
# THESIS

## THE NOVEL BOTANICAL INSECTICIDE, ALPHA-MANGOSTIN FROM MANGOSTEEN PERICARP EXTRACTS, FOR CONTROL OF *Nilaparvata lugens* (Stal.)

**VASAKORN BULLANGPOTI**

**GRADUATE SCHOOL, KASETSART UNIVERSITY**

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# THESIS APPROVAL

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**NAME**: Miss Vasakorn Bullangpoti

**THIS THESIS HAS BEEN ACCEPTED BY**

**THESIS ADVISOR**

( Associate Professor Suraphon Visetson, Ph.D. )

**COMMITTEE MEMBER**

( Assistant Professor John Milne, Ph.D. )

**COMMITTEE MEMBER**

( Assistant Professor Somchai Pornbanlualap, Ph.D. )

**COMMITTEE MEMBER**

( Mrs. Manthana Milne, Ph.D. )

**COMMITTEE MEMBER**

( Mr. Chaiwud Sudthongkong, Ph.D. )

**DEPARTMENT HEAD**

( Associate Professor Sompob Naveepab, M.S. )

**APPROVED BY THE GRADUATE SCHOOL ON** .....

**DEAN**

(Associate Professor Vinai Artkongharn, M.A. )

**THESIS**

# THE NOVEL BOTANICAL INSECTICIDE, ALPHA- MANGOSTIN FROM MANGOSTEEN PERICARP EXTRACTS, FOR CONTROL OF *Nilaparvata lugens* (Stal.)

VASAKORN BULLANGPOTI

**A Thesis Submitted in Partial Fulfillment of**
**the Requirements for the Degree of**
**Doctor of Philosophy (Zoology)**
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Vasakorn Bullangpoti 2007: The Novel Botanical Insecticide, Alpha-Mangostin from Mangosteen Pericarp Extracts, for Control of *Nilaparvata lugens* (Stal.). Doctor of Philosophy (Zoology), Major Field: Zoology, Department of Zoology. Thesis Advisor: Associate Professor Suraphon Visetson, Ph.D. 116 pages.

This research was done to evaluate the efficiency of mangosteen (*Garcina mangostana* L.) pericarp extract as an alternative control of the Brown planthopper, *Nilaparvata lugens* Stal (BPH) (Thailand strain). The pericarp of mangosteen fruit was extracted using ethanol, hexane, acetone and dichloromethane as separate solvent systems. The topical sprayer method was used to apply extracts against various stages of nymph and adult BPH to determine toxicity. The highest yield, *ca.* 29.46% w/w, was obtained for ethanol and exhibited LC50 at 4.5% w/v ($r^2$=0.95) against 3rd instar BPH. The active ingredient compound, alpha-mangostin (2.956% w/w) showed LC50 at 5.44% w/v ($r^2$=0.88). The ethanolic mangosteen pericarp extract produced less toxicity than imidacloprid, which showed LC50 at 0.0042% w/v ($r^2$=0.99) against the same stage of BPH. The toxicity to non-target organisms was determined with guppies (LC50 = 2.53 and 4.27 ppm for females and males, respectively; $r^2$ =0.97 and 0.97, respectively) and bees (LC50 = 4.38% w/v, $r^2$ =0.95). Furthermore, mice showed no acute toxic effects via oral injection and no dermal inflammation was recorded. On the other hand, they showed temporary eye irritation for 1 day. The *in vitro* detoxification enzyme activities of carboxylesterase, acetylcholinesterase and glutathione-s-transferase from BPH after 24 hours exposure were also observed. Carboxylesterase showed stronger activity than other enzymes. Toxicity in terms of LC50 values of both the extract and imidacloprid treatments increased in each generation. The LC50 values for each generation ca. 4.22 – 6.67 after sequential spray. Comparisons of carboxylesterase gene sequences between control, Genbank, imidacloprid treatment (F8) and mangosteen fruit extract treatment (F8) showed that sequences were homologous. After the ethanol extract was kept at 4 °C, room temperature and 55 °C for 3 months, the quantity of alpha-mangostin and the BPH control efficiency was lower at 55 °C than those for other temperatures.

_____ _____ ___ / ___ / ___

Student's signature Thesis Advisor's signature

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# THE NOVEL BOTANICAL INSECTICIDE, ALPHA-MANGOSTIN FROM MANGOSTEEN PERICARP EXTRACTS, FOR CONTROL OF *Nilaparvata lugens* (Stal.)

## INTRODUCTION

Rice has played an important role to the Thai people society for thousand of years dating back to the King Ramkamhang the Great in the 18th BE when he pronounced "In the water, there is fish, in the field, there is rice". Currently, Thailand has been one of the major rice exporters in the world showing the income of approximately 2,500 million dollars a years (Agro-economic, 2003).

Rice belongs to the grass family of Gramineae or Poaceae and is classified as *Oryza* genus. Rice grows well from the hot through the warm climate, covering from 43 degree north latitude to 35 degree south and can be grown on the various altitudes ranging from sea level to 2500 meters above sea level. Although, there are at least 23 species of rice grown in the world, only 2 species of rice, Asian rice (*Oryza sativum* Linn.) and African rice (*Oryza glaberrima* Steud.) are commonly cultivated for local consumption and exportation in the world. Of these two species, *Oryza sativum* Linn. is best known and commercially grown in Thailand.

Currently, Thai rice has faced a lot of problems, low quality soils, bad climate, out break of disease and most of all are the insect pest. There is brown planthopper (BPH) *Nilaparvata lugens* (Stål) (Homoptera: Delphacidae), whitebacked planthopper (WBPH) *Sogatella furcifera* (Horvath) (Homoptera: Delphacidae), green leafhopper (GLH) *Nephotettix virescens* (Distant) (Homoptera: Cicadellidae), Asian rice gall midge *Orseolia oryzae* (Wood-Mason) (Diptara: Cecidiomyiidae), and yellow stem borer (YSB) *Scirpophaga incertulas* (Lepidoptera: Pyralidae) (Vungsilabutr *et al*., 1995). Some of the insect pests mentioned above decrease both quantity and quality of rice products by transmitting of disease virus such as ragged stunt diseases and grassy stunt disease. Thailand lost ca.1.5 -1.8 million tons or ca. 5,000 – 6,000 million Bath causing by brown planthopper (Entomological and Zoological Division, 2000). Because Thai farmer have been using too many synthetic pesticides, so a lot problems occurred. There are cereal contamination, soil, water and air pollution, and most of all pest resistant.

In addition to the troublesome mentioned above, farmer health problems and insecticide effects on non target organisms as well as the competitiveness in the world rice markets are the most public concerns in Thailand. The government are trying to minimize the use of such the dangerous synthetic pesticides and introduce other alternatives namely, bio-botanical pesticides as the rice pest control. The crude extracts of insecticidal plants are the main targets. Unlike almost synthetic pesticides, they do not possess contaminants because they are biodegradable (Visetson *et. al*, 2002), giving no toxic effect to human beings (Udomchoak, 1985).

The successful researches on plant extracts against some key agricultural pests have been developing by many scientists. For example, in Thailand, a lot of

insecticidal plants have revealed good tendency for insect control, for example, Chili (*Capsicum frutescans* L.) for the control of *Sitophilus zeamais* Motschulsky (Bullangpoti *et al.,* 2002), derris (*Derris elliptica* L.) for the control of *Plutella xylostella* L. (Visetson, *et al*. 2001), lemon grass (*Cymbopogon winterinus*, Jewitti) for the control of *Culex pipien quinquefasciatus* (Thummasarangkoon, 2000), nudsage (*Cyperus rotandus* L.) for the control of *Pomacea canaliculata* L. (Ruamthum,2002), The fruit rind of mangosteen (*Garcina mangostana* L.), rambutan's seed (*Nephilium lappaceum* L.) and kaffir lime's leave (*Citrus hystix* DC.) for the control of *Sitophyllus oryzae* L. (Bullangpoti, 2004).

After trying to measure efficacy of all insecticidal plants for years, I have found that the pericarp of mangosteen fruit (*Garcina mangostana* L.) extract has been revealed promising efficiency on the rice weevils (*Sitophyllus oryzae* L.) and 3rd nymph brown planthopper under laboratory condition (Bullangpoti *et al.*, 2004 and Bullangpoti, 2004). Therefore in this research I was study the effect of mangostin from mangosteen pericarp compare efficiency with synthetic insecticides, imidacloprid as controlling of brown planthopper in terms of $LC_{50}$ moreover, the mechanisms of detoxification enzyme activities namely, acetylcholinesterase, carboxylesterase and glutathione-S-transferase, from all treatment were trailed using enzyme-substrate assays with spectrophotometer and the PCR method for determine the mechanism which insect use to be resistant. I am also study the toxicity of such extracts to non-target organisms such as fishes, bees and mouse in order to determine the safety commercial standard for the product. Finally, I was investigated the yield percentage and its efficiency of the extract when kept in the different temperature for determine the suitable temperature to keep the extract. The main objective of this research is to develop new botanical insecticide, mangostin from pericarp of mangosteen fruit, for controlling brown planthopper. Before developing it to be a commercial product, the efficiency of this extract must be investigated such as toxicological, physiological and biochemical data to the pest and non-target organisms. In short, the main objectives of this research can be concluded below;

1. To determine the toxicity of both crude extract, imidacloprid and purified ingredient from pericarp of mangosteen fruit compared with the synthetic insecticide, imidacloprid, against brown planthopper.
2. To identify detoxification mechanisms of brown planthoppers against crude extract and purified ingredient from mangosteen pericarp extract and against imidacloprid
3. To analyze changes in the detoxification enzyme gene, carboxylesterase, after sequential use of mangosteen pericarp extract and imidacloprid against brown planthopper populations.
4. To determine toxicity of mangosteen pericarp extract on non-target organisms such as fishes, bees, and mice.
5. To determine the change in active ingredient concentration and toxicity against brown planthopper of mangosteen pericarp extract after being stored at different temperatures.

