper (SNIP): 0.655
SCImago Journal Rank (SJR): 0.17
Print ISSN: 0974–2441