RESEARCH NOTE

POSSIBLE SITE OF ACTION OF *KAEMPFERIA GALANGA* IN KILLING *CULEX QUINQUEFASCIATUS* LARVAE

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Mosquito-borne disease is still a major problem of the world, particularly, in tropical and subtropical regions. In Thailand, there are at least 24 species belonging to 4 genera of mosquitos that play an important role in transmission of (a) malaria : *Anopheles dirus, An. minimus, An. maculatus, An. pseudowillmori, An. aconitus, An. sundaicus;* (b) filariasis, including zoonotic filarial infection : *Aedes harinasutai, Ae. desmotes, Ae. annandalei, Ae. imitator, Ae. aegypti, Ae. albopictus, Culex quinquefasciatus, Mansonia annulata, Ma. bonneae, Ma. indiana, Ma. uniformis, Ma. dives;* (c) Japanese encephalitis : *Culex fuscocephalus, Cx. gelidus, Cx. pseudovishnui, Cx. tritaeniorhynchus, Cx. vishnui;* (d) dengue hemorrhagic fever : *Ae. aegypti, Ae. albopictus* (Gould *et al,* 1982; Choochote *et al,* 1992; Rattanarithikul and Panthusiri, 1994; Division of Filariasis, 1995).

Even though the vector control programs have been established in Thailand for a long time, the diseases are endemic year by year. The failure for control were partly due to the refusion of insecticide house spraying, changing to biting habit of vactors, tolerance or resistance to insecticides, etc (Nontananda, 1972; Ismail *et al,* 1978; WHO, 1992). According to the failure of vector control, therefore, the search for alternative methods for control has become of great importance. The control of mosquito vector through exploitation of innovative strategy has been proposed, *ie.* phytochemicals, biological agents, genetic manipulations, etc. Recently, Pitasawat *et al* (1998) investigated the ethanolic extracts of ten species of carminative plants on killing *Cx. quinquefasciatus* larvae, *ie* Krawan fruit *(Amomun krervanh* Pierre), Khum foi flower *(Carthamus tinctorius* L.), Phak chee fruit *(Coriandrum sativum* L.), Kaan phluu flower buds *(Eugenia caryophyllata* Thunberg), Pooi kak fruit *(Illicium vernum* Hooker), Proh hom rhizome *(Kaempferia galanga* L.), Kaeo leaf *(Murraya paniculata* L.), Chan thet arillode *(Myristica fragrans* Houtt), Horaphaa chaang leaf *(Ocimum gratissimum* L.), and whole part of Phak Khraat huawaen *(Spilanthes acmella* Murr). The results have indicated that there are at least three carminative plants, *ie. K. galanga, I. vernum* and *S. acmella* having promising larvicidal activity of which LC_{50}; LC_{95}; LC_{99}; values were 50.54, 54.11 and 61.43 ppm; 73.61, 159.00 and 194.26 ppm; 90.88, 290.95 and 370.40 ppm; respectively. As an adjunct to the previous study, we report herein the possible site of action of *K. galanga* on killing *Cx. quinquefasciatus* larvae.

The rhizomes of *K. galanga* were purchased in Chiang Mai Province. For ethanolic extraction, 1.5 kg of dried, powdered rhizome was macerated with 5 liters of 95% ethanol at room temperature for 2 days. The mixture was suction filtered through a funnel and residue was reextracted with 95% ethanol for 2 times. The filtrate obtained was combined and evaporated by using a rotary evaporator at 60°C, lyophilized and kept at -20°C. Larvicidal test was assessed by following the modification method of WHO (1970) as described by Pitasawat *et al,* 1998 Briefly, 95% ethanol was used as solvent to dilute the ethanolic extract to an appropriate test concentration. For experimental treatment, one ml of the test solution was completely mixed with 224 ml of distilled water in an enamel bowl of 10 cm in diameter and 8 cm in depth. Then 25 healthy, early 4^{th} larvae of laboratory-raised *Cx. quinquefasciatus* (Chiang Mai strain), the natural vector of *Dirofilaria immitis* (Choochote *et al,* 1992) and potential vector of nocturnally periodic and subperiodic *Wuchereria bancrofti* (Jitpakdi *et al,* 1998) were transferred to that bowl. Each experiment was done in 4 replicates with the final total number of 100 larvae for each concentration. Each replicate set contained one control which consisted of 1 ml of 95% ethanol and 249 ml of distilled water and one untreated which contained only 250 ml of distilled water. After a period of 24 hours, the mortality counts were performed.