# LITERATURE REVIEW

## The Importance of Brown Planthopper in the World

Brown planthopper (Figure 1) is distinguished from other hopper on the basis of small spines on the first tarsal segment (Mochida and Okada, 1979). The color of adult brown planthopper is yellowish-brown to dark brown. Genitalia of male are distinctive, with a slender and distinctly shaped.

(a) (b)

**Figure 1** Adult Brown planthopper: (a) macropterous (b) brachypterous
Source: Department of Agriculture Extension (2004)

The brown planthopper, *Nilaparvata lugen* (Stal) is classified as;

Phylum Arthopoda

Class Insecta

Order Hemiptera

Family Delphacidae

Genus *Nilaparvarta.*

The adult has two wing forms, short- winged (brachypterous) and long winged (macropterous) (Figure 1). The macropterous form is migratory and adapted to finding a new habitat, and branchypterous form is sedentary and adapted to breeding in a suitable habitat. Nymphs and brachypterous adults move by walking and hopping although macropterous adults move by flying, walking and hopping. Macropterous adults fly into a newly transplanted field and produce a small generation. The second generation is larger, with mostly brachypterous adults developing. These produce the third and occasionally most damaging generation (Mochida and Dyck 1977).

This insect is widespread in rice paddies throughout southern and eastern Asia as Bangladesh, Brunei, China, India, Indonesia, Japan, Kampuchea, Korea, Malaysia, Nepal, Taiwan, Philippines, Vietnam and Thailand. Not only in Asia, brown

planthopper widespread in Australia (Queensland) and Pacific Island (Caroline Islands, Fiji, Guam and Solomon Islands) (figure 2).

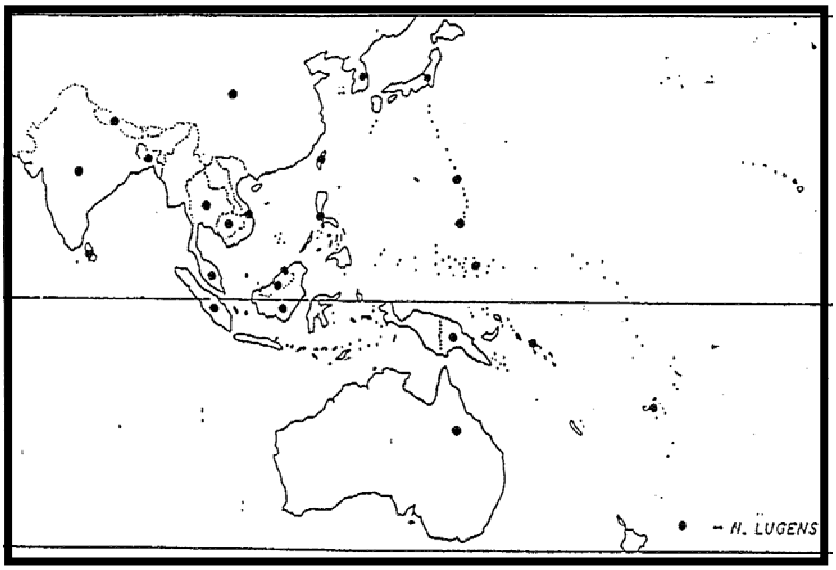



**Figure 2** Distribution of brown planthopper
Source: Tripop (1997)

The eggs of brown planthopper are laid in groups. The females usually lay their eggs in the tissue of the lower part of rice plants, mostly in leaf sheaths of rice plant. In populations with a high adult density eggs can be found in the upper part of rice plant (Mochida and Okada 1979). In the topics, the egg stage persists about 4 to 8 days (Figure 3). The effect of temperature on the development of the egg stage of brown planthpper that the shortest development time was at about 28 °C (Mochida and Okada, 1979). Pathak (1977) found that the brown planthopper eggs usually will not hatch at temperature greater than 33 °C.

The average nymph stage takes 15-16 days (Figure 3). Upon hatching, the lengths of the nymphs were about 0.6 mm. The nymphs molt 5 times before becoming adult (Vungsilabutr, 2002) and usually stay on the lower parts of the rice plants. The adult stage can survive up to 20 days (Figure 3). The brachypterous female takes about 16.5 days then lay their eggs *ca*. 485.8 eggs. The preoviposition period averaged 3 or 4 days for brachypterous female and 3 to 10 days for macropterous females.

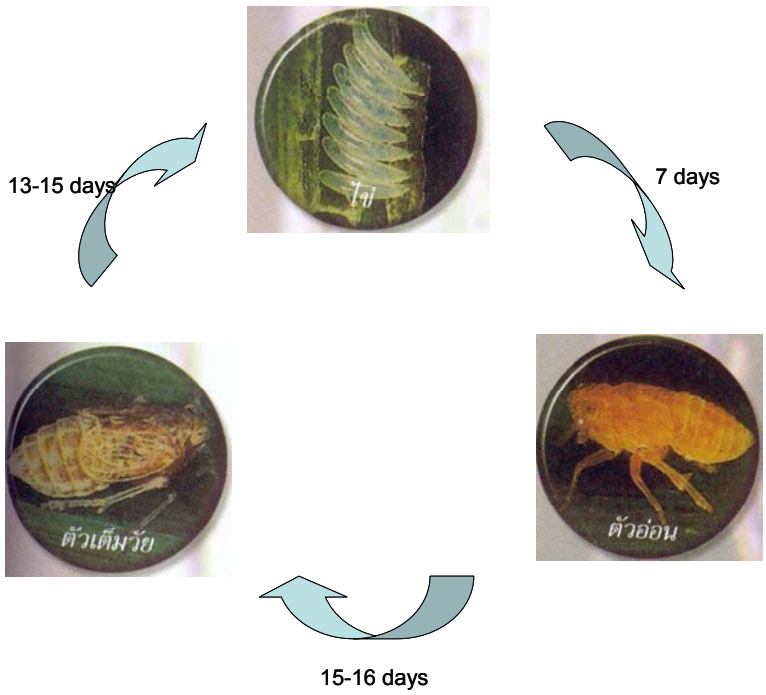



**Figure 3** Brown planthopper Life cycle
Source: Entomological and Zoological Division (2002)

Both nymphs and adults of the brown planthopper insert their sucking mouthparts into the plant tissue to remove plant sap from phloem cells. During feeding, brown planthopper secretes feeding sheaths into the plant tissue to form feeding tube or feeding sheaths. The removal of plant sap and the blockage of vessels by the feeding tube sheaths cause the tillers to dry and turn brown, a condition called "Hopperburn" (Figure 4). Moreover, the brown planthopper removes more plant sap than it digests. The excess plant sap, which high in sugars, is expelled from the body as honeydew. The honeydew is dropped on the base of plants and is turn black caused by the infection of sooty mold fungus.

Moreover, this insect also transmit virus as rice ragged stunt virus or grassy stunt virus to rice plants. Every problem causing from brown planthopper induce economic loss. For example, Thailand loss paddy yields to about 1.5 -1.8 million tons or about 5,000 -6,000 million baht in 2000 causing by brown planthopper (Vungsilabutr, 2002) or in 1990, the brown planthopper and the ragged stunt virus cause about 1 million tons (Sindhusake, 1990).

**Figure 4** Hopperburn caused by brown planthopper
Source: Entomological and Zoological Division (2002)

Currently, there are many methods which developed by scientists for control brown planthopper such as rice varieties and synthetic insecticides. Another method to control brown planthopper is the use of natural enemies such as *Anagrus optabilis* (Perkins) (Mymaridae), *Oligosita* sp. (Trichogrammatidae), *Tetrastichus* sp. (Eulophidae), *Elenchus yasumatsui*, *Conidiobolus coronatus* (Zygomycetes: Entomophthorales), *Cyrtorhinus lividipennis* Reuter (Miridae), *Lycosa pseudoannulata* (Bosenberg & strand) (Lycosidae) and *Tetragnatha* sp. (Tetragnathidae).

However, most method had little success to use in rice field at the long time because brown planthopper can become resistant to insecticides, there is limited control by natural enemies and also become adapted to resistant rice varieties as shown in many reports such as the report in Japan, benzene hexachloride (BHC) has been widely used for control brown planthopper since 1949. In 1967, brown planthopper collected from locations within the Hiroshima and Kyushu prefecture had a maximum of 9 fold resistance (Kimura *et al*., 1973; Nagata and Moriya, 1969), the cross resistance to dieldrin and fenitrothion was observed in a BHC –resistant strain of the brown planthopper, but no apparent cross resistance was observed to DDT and carbaryl (Kimura *et al*., 1973; Nagata and Moriya, 1969).

In 1988, the populations of brown planthopper which collected from Chachengsao, central region of Thailand, were found resistance to MIPC and BPMC (Budhasamai, 1990). In Taiwan reported that field strains of brown planthopper were highly resistance to permethrin, propoxur, permethrin, pyrethroids, parathion (Sun and Chung, 1982; Sun and Chen, 1986). In Indonesia study on brown planthopper resistance to insecticide that found brown planthopper was resistance to diazinon, carbaryl, fenitrothion, fenthion and isoprocarb (Sutrisno, 1987; Kilin *et al*., 1979; Soekarna and Kilin, 1988)

Many scientists believed that the resistance of the insect against the insecticides comes from the detoxification enzymes that the insect produces after exposure to the insecticides for a long period of time (Chen and Sun (1994), Small and Hemingway (2000), Vontas *et al*. (2001).

## Detoxification Enzymes as Indicators of Resistant Mechanisms

The metabolic transformation of detoxification enzyme mechanism is divided by toxicologist into two types of reactions known as metabolic transformation (Phase I) and conjugation (Phase II reaction). These reactions are catalyzed by microsomal enzymes. The systems of detoxification enzymes work like common enzymes. After xenobiotics enter into our body, they will be metabolized by microsomal enzymes into highly water soluble substances and then secreted out from body via bile, urine and sweat (Visetson, 1991). Many researchers reported that the major sites of detoxification enzymes in vertebrates are the liver, lung and intestine. But in insects these enzymes are accumulated in the intestine and/or adipose cells. The detoxification enzymes work on various substrates to reduce poisons. When insects are exposed to chemicals, they may change behavior known as behavior avoidance to decrease exposure to chemicals. If insects do not die, they collect substance in adipose tissue and other tissue. Insects have various behavior avoidance and various detoxification systems. Insects use detoxification enzymes to decrease poisons because it is very fast mechanism (Visetson, 2001).

### 1. Esterase

Esterase is the one of important detoxification enzyme in phase I. It is classified into main two group; A- type esterase including arylesterase that are not inhibited by organophosphate. Another one is B-type esterase such as carboxylesterase, aliesterase and cholinesterase. B-type esterase is inhibited by organophosphate due to irreversible phosphorylation of the active serine site (Dauterman, 1985)

B-type such as carboxylesterase and acetylcholinesterase are almost studied from many scientists because it plays important role which insect use to be resistant. For the example, Organophosphates usually process high levels of these enzymes (Mackness, 1983). Cholinesterases are capable of splitting acetylcholine to yield acetate and choline. The properties of cholinesterase in insects are similar to mammals. It is therefore called either acetylcholinesterase or cholinesterase. These enzyme is very sensitive to organophosphate inhibition (Visetson, 1991).

In insect, esterase founds in cytosol, microsome as well as mitochondria and nuclei. There are many report shows esterase play a significant role in the metabolism in organophosphate, pyrethroid and chitin inhibitor. In this thesis, I focus on acetylcholinesterase and carboxylesterase which many reports show it play important role in resistant.

#### 1.1 Acetylcholinesterase

1.1.1 Type of reaction. Acetylcholinesterase (AchE) insensitivity can also be a resistance mechanism in insects. Organophosphates and carbamate irreversibly bind to cholinesterase causing the phosphorylation and deactivation of acetylchoinesterase. The subsequent accumulation of acetylcholine at the neural

synapse causes an initial overstimulation followed by eventual exhaustion and disruption of neural transmission in the central and peripheral nervous systems

1.1.2 Role in resistance. It has been accepted that reduce sensitivity of acetylcholinesterase is significant factor in resistance to organophosphate and carbamate compounds (Hama, 1983). Insensitivity of AchE have been reported in a number of organophosphate and carbamate resistant strain of insects such as *M. domestica* (De Jersey *et al.,* 1985), *C. pipiens* (Tang *et al.,* 1990) and *L. hesperus* (Zhu and Brindly, 1990).

1.1.3 Method to determine activity. In this experiment, neither the substrate nor the products of enzyme-catalyzed reaction are easily measured, so an alternate reaction is used, where the artificial substrate acetylthiocholine iodide (ATCh) is hydrolyzed into acetate and thiocholine. The sulfhydryl group in thiocholine reacts with dithiobisnitrobenzoate (DTNB) to produce two compounds, 2-nitrobenzoate-5-mercaptoathiocholine and 5-thio-2-nitrobenzoate. The latter compound has a yellow color and can be measured in a spectrophotometer. The second reaction is rapid and complete. Therefore, the concentration of the final colored end product is a direct measure of the concentration of thiocholine produced.

1.2. Carboxylesterase

1.2.1 Type of reaction. carboxylesterase is one of detoxification enzymes in phase. Zhu and Brindley (1990) showed the highest specific activity of carboxylesterase was found in the mitochondrial fraction followed by the microsomal fraction; the activities in nuclei, cell debris and in the soluble fraction were relatively low. The mechanism of this esterase type to transforming the ester bond of xenobiotics to acid and alcohol is shown in Figure 5.

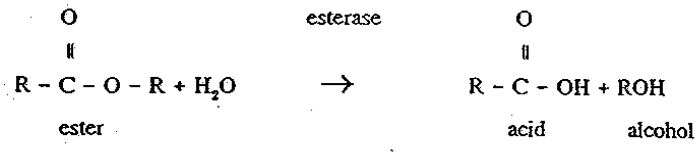



**Figure 5** Reaction catalyzed by esterase
Source: Visetson (1991)

1.2.2 Role in Resistance. There are many research that study role of carboxylesterase to detoxify toxic in brown planthopper and other insect pests such as Chang and Whalon (1987) observed that carboxylesterase hydrolysis was closely involved in Brown planthopper resistance to malathion while Dai and Sun (1984) subsequently proposed that this enhanced esterase activity also confirmed a major part of brown planthopper resistance to permethrin and other pyrethroids of primary alcohol esters, such as phenothrin. Furthermore, Chang and Whalon (1987) resolved eight esterases with pI ranging between 4.3 and 5.3 from multiresistant brown planthopper homogenate using isoelectric focusing and all eight forms were able to

hydrolyze alpha- and beta- naphthyl acetate, malathion, cis- and trans- permethrin at different rates.

Chen and Sun (1994) studied characteristic of esterase (carboxyesterase) in brown planthopper. They purified carboxyesterase from brown planthopper and suggested carboxylesterase of brown planthopper appeared to exert in the detoxication of insecticides such as catalytic protein for the hydrolysis of some insecticides such as malathion and trans-permethrin, and a binding protein for the oxons of some Organophosphates such as paraoxon and malaoxon, and possibly some carbamates and pyrethroid. Moreover, they suggested that gene encoding 3 isozymes, $E_1$, $E_2$and $E_3$ (and possibly other less active isozymes) was expressed to a greater extent in resistant strains than in susceptible ones. The isozymes might represent the products of different posttranslational modifications of the nascent protein.

1.2.3 Method to determine activity and purification The most common method for study multiple forms of insect esterase are by electrophoretic separation and determination of enzyme activity with color forming product. By using ion exchange chromatography, Kao *et al*., (1985) found multiple forms of four types of esterases. They presented the molecular weights of being 220 kDa. Chen and Sun (1994) use gel permeation/ chromatofocusing chromatography for purified carboxyesterase of a rice brown planthopper and used alpha-naphthyl acetate as substrate that observed molecular mass between 62 and 64 kDa and no difference in sensitivity toward the inhibition of paraxon, methyl paraoxon and malaoxon. Because all forms of esterase catalyzed hydrolysis reaction, therefore the uses of simple substrates are not likely to reveal differences in the level of each enzyme activity. On the other hand, Small and Hemingway (2000) purified BPH esterase by using Q-Sepharose, phenyl Sepharose and hydroylapatile column chromatography then were pooled and concentrated in an Amicon Centripep 10unit to a volumn of 25 ml. after purified they store enzymes at -20 °C that BPH esterase were stable for several months.

There are many substrates (*in vitro*) such as pNPA (paranitrophenyl acetate), ANA (Alpha naphthylacetate) , phenylacetate, MTB (Methyl thiobutylate) to detect levels of general esterase activity. Each Assay using different substrate can investigate by spectrophotometer with different absorbance. For the example, pNPA (for carboxylesterase) is 400 nm, phenyl acetate (for arylesterase activity) is 510 nm and MTB is 412 nm.

## 2. **Glutathione-S-transferases (GSTs).**

2.1 Type of reaction.

Glutathione –S- transferase is an important detoxification enzyme in phase II This enzyme catalyzes the conjugation of reduced glutathione with various compounds possessing an electrophilic center, include insecticides. GSTs are found in mammals, insects, protozoa, algae, fungus, and bacteria also in plants (Jakoby, 1978). Structure of GST consists of three amino acid, glutamic acid, cysteine and glycine.

They metabolized by several steps and excreted to form mercapturic acids for increase solubility in water which make the xenobiotic rapidly excretion out of the body. At the results, compounds may be dealkylated, dehalogenated or cyanide may be released from organic thiocyanates.