To determine the possible site of action of *K. galanga* on killing *Cx. quinquefasciatus* larvae, symp-

tomatological and specific target organ were observed. Symptomatological observations were carried out continuously until treated larvae died. Symptoms of test larvae such as convulsion, unnatural positions, tremor, incoordination, rigor, slugglish movement, failure of body to balance in water and lack of feeding (immotile mouth brushes) were recorded at time-intervals until all of them died. Some of dead larvae were further studied for toxic effects on specific target organs. For toxic effects on specific target organs, the external organs of dead larvae, *ie* body cuticle, head, thorax and abdomen, eye, antennae, mouth brushes, setae, saddle, anal gills, siphon and spiracles were scrutinized under both light and scanning electron microscopes. Larvae fixed in 2.5% glutaraldehyde were mounted with Hoyer's medium on the slides for light microscopic observations, whereas they were processed following the manner of Iwaki and Choochote (1991) for scanning electron microscopic examinations. For cellular changes, both dead and control larvae were immediately dissected and the target organs were pulled out without any rupture in normal saline solution on the cavity slides. The alimentary canal and Malpighian tubes were separated and placed in a drop of normal saline solution on a clean glass slide, covered with coverslip, and then examined freshly under compound microscope. The brain stem was investigated after passage through fixing and conventional staining of Giemsa. It was removed from head capsule in 0.85% sodium chloride solution, fixed in one drop of 15% acetic acid for 2 minutes, transferred to one drop of 45% acetic acid on a clean slide, teared apart, smeared to make monolayer, air dried, stained with 10% Giemsa in phosphate buffer (pH 6.8) 30 minutes, and mounted in Permount (Fisher) for permanent preparation.

Immediately after exposure to LC_{99} of ethanolic extract of *K. galanga,* all larvae were still active and exhibited in a normal appearance, siphon rested up and head hung down. The process of larval feeding, both collecting-filtering in the water column and collecting-gathering at submerged surfaces, were obviously seen, Between 5 and 15 minutes after treatment some of larvae were restless, frequently sank down and quickly floated up. At 30 minutes, the restlessness still persisted, and tremor and convulsion at the bottom of container were observed with approximately 2-3 larvae. Similar evidence of restlessness, tremors, convulsions followed by paralysis was clearly seen at 45 minutes with approximately 4-5 larvae. For as long as 60 minutes after treatment, approximately one third of the larvae were paralyzed and sunk to the bottom of the bowl. More and more larvae exhibited toxic symptoms during 2 to 3 hours. Subsequently, all of them died within 4 hours.

Investigations on the cuticular sculpturing and ornamentation of the dead 4th larvae after treatment with ethanolic extract of *K. galanga* and normal larvae revealed the similarity in the structures of head capsule including antennae, compound eyes, clypeal, frontal, sutural, transutural, lateral and ventral hairs; simple and branched hairs of prothorax, mesothorax and metathorax; simple and branched hairs of nine abdominal segments; siphon; saddle; and ventral brush. The distinct different points were the structures of anal gills or papillae. Untreated larvae showed normal intact cuticle but in treated larvae, shrunken cuticle had been found (Fig 1A,B). Similar results were also found in the examinations under scanning electron micorscope (SEM), except the more details of damaged cuticle of anal gills had clearly seen. Anal gills were cone-shaped, and in the control larvae, the surface topography was seen as irregular ridge-like reticulum (Fig 1C), whereas the reticulum disappeared in the treated larvae (Fig 1D).

Examination of alimentary systmes of treated larvae, *ie* cecum; anterior, middle and posterior parts of stomach; pylorus; anterior intestine and rectum; and Mulpighian tubes revealed the normal contour of organs and intact epithelial cell lines without evidence of vacuolization and/or degeneration (Fig 2A). The examination of brain stem stained with Giemsa revealed that most of the neuroblasts and neurons were intact cells without evidence of vacuolization and/or degeneration. The cytoplasm was spongy-like and dark blue color of Nissl substance was distributed throughout the cytomplsm of cell body, except at the region of axon hillock (Fig 2B). Similar results were also observed in the control.