2.2 Role in Resistance.

High levels of glutathione – S - transferase activities are important in resistance to organophosphate and organochlorine (Motoyama and Dauterman, 1980). Vontas *et a*l. (2001) studied in brown planthopper with the pyrethroid permethrin resistance. Biochemical analysis and synergistic studies with metabolic inhibitors in their study indicated that elevated glutathione –S- transferase (GSTs) with a predominant peroxidase activity conferred resistance pyrethroid. Thus they hypothesize that the main role of elevated GSTs in conferring resistance in *N. lugens* is through protecting tissues from oxidative damage.

2.3 Method to determine enzyme activity and purification.

Vontas *et al*. (2001) purified GSTs from brown planthoppers using Q-Sepharose and S-hexylglutathione-agarose affinity chromatography. GSTs bound to the S-hexylglutathione-agarose were eluted with 5mM S-hexylglutathione. Fractions certaining enzyme were pooled and concentrated by Amicon Centriprep and buffers were exchanged using a PD-10 column.

3. **Enzyme preparation conditions**.

Enzyme activity from insect is usually inhibited by a number of endogenous inhibitors for example xanthommatin in the heads of houseflies, quinine in house flies (Motoyama et al., 1978). Several inhibitors are presented in whole homogenates of different insect species, sex, tissue and organ (Visetson, 1991). A variety of chemicals as thiols can protect activity of enzymes during homogenization. They are DTT, PVPP, EDTA, PVP, PMSF, reduced gluthathione, mercaptoethanol etc. Moreover, the accuracy of enzyme activity analysis depends on temperature, species, age, exposure time, sex, substrate, organ, behavior feeding, nutritional status in insects and solvent system (Visetson *et al.*, 2004) and Tyler and Binns (1982) also suggested that the most appropriate temperature for laboratory toxicity evaluation of insecticides appears to be less than 25°C for most insects.

Currently, there are many assays for the determination of protein concentration in a solution such as the Biuret, Lowry and Bradford assay. The criteria for choice of a protein assay are usually based on convenience, availability of protein for assay, presence or absence of interfering agents, and need for accuracy. For example, the Lowry method is very sensitive but is a two step procedure that requires a minimum of 40 minutes incubation time. The Bradford assay is more sensitive and can be read within 5 minutes, however proteins with low arginine content will be underestimated.

In this thesis, the Bradford assay is used for determine protein concentration. This method is very fast and uses about the same amount of protein as the Lowry assay. The assay is based on the observation that the absorbance maximum for an acidic solution of Coomassie Brilliant Blue G-250 shifts from 465 nm to 595 nm when binding to protein occurs. Both hydrophobic and ionic interactions stabilize the anionic form of the dye, causing a visible color change. The assay is useful since the extinction coefficient of a dye-albumin complex solution is constant over a 10-fold concentration range. It is fairly accurate and samples that are out of range can be retested within minutes. The Bradford assay is recommended for general use, especially for determining protein content of cell fractions and assessing protein concentrations for gel electrophoresis.

## 4. Kinetic of enzyme inhibition

There are three types of reversible inhibitor. The binding of these inhibitors to the active site of the enzyme may result in decrease of Km or/ and Vmax. Km is the concentration of substrate at which half the active site of the enzyme is full or V= 1/2 Vmax and can obtained experimentally from the graph. Low Km value means that substrate bind tightly to enzymes. Vmax is the maximum rate or velocity of an enzyme in a defined reaction mixture. Vmax is achieved only when all of the active site of enzyme is saturated or occupied by substrate. In other words, Vmax obtained must be equal to the total number of enzyme ($[E]_t$) existed in the reaction mixture. However, Vmax can be determined by measuring the enzyme activity at various substrate concentrations and its value can be obtained directly from plot or graph. (Pornbanlualap, 2003; David and Michael, 2005).

### 4.1 Competitive inhibitor

This inhibitor is molecule that binds to the enzyme in such a way that prevent substrate binding or mutually exclusive binding at active site of enzyme. A competitve inhibitor is a non-metabolizable (cannot converted to product) and structurally similar to the substrate, transition state or product of the reaction. The equation for competitive inhibitor can be converted into double reciprocal form that shown affects Km only and not Vmax, because infinitely high concentration of substrate can displace all the inhibitor from the active site of enzymes.

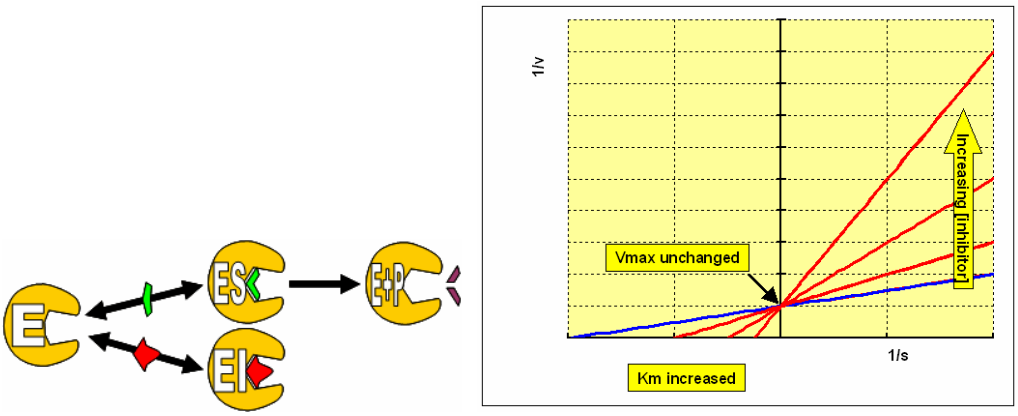



**Figure 6** Characterization of competitive inhibitor

4.2 Non- competitive inhibitor

This inhibitor bounds to enzymes, decrease the enzyme activity but no effect on substrate binding. In other word, non competitive inhibitor affects Vmax but not Km (Vmax change only). Non competitive inhibitor and substrate bind reversible to enzyme at a different site with same affinity. Similar, substrate can bind to both free enzyme (E) and enzyme-inhibitor complex (EI) with same affinity. Although binding of inhibitor to the enzyme has no effect on binding constant of substrate, however, the resulting complexes, EI and ESI, is catalytically inactive. When bound to the enzyme in E or ES, inhibitor causes distortion in catalytic sute of enzyme so that the catalytic site is not proper position to attack the substrate. Thus, EI and ESI are catalytically inactive. Moreover, in double reciprocal plot, [I] appears only in both intercept and slope.

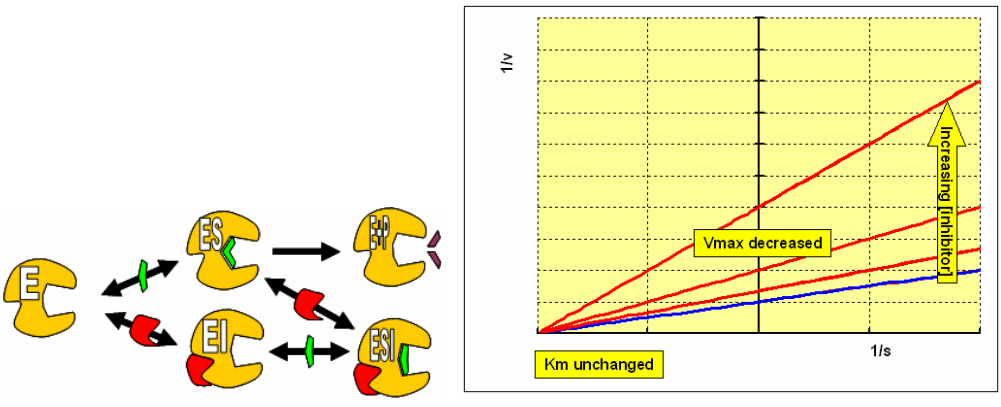



**Figure 7** Characterization of non-competitive inhibitor

4.3 Uncompetitive inhibitor

An inhibitor that binds only enzyme-substrate complex ([ES]) but not free enzyme is called an uncomtitive inhibitor. Uncompetitive inhibitor binds reversibly to the ES complex and resulted in active ESI complex. There is extremely few pure uncompetitive inhibitor found in biological system, a rare example of this inhibitor type of a single substrate enzyme is the inhibition of alkaline phosphtase by L-phenylalanine. This inhibitor binds at one site the substrate at the other. This uncompetitive inhibitor affects both Vmax and Km (Vmax and Km change). Thus, [I] appears only in intercept of double reciprocal.

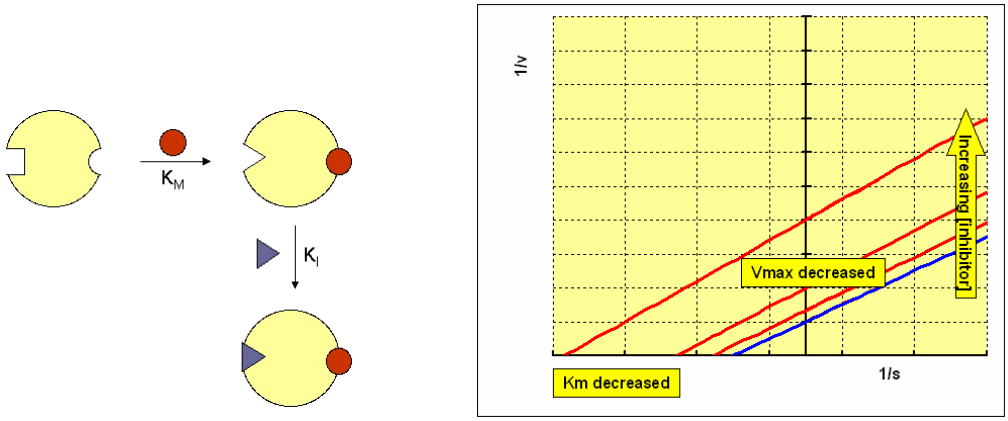



**Figure 8** Characterization of competitive inhibitor

## 5. The molecular study of gene responsible for detoxification enzyme expression

In the present, molecular analysis techniques are almost study in entomological study because they display single or multi locus banding pattern of generally easily interpretable Mendelian nature and the statistics for their analysis are well established. Molecular techniques are also suitable for use with small amounts of insect material and can be used with store, dry or old samples (Loxdale and Lushai, 1998). Insecticide resistant of insect can occur from mutation of gene as show in many reports such as Small and Hemingway (2000) and Feyereisen (1995).

The detection of insecticide resistance can be done by using molecular techniques with resistant gene compare with the susceptible. The method which almost use for detect insectide resistance is the method using PCR technique (Changsang, 2003). The biochemical/ physiological mechanisms of resistance can be categorised as target site resistance, increase metabolic detoxification and sequestration or lowered availability of the toxicant. There are achieved many method show the detection at the molecular level such as point mutation, amplification in gene cuases the resistant in insect pests; for the example, there are some report show amplification of an identical gene to that in insecicide-susceptible insects (Devonshire, 1977), the amplified esterase in mosquito (resistance stain) differ form, and code for protein which have a affinity for organophosphate than susceptible

equivalents (Small *et al,* 1998). In several cases, the selection of precisely homologous mutation has been observed in different insect species (Feyereisen, 1995; Enayati *et al.,* 2003; Soderlund and Knipple, 2003).

The elucidation of the molecular basis of insecticide resistance to many synthetic insecticides, target gene mostly in the nervous system sush as GABA gene or Acetylcholinesterase gene have been identified. Anyway, in the present, the cloning of resistance genes has enabled to address fundamental questions relevant to the selection if these adaptive traits (Richard *et al*, 2004).

Non-silent point mutation within structure genes are the most common cause of target site resistance. For selection of the mutation occur, the resultant amino acid change must reduce binding sites of the insecticide without causing a loss of primary functional of the target site. Therefore the number of possible amino acid subsitutions is very limited. Thus, identical resistance associated mutations are commonly found across highly diverged taxa. The degree to which function is impaired by the resistance mutation is reflected in the fitness of resistant individuals in the absence of insecticide selection. This fitness cost has important implifications for the persistence of resistance in the field.

In this research, I studied the molecular changing of carboxylesterse gene after sequential use the mangosteen pericarp fruit extract and imidacloprid on brown planthopper (F0 and F8). The carboxylesterase gene sequence of brown planthopper was obtained from the Genbank data from the report of Small and Hemingway (2000) (acc: 30277).

## Mangosteen

Mangosteen (*Garcina mangostana* L.) is classified in family GUTTIFERAE, genus Garcina and Thai people called mangosteen as Mag kut (Smitinand, 2001). Mangosteen is one of praised tropical fruits. This kind of fruit is usually eaten as dessert; furthermore, they can be made processed products such as juice, jam, jelly and sugar. The seeds are sometimes eaten alone after boiling or roasting.

### 1. Pericarp active ingredient and pharmacological data.

Asai *et al.* (1995) described chemical constituents in pericarp of mangosteen consist of xanthone derivative as alpha mangostin, beta mangostin, gamma mangostin, gartanin, garcinone E, 1,5-dihydrozy-2-(3-methylbut-2-enyl)-3-methoxy-, 1-7-dihydroxy-2-(3-methylbut-2-enyl)-3-methoxyxanthone and mangostinone (Figure9).

Chairungsrilerd *et al.* (1996) described the fruit hull (pericarp) of mangosteen is used as an anti-inflammatory agent, astringent and in the treatment of diarrhea.

They reported the yellowish excretion of the fruit hull, alpha mangostin, gamma mangostin and mangostanol showed an inhibitory effect on cAMP phosphodiesterase.

Moreover, extracts of the pericarp of the ripe fruits have been show to have immunomodulating (Chanarat *et al.*, 1997; Gopalakrishnan *et al.*, 1980), anti-bacteria (Iinuma *et al.*, 1996), anti-mutagenic (Edenharder and Tang, 1997), anti-cancer (Liou *et al.*, 1993; Lin *et al.*, 1996; Chi *et al.*, 2002).

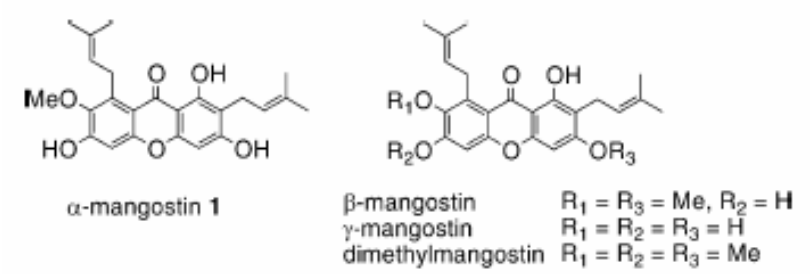



**Figure 9** Structure of Mangostin and its derivatives

Source: Hamada *et al*. (2003)

**2. Method for purification of active ingredient**.

Currently, the common methods that had been developed to purify active ingredient from mangosteen generally involved using silica gel column chromatography. Chairungsrilerd et al. (1996) extracted fresh fruit hull (Pericarp) of *G. mangostana* with n- butanol and purified the extract on silica-gel column then eluted with $CH_2Cl_2$, $CH_2Cl_2$-MeOH (9:1), $CH_2Cl$-MeOH (4:1) and $CH_2Cl_2$-MeOH (1:1), respectively. Relatively less polar fractions gave known compound as alpha-mangostin, gamma mangostin and gartanin *etc.* They identified known compounds from their MS, IR, 1H and 13C NMR Spectra. Successive purification of polar fraction by reverse phase HPLC using aq. MeOH as an elution gave mangostanol.

Govindachart et al. (1971) used silica gel column to purify active ingredient of different part of mangosteen with various elution. For example, partially ripe fruit was chromatographed using silica gel and eluted with benzene. First 200 ml of eluted gave no material. The next 250 ml gave beta mangostin and the later 300 ml gave mangostin.

Sakagami *et al*. (n.d.) extracted and isolated alpha-mangostin and beta mangostin from stem bark of *G. mangostana* which was dried, powdered and extracted with hexane, methylene chloride and methanol, respectively then used silica

gel column chromatography with hexane, methylene chloride and methanol as solvent. The hexane extract and methylene chloride extract gave two major compounds, alpha mangostin and beta-mangostin as yellow needles.

Chi *et al.*, (2002) described isolation method which the hull (pericarp) of mangosteen fruit was first dried at 60 ºC in an oven for 24 hour and 3 kg batches of the dried hulls were extracted with ethanol (25l) at 50 ºC for about 16 hours. The extracts were pooled, and concentrated by evaporation and then allowed to partition into aqueous and organic (EtOAc) phase. The EtOAc soluble fraction was then loaded into a silica gel column and eluted with gradient solvent system consist of: 10 l aliquots each of n-hexane-EtOAc (20:1, 10:1 and 5:1), 7 l aliquots each of $CH_2Cl_2$-$Me_2CO$ (10:1, 5:1 and 0:1) and aliquots each $CH_2Cl_2$-MeOH (10:1, 5:1 and 0:1) to yield 22 (~3 l) fractions. Fraction 13, the $CH_2Cl_2$- $Me_2CO$ (10:1) eluted fraction, was further chromatographed on a silica gel column equilibrated and eluted with n-hexane –EtOAc (5:1) to yield alpha mangostin or in Sephadex LH20 column equilibrated with MeOH-$H_2O$ (5:1) to yield gramma- mangostin.

For the isolation method in this research, above method from various scientists are modified. After trying to isolate constitutes from pericarp of mangosteen fruit, each constituents will study their efficiency to controlling brown planthoppers and will try to formulation as pesticide or other purpose.

## Imidacloprid

Imidacloprid is a widely used insecticide with relatively low human toxicity. It has raised concerns because of its possible impact on bee populations, ability to cause eggshell thinning in birds, and reduced egg production and hatching success. (Buffin, 2003) This synthetic insecticide is a systemic, chloro-nicotinyl insecticide with soil, seed and foliar uses for the control of sucking insects including rice hoppers, aphids, thrips, whiteflies, termites, turf insects, soil insects and some beetles. It is most commonly used on rice, cereal, maize, potatoes, vegetables, sugar beets, fruit, cotton, hops and turf, and is especially systemic when used as a seed or soil treatment. The chemical works by interfering with the transmission of stimuli in the insect nervous system. Specifically, it causes a blockage in a type of neuronal pathway (nicotinergic) that is more abundant in insects than in warm-blooded animals (making the chemical selectively more toxic to insects than warm-blooded animals). This blockage leads to the accumulation of acetylcholine, an important neurotransmitter, resulting in the insect's paralysis, and eventually death. It is effective on contact and via stomach action (Kidd and James, 1991).