In general, the major pathway of insecticide entry into insects is usually cuticular penetration. Because of the large proportion of cuticular surface in relation to the body surface area, the penetration of insecticide entry. However, insects do possess several vulnerable parts exposed to the outside through which insecticides may enter, *eg,* intersegmented region, the respiratory system, the mouth, or any other exposed sensory organs such as the antennae, eyes and tarsi. In such cases, insecticides having entered by these routes may pass throughout the interior of insect to its site of action (Matsumura, 1975).

To determine the specific site of action of ethanolic extract of *K. galanga,* morphological observations of dead 4th larvae of *Cx. quinquefasciatus*

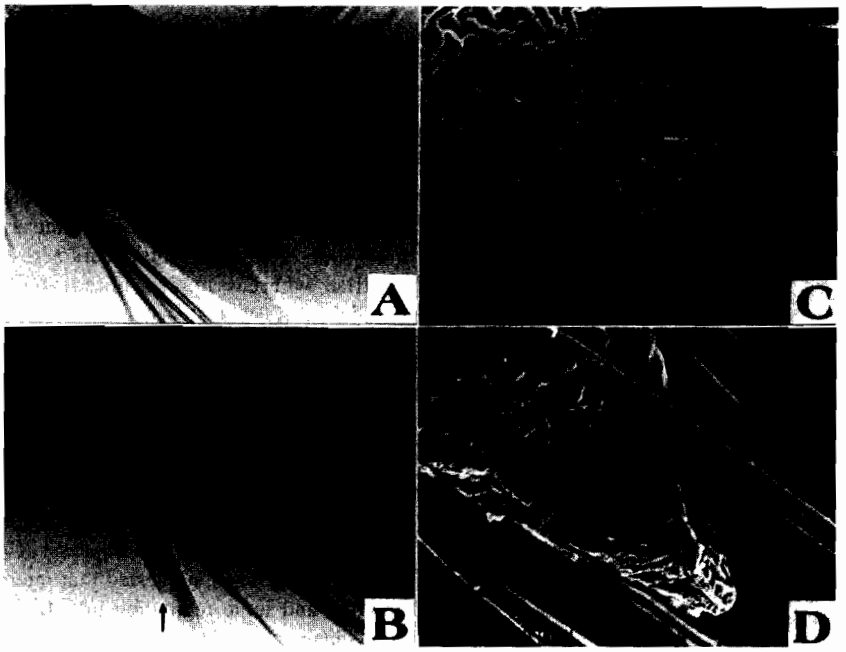

Fig 1–Four anal gills of *Cx. quinquefasciatus* larvae mounted in Hoyer's media. Control : normal gills with intact cuticle (A). *K. galanga* treated; shrinkage cuticle border (arrow) (B). Anal gill of *Cx. quinquefasciatus* larvae examined under SEM. Control : cone-shaped anal gill of normal larva showing irregular ridge-like reticulum of outer surface (C), x1,600. *K. galanga* treated : damaged surfaced and lost of ridge-like reticulumn (D), x1,600.

after treatment with the ethanolic extract under light and scanning electron microscopes were carried out and compared with the control larvae. Cellular observations of various target cells or organs under fresh and staining conditions were also included. Investigations on morphological features and cuticular topography of treated 4th larvae under light microscope revealed that most organs, except anal gills were normal in structure and similar to those of the control. The surface cuticles of head, thorax, abdomen, siphon and saddle were still intact. The features of anal gills, on the other hand, differed markedly from those of the control. Their cuticles were shrunken. Morphological changes of treated larvae were confirmed by diagnostic electron microscopy. The purely simple shrinkage of anal gills observed under light microscopy was demonstrated clearly as the destruction of irregular ridge-like reticulum on their surface under SEM. However, the osmolarity of the test solution (ethanolic extract) was found to be 71 mOsm/kgH_2O which was lower than that of the intracellular fluid (280 mOsm/kgH_2O (Guyton and Hall, 1995). It is therefore suggested that the osmolarity of the test solution is not involved in eliciting anal gill shrinkage.