Imidacloprid based insecticide formulations are available as dustable powder, granular, seed dressing (flowable slurry concentrate), soluble concentrate, suspension concentrate, and wettable powder. Typical application rates range from 0.05 - 0.125 pounds/acre. These application rates are considerably lower than older, traditionally

used insecticides. It can be phytotoxic if it is not used according to manufacturer's specifications, and has been shown to be compatible with fungicides when used as a seed treatment to control insect pests

**1. Trade name.**

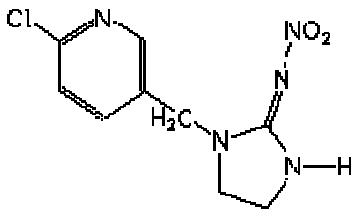
Imidacloprid is found in a variety of commercial insecticides. The products Admire, Condifor, Gaucho, Premier, Premise, Provado, and Marathon all contain imidacloprid as the active ingredient

**2. IUPAC name**

(*EZ*)-1-(6-chloro-3-pyridylmethyl)-*N*-nitroimidazolidin-2-ylideneamine

**3. Chemical structure**

$C_9H_{10}ClN_5O_2$




**4. Toxicity** (Buffin, 2003)

4.1 Acute toxicity.

The imidacloprid active ingredient is considered by the World Health Organisation to be moderately toxic. In laboratory animals, symptoms of acute (short term) oral exposure to imidacloprid included apathy and laboured breathing which lasted for five days. The $LD_{50}$ for imidacloprid (an oral dose that results in mortality to half of the test animals) is 450 mg/kg body weight in rats and 131 mg/kg in mice. The 24-hour dermal $LD_{50}$ in rats is >5,000mg/kg. It is considered non-irritating to eyes and skin from tests on rabbits.

Symptoms following acute exposure to the agricultural imidacloprid formulation (imidacloprid and inert product) included reduced activity, lack of coordination, tremors, diarrhoea and weight loss. Some symptoms lasted up to 12 days after exposure, twice as long as the symptoms of exposure to the active ingredient imidacloprid alone.

4.2 Chronic toxicity.

Chronic feeding studies with rats showed that the thyroid is especially sensitive to imidacloprid. Thyroid lesions were caused by doses of 17 mg/kg of body weight per day in males. Slightly higher doses of 25 mg/kg per day reduced weight

gain in females. At still higher doses such as 100mg/kg per day, effects included atrophy of the retina in females.

4.3 Mutagenic effects.

Imidacloprid may be weakly mutagenic. In tests of the ability of imidacloprid to cause genetic damage submitted to the EPA as a part of the registration process, no evidence of genetic damage was found, or evidence only at high exposures. However, a new technique that looks at the ability of a chemical to cause genetic damage by chemically binding to DNA found that the imidacloprid insecticide Admire, increased the frequency of this kind of damage. DNA adducts (the binding of a chemical to DNA) were five times more common in calf thymus cells exposed to Admire than in unexposed cells.

4.4 Reproductive effects.

Laboratory studies on imidacloprid have shown it can have an impact on reproduction. Imidacloprid fed to pregnant rabbits between the sixth and eighteenth days of pregnancy caused an increase in the frequency of miscarriages and an increase in the number of offspring with abnormal skeletons. These effects were observed at a dose of 72mg/kg per day. In rats, a two-generation feeding study found that rats fed imidacloprid gave birth to smaller offspring; their weight was reduced at a dose of 19 mg/kg per day.

**5. Usage**

Imidacloprid is a relatively new insecticide, having first been registered for use in the UK in 1993 and in the United States in 1994. It is a systemic insecticide, chemically related to the tobacco toxin, nicotine. It works by blocking the elements of the insect nervous system which are more susceptible to the toxic effects of imidacloprid than those of warm blooded animals.

Imidacloprid is manufactured by Bayer CropScience. Since its launch in 1991, products containing imidacloprid have gained registrations in about 120 countries and are marketed for use on over 140 agricultural crops. With annual sales of more than 600 million Euro in year 2001, imidacloprid is one of the top selling products of Bayer CropScienc. It is marketed under a variety of names including Gaucho, Admire, Confidor and Winner (Buffin, 2003).

Imidacloprid has a wide range of uses – soil, seed and foliar. It is used to control sucking insects such as rice-, leaf- and plant hoppers, aphids, thrips and whitefly. It is also effective against soil insects, termites and some species of biting insects, such as rice water weevil and Colorado beetle but has no effect on nematodes or spider mites. It can be used as seed dressing, as soil treatment and as foliar treatment in different crops including rice, cotton, cereals, maize, sugar beet, potatoes, vegetables, citrus fruit, apples and pears, and stone fruit. In European countries such as France, UK, and Holland, imidacloprid is widely used as an insecticide in sugar

beet crops (Buffin, 2003). However, imidacloprid can be phytotoxic (toxic to plants) if not used according to manufacturers instructions and it has a tendency to reduce seedling emergence.

**6. Ecological effects**

The acute toxicity of imidacloprid varies widely among bird species. It is highly toxic to certain species including house sparrow, Japanese quails, canaries and pigeons. Based on these tests, the ecological effects branch of the US Environmental Protection Agency (EPA) concluded that their levels of concern were exceeded for both non-endangered and endangered songbirds. Imidacloprid causes abnormal behaviour such as lack of coordination, lack of responsiveness and an inability to fly, even in birds for which it is not highly toxic, such as mallards. Other problems include eggshell thinning (at exposures of 61mg/kg), decreased weight (at exposures of 150 parts per million (ppm) in food) and reduced egg production and hatching success (at exposure of 234 ppm in food). In studies with red-winged blackbirds and brown-headed cowbirds, the birds learned to avoid imidacloprid treated seeds after experiencing transitory retching and loss of co-ordination (Buffin, 2003; Kidd and James, 1991).

6.1 Aquatic species.

Imidacloprid is acutely toxic to adult fish at relatively high concentrations (over 80 ppm) with juvenile fish being considerably more susceptible. It is however extremely toxic at low concentrations to some species of aquatic animals, including the freshwater crustacean Hyalella aztecais, and the estuary crustacean Mysidopsis bahia.

6.2 Beneficial insects.

The application of imidacloprid by foliar spraying, is highly toxic to honey bees. Imidacloprid is acutely toxic to earthworms, for example the LC50 (the lethal concentration required to kill 50% of a test population) of the species Eisenia fetida is between 2 and 4 ppm in the soil. At lower concentrations, the activity of the enzyme cellulase in the soil, that allows the break down of plant litter is reduced by imidacloprid concentrations of 0.2 ppm.

Soil application of granular imidacloprid is the most common. This is less harmful than the foliar spray because it is less likely to come into direct contact with non-target insects. However many natural enemies supplement their diet by feeding on plant material. As imidacloprid is systemic it can be translocated to the surface of the plant, increasing the chances of direct contact with insects on the plants. Laboratory tests have also shown that imidacloprid is acutely toxic to a variety of predatory insects including mirid bugs, ladybirds and lacewings.

6.3 Food contamination.

Little information about monitoring of imidacloprid in food crops is publicly available. The US Department of Agriculture and the Food and Drug Administration (FDA) do not include imidacloprid in their food monitoring programs. However, two published studies from Spain have found, in one case, that all the greenhouse vegetables tested one week after treatment contained residues and, in another case found imidacloprid in tomatoes, potatoes, peppers, carrots, eggplants, pears and melons. Twenty-one percent of the samples were contaminated.

6.4 Water contamination.

According to the US EPA, imidacloprid, has the potential to leach to ground water. In addition, high solubility and mobility are concerns for transport to surface water by dissolved runoff. The half life (the amount of time required for half of an applied pesticide to break down or move away from the test site) of imidacloprid in water was much greater than 31 days at pH 5, 7, and 9. Its ability to move through soil has been tested, along with other widespread water contaminants and it was found to be the quickest. Nevertheless, the EPA did not classify imidacloprid as a restricted use product, probably for economic reasons.

**7. Physical Properties.**

This chemical has appearance as colorless crystals with a weak characteristic odor with molecular weight 255.7. The water Solubility is 0.51 g/l (20 degrees C) and the solubility in other Solvents at 20 degrees C: dichloromethane - 50.0 - 100.0 g/l; isopropanol - 1.0-2.0 g/l; toluene - 0.5-1.0 g/l; n-hexane - <0.1 g/l; fat - 0.061 g/ 100g. Melting point data is 136.4-143.8 degrees C., 143.8 degrees C (crystal form 1) 136.4 degrees C.

My research is concentrate on the determination of the toxicity of both crude and purified pericarp of mangosteen fruit extract in term of $LC_{50}$ against every stage nymph and adult BPH compare toxicity with representative chemical insecticide, imdacloprid which widely use in Thailand. The identification of detoxification mechanisms against all treatments were going to be done.