All larvae died withing 4 hours after exposure to ethanolic fraction, suggesting a delayed type of larval killing. The symptoms observed in treated larvae were similar to those cause by nerve poison, *ie* excitation, convulsions, paralysis and death. However, the symptoms caused by nerve poisons occoured more rapidly by than those observed in larvae treated with the ethanolic fraction. Microscopic examinations of brain stem revealed that both neuroblasts and neurons were intact without evidence of vacuolizaion and/or degeneration. It is therefore indicated that the site of action is unlikely on the nervous system.

The ethanolic extract dissolved in the medium entered through the mouth by the process of larval feeding both collecting-filtering in water column and collection-gathering at submerged surfaces (Clements, 1992) and then reached the alimentary tract of larvae. In order to find out whether it affected the alimentary system and/or Malpighian tubes, cellular obervations on these target cells or organs of the treated dead 4th larvae were conducted under fresh

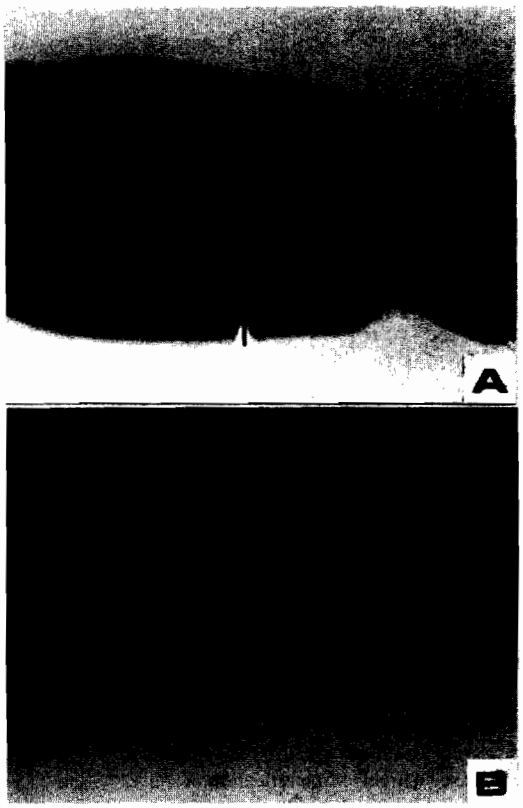

Fig 2– An alimentary canal and associated organs of 4th larva of *Cx. quinquefasciatus* after treatment with ethanolic extract of *K. galanga.* (A) Anterior part of stomach with intact epithelial cell lines (arrow). Note the middle, dark line in lumen is compact of food particles. The brain stem of 4th larva of *Cx. quinquefasciatus* after treatment with ethanolic extract of *K. galanga.* (B) Intact early stage of neuron stained with Giemsa, showing the dark blue color of Nissl substance (NSS) which is dustributed throughout the cytoplasm of cell body, except at the region of axon hillock (AH).

condition. Examinations of alimentary canal and Malpighian tubes revealed the normal contour of organs and intact of epithelial cell lines without evidence of vacoulization and/or degeneration. It is therefore demonstrated that the alimentary canal and Malpighian tubes were relatively unaffected. This finding excludes the alimentary system as the site of action. Thus, it could be concluded that the ethanolic fraction has a specific site of action on the anal gills, by destruction of the irregular ridge-like reticulum on the surface of gills which function as an ionic regulator.

It is known that the mosquito larvae in fresh water are stressed by water overload and scarcity of salts. Feeding and the flow of water into the animal across the cuticle results in excess water that the larvae must eliminate through Malpighian tubes and rectum. Scarcity of salts is compensated by taking up salts via the anal gills. Uptake of most ions occurs via this route. Ninety percent of the active exchange of sodium and chloride occurs across the anal papillae whereas uptake of potassium is passive (Clements, 1992; Beaty and Marquardt, 1996). The lower limits of ion concentrations that permit survival have not yet been established. The active uptake of ions by the anal gills. however, is an important aspect of ionic regulation. Dysfunction of anal gills by ethanolic extract treatment may be the principle cause of larval death. Green *et al* (1991) reported the highly swollen anal papillae of dead *Ae. aegypti* larvae after treatment with whole oil of *Tagetes minuta* (Marigold). They suggested that interruption of osmotic and ionic regulation may be at least part of mechanism causing the death of mosquito larvae.

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