Toxicity to non-target organisms such as bees, fishes and mouse were observed after treated with the crude extracts which will be develop as formulated product. The formulation was analyses as quality and quantity of product after time changing. Moreover, the sequential use of formulated product will compare efficiency with imidacloprid to estimate trend to be resistant via toxicity data and detoxification enzyme mechanism analysis which using in vitro analysis and molecular analysis. Thus, this research will evaluated the tendency of this extract in commercial scale in the future.

# MATERIALS AND METHODS

## Materials

1. Laboraratory animal uses
   1.1 Brown planthoppers ( *Nilaparvata lugens* Stal.) were received from the Pathumthani Rice Research Center, Ministry of Agriculture and Cooperative, Pathumthani, Thailand.
   1.2 ICR mouse from Animal Laboratory Institute, Mahidol University
   1.3 Guppies (*Poecilia reticulata*) from Noi Farm, Pathumthani province, Thailand
   1.4 Honey Bee (*Apis melifera*) in Kasetsart University, Bangkok

2. Material and Equipment for massing brown planthopper
   2.1 Paddy rice, RD7 from the Pathumthani Rice Research Center, Ministry of Agriculture and Cooperative, Pathumthani, Thailand.
   2.2 Plastic boxes (20 x 10 x 4 cm)
   2.3 Cage (24 x 24 x 24 inch)

3. Material and Equipment for extraction and isolation
   3.1 Ethanol
   3.2 Hexane
   3.3 dichloromethane
   3.4 Acetone
   3.5 Soxhlet apparatus
   3.6 Rotary evaporator (Buchi B-850)
   3.7 Mixer (Buchi Model 5000)
   3.8 Thimble
   3.9 Hot air oven (Memmert-600)
   3.10 Mangosteen fruit from Neuw Suthi Farm, Nakhon Si Thammarat province, Thailand.
   3.11 Column chromatography
   3.12 filter paper
   3.13 Silica gel (Merck)
   3.14 Cotton
   3.15 HPLC (Hewlett Packard series1100) with column (Synergi 4u hydro –RP 80A, 150x4.06 mm 4 micron)
   3.16 Nuclear Magnetic Resonance (AVANCE 300 MHz)
   3.17 Infared Spectrocopy (Bruker)
   3.18 Mass spectrophotometer (microTOF 72)
   3.19 Tank of TLC analysis

4. Material and Equipment for toxicity to brown planthopper and non-target organisms analysis
   - 4.1 Spreader 100ml
   - 4.2 Glass plate
   - 4.3 Filter paper
   - 4.4 Refrigerator
   - 4.5 Tank for fish experiment (30 X 30 X 30 cm)
   - 4.6 Box for Bee experiment (7 x 5 x 4 cm)
   - 4.7 Cage and room for mouse experiment 25°C, 70%RH day: night period (16:8)
   - 4.8 Imidacloprid (Confidor® 100 SL, Bayer CropScience)
   - 4.9 Distilled water

5. Material and Equipment for detoxification enzyme analysis
   - 5.1 Micropipette (NICHIRYO)
   - 5.2 1.5 ml polystylene cuvette (Bio-Rad)
   - 5.3 Refrigerate high speed centrifuge
   - 5.4 Spectrophotometer with Kinlab program
   - 5.5 Albumin from bovine serum (Sigma)
   - 5.6 CDNB (1- Chloro -2, 4- dinitrobenzene) (Sigma)
   - 5.7 Coomassie Brilliant Blue R (Sigma)
   - 5.8 Ethylene diamine tetra acetic acid (EDTA) (Fluka)
   - 5.9 Glutathione reduced form (Sigma)
   - 5.10 Paranitrophenyl acetate (p-NPA) (Sigma)
   - 5.11 Polyvinylpolypyrolidone (PVPP) (Sigma)
   - 5.12 Phosphoric acid (Sigma)
   - 5.13 Potassium Phosphate (Sigma)
   - 5.14 DCNB (1, 2- dichloro- 4,nitrobenzene) (Fluka)
   - 5.15 Mortar

6. Material and Equipment for molecular analysis
   - 6.1 Autoclave
   - 6.2 Thermocycler (GeneAmp ® model 9700)
   - 6.3 1.5, 0.2 ml polystylene tube (PCR- Tube) (Corling)
   - 6.4 Agarose (molecular biology grade) (Research organics, USA)
   - 6.5 Ethanol Absolute (Sigma)
   - 6.6 Ethidiumbromide (Bio-Rad)
   - 6.7 TAE (Tris – Acetate – EDTA Buffer) (Bio –Rad)
   - 6.8 Trizol (Invitrogen®)
   - 6.9 RNase-free water for RNA elution (not DEPC treated) (Invitrogen)
   - 6.10 Isopropanol (Sigma)
   - 6.11 BCP (1-bromo-3-chloropropane)
   - 6.12 Superscript III reverse transcriptase one step (Invitrogen®)
   - 6.13 Primer CarF and Car R (Operon®)
   - 6.14 Depth freeze

## Methods

### 1. Mass rearing of brown planthopper

The adult brown planthopper was received form the Pathum Thani Rice Research Center, Ministry of Agriculture and Cooperative, Pathumthani province, Thailand (Figure8). They were kept in the cages (24 x 24 x 24 inch) at Department of Zoology, Faculty of science, Kasetsart University at 28 – 33°C and day/night period was adjusted about 16:8 hours (Figure 11 and 12). A rice variety susceptible to *Nilaparvata lugens* (Stal.) i.e., RD7from Pathum Thani Rice Research Center was planted in the plastic boxes (20 x 10 x 4 cm) with 4 cm depth of soil and was placed in the cage when the rice seedling was about 10 days old (Figure 10). The rice seedling was changed when the leaf turn yellow and dry up. All stage of nymphs and adults brown planthopper were used in this thesis.

**Figure 10** RD7 Rice for mass rearing brown planthopper

**Figure 11** Brown planthopper on Rice Plant

**Figure 12** Cage for mass brown planthopper at Department of Zoology, Faculty of Science, Kasetsart University.

**2. Extraction of compounds from the pericarp of mangosteen fruit**

Pericarps of mangosteen fruit (20kg) collected on May 2004 from Neuw Suthi Farm, Nakhon Si Thammarat (600 kms south of Bangkok, Thailand) were dried under a hot air oven (Memmert- 600) and were powdered by mixer (BUCHI Mixer model 5000) then were extracted using Soxhlet extractor (Figure 13-14). Ethanol, acetone, dichloromethane and hexane were used as solvent systems (15g Dried mangosteen pericarp powder/ 300 ml solvent/ extraction). The temperature of extraction process was set at 75 °C for 8 hours. Each extract were concentrate in a rotary evaporator (BUCHI- B850) to give a brown sticky semi-solid for ethanol and acetone and yellowish powder for n-hexane and dichloromethane (Figure 15). The best control efficiency of the crude extracts was isolate using column chromatography and thin layer chromatography.

**Figure 13** Dried Pericarp of mangosteen fruit

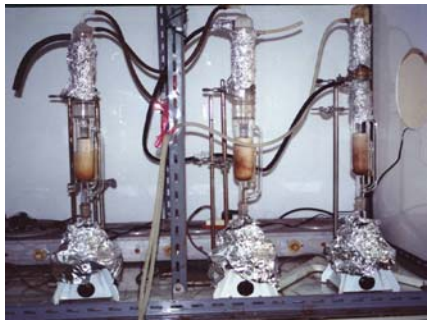
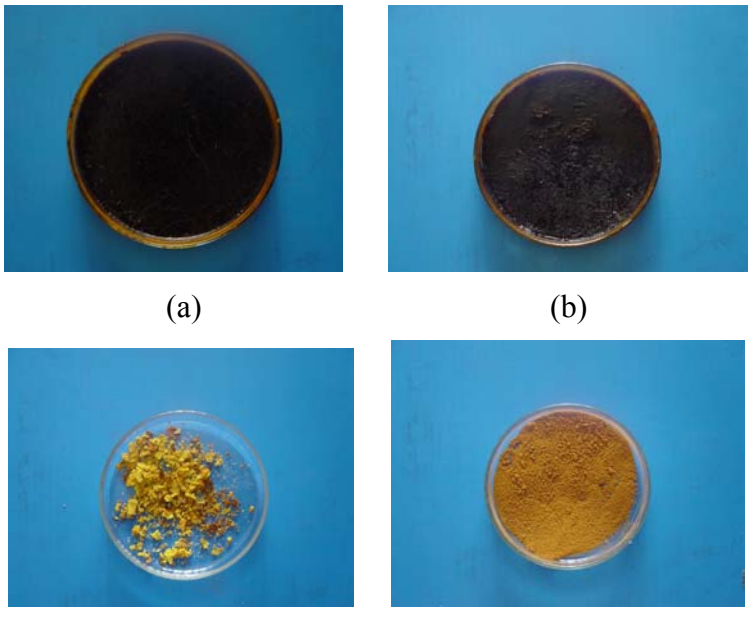


**Figure 14** Soxhlet apparatus



(a) (b)

(c) (d)

**Figure 15** Pericarp of mangosteen fruit extract which extracted with various solvents: (a) ethanol (b) acetone (c) hexane and (d) dichloromethane

**3. Isolation of active compounds**

After test efficiency in each extract, the extract with best control efficiency was isolated for analyze the active ingredient compound ans this extract was used throughout this research. The method to isolate compounds from pericarp of mangosteen fruit procedure was based on those of Chirungsrilerd et *al*. (1996), Sakagami *et al.* (n.d.) and Chi *et al*.(2002) (Figure 16).

The crude extract (12g) was subjected to column chromatography (gravity) over silica gel (60-120 mesh, 120g) to yield twelve fractions, 1-12, on the successive elution with dichloromethane: hexane system, of 20:80, 25:75, 30:70, 40:60, 50:50, 60:40, 80:20 and 100:0, respectively. Fraction 6-8 was re-chromatograph (gravity) over silica gel (60-120 mesh, 10g) with dichloromethane: hexane (50:50) to yield nine fraction, 1-9, fraction 6 (2.956%w/w) gave the main component, a yellowish crystalline compound (Figure 17). This compound was confirmed the purification by Thin Layer Chromatography with dichloromethane: hexane system (50:50) under UV condition and continue to elucidate the structure by 2D-NMR, IR and MS at department of chemistry, Mahidol University and compare structure spectrum data with previous paper.

**Figure 16** Isolation experiment using column chromatography as mentioned in the texts.

**Figure 17** Alpha Mangostin compound after isolated crude mangosteen pericarp fruit extract by column chromatography method

**4. Toxicity analysis method for mangosteen pericarp extract and imidacloprid.**

This research uses Completely Randomized Design with 5 replicates and 60 sampling of *Nilaparvata lugens* (Stal.) unit each replicate. In the experiments, 5 concentrations of crude, active ingredient from mangosteen pericarp extract, control treatments (distilled water) and Imidacloprid were treated with all stages of nymph and adult *Nilaparvata lugens* (Stal.). The differences of mean mortality from each treatment were compared. Data was determined to be normally distributed and variances were homogeneous. Thus, analysis of variance was used with SPSS (Statistical Package for the Social Sciences) for windows version 11.0 and Sigma plot 2000 program.

To estimate the mortality dose response of the crude, pericarp mangosteen extract and imidacloprid, method of Jansiri (1994) was applied. The 1 day old of all stages of nymph and adult brown planthoppers were treated with crude and purified pericarp of mangosteen fruit extract and imidacloprid.

The mangosteen pericarp extract and imidacloprid were diluted into 5 concentrations with distilled water contained in 100 ml of the sprayer. The 1 day old in each stage of brown planthoppers (60 samplings in replicate) were released from the cage, knock it by refrigerator (10 sec.) and put it on the glass plate then treated with each concentration by sprayer (5 seconds). After that the brown planthoppers were transfer to the Rice plant (RD7, 10 days old) in the test tube (ø 1.5 cm, 15 cm) and were kept at 28 – 33°C and day/night period was adjusted about 16:8 hours. For the control treatment (absence insecticides), the 1 day old in each stage of brown planthoppers (60 samplings in each replicate) were sprayed with distilled water by spreader.

The insect which did not move and/ or lay down in the rice box is inferred as this insect is dead. The Mortality was recorded for each experiment in 5 replicates 24 and 48 hours after treatment. If the mortality data in the control treatment appeared, the data would be corrected by Abbott's formula (Abbott, 1925) as shown below. Raw bioassay data was analyzed using regression equation to obtain $LC_{50}$ by Sigma plot 2000 program.

Abbott's formula:

$$\% \text{ corrected mortality} = \frac{[(\% \text{ treatment mortality} - \% \text{ control mortality}) \times 100]}{(100 - \% \text{ control mortality})}$$

**5. Detoxification mechanisms of treated nymphs and adults *Nilaparvata lugens* (Stal.) with crude and purified pericarp of mangosteen fruit extract.**

5.1 Insect treatment for enzyme extraction.

Live Nymphs and adults of brown planthopper (30 sampling in each replicate) after treated with crude extract, purified mangosteen pericarp extract and imidacloprid (same toxicity analysis method as describe above) were separated and collected from the dead ones at 24 hour after exposure and were used for enzyme extraction.

5.2 Extraction of detoxification enzymes method.

This research use the method modify from Yang *et al*. (2004) and Visetson and Milne (2001) that uses survival brown planthopper. All nymph stage and adult insects (30 sampling in each replicate) were placed in ice and were homogenized on cool mortar in homogenization buffer (1ml, 0.1 M potassium phosphate, containing 1 mM EDTA, pH 8.0 and 1 ml, 0.1 M potassium phosphate, containing 10 mM glutathione reduced form, pH 8.0). The homogenate were centrifuged at 4° C, 18,000 rpm for 5 minutes. The supernatant were decanted into a clean 1.5 ml microtube, placed on ice and use immediately for acetylcholinesterase (ACTh), glutathione-S-transferase (GST) and carboxylesterase assays with spectrophotometer as shown in figure 15.

5.3 Enzyme activity analysis.

The pH optimum of all enzymes activity detections were preliminary test which was elucidated with various pH of potassium phosphate buffer (pH 4-13). The activities were test with enzymes of 30 3$^{rd}$ nymph brown planthopper with paranitrophenylacetate as substrate for carboxylesterase, acetylthioiodide for acetylcholinesterase and 1, 2-dichloro-4- nitrobenzene (DCNB) and 1- chloro-2,4-dinitrobenzene (CDNB) for glutathione-s-transferase. The pH which shows the highest activity level of substrate detected of each enzyme was selected to apply in this study.

The actylcholinesterase was measured using the Acetylthiocholine iodide assay which modified from Visetson (2001) as shown in appendix A6. The incubation mixture contained 2765 µl, 0.1 M potassium phosphate buffer pH 8.0, 50 µl enzyme. The reaction was initiated by addition of substrate, 60 µl 0.1 M acetylthiocholine iodide (ATCL), to the mixture at 28 -30°C of both the sample and reference (no enzyme) cuvettes. The reaction is generated by 75 µl 0.01 M DTNB (5, 5'-dithiobis

(2-nitrobenzoic acid)) which shows the yellow color and was detected by the change in absorbance at 412 nm using a spectrophotometer and Printer (Perkin Elmer- Lamda 25) with Winlab program.

Carboxylesterase activity was measured using the PNPA assay which modified from Visetson (2001) and Visetson *et al*. (2003, 2004) as shown in appendix A3. The 3 ml incubation mixture contained 2900 µl, 0.1 M potassium phosphate buffer pH 8.0 and 50 µl enzyme. The reaction was initiated by addition of 50 µl substrate, pNPA (paranitrophenylacetate) to the mixture at 28-30°C of both the sample and reference (no enzyme) cuvettes. The hydrolysis of paranitrophenylacetate shows the yellow color of paranitrophenol generated and was detected by the change in absorbance at 400 nm using a spectrophotometer and Printer (Perkin Elmer- Lamda 25) with Winlab program.

Glutathione-S-transferase (GST) activity, the CDNB assay is modified the method of Visetson *et al.* (2002, 2003) and Bullangpoti (2002) as show in appendix A4 and A5. The reaction mixture consists of 10 µl, 150 mM - chloro-2, 4-dinitrobenzene (CDNB) and enzyme solution, 130 µl of 10 mM Glutathione reduced form in 0.1 M potassium phosphate buffer pH 8.0 to give a final glutathione concentration of 1 mM, and 1150 µl, 0.1 M potassium phosphate buffer pH 8.0 (containing no Glutathione) to give a final volume of 1.31 ml. Reaction is initiated by addition of 150 mM - chloro-2,4-dinitrobenzene (CDNB) to both the sample and reference (no enzyme) cuvettes. Change in absorbance at 340 nm is recorded using a spectrophotometer and Printer (Perkin Elmer- Lamda 25) with Winlab program.

Moreover, DCNB assay which have dichloronitrobenzene as substrate was used for analyse glutathione-s-transferase activity compare with CDNB assay. The 1.4 ml incubation mixture contained 1100 µl, 0.1 M potassium phosphate buffer containing glutathione pH 8.0 and 200 µl enzyme. The reaction was initiated by addition of substrate, 10 µl 150 mM 1,2-dichloro-4,nitrobenzene (DCNB) to the mixture at 28-30°C to both the sample and reference (no enzyme) cuvettes. The reaction was detected by the change in absorbance at 344 nm using a spectrophotometer and Printer (Perkin Elmer- Lamda 25) with Winlab program.

### 5.4 Total protein concentration.

Protein content was determined by the Bradford method using bovine serum albumin (sigma) as a standard followed the method in appendix B. The 5.5 ml incubated mixture contained 0.5 ml sample and 5 ml Bradford solution (100mg coomassie brilliant blue R250 in 50ml 95% ethanol then add 100 ml 85% Phosphoric acid and make final volume to 1 L by distilled water). Incubated the mixture at room temperature for 5 minutes then the reaction was detected by the change in absorbance

at 595 nm using a spectrophotometer and Printer (Perkin Elmer- Lamda 25) with Winlab program.


**Figure 18** Doing enzyme activity analysis experiment

The chemical and protocol for analyze activity of acetylcholinesterase, carboxylesterase and glutathione-S-transferase and protein concentration were described in appendix A and B.

**6. Evaluation effect to non-target organisms**

6.1 Effect of mangosteen pericarp extract on ICR mouse.

This research uses Completely Randomized Design with 3 replicates and 6 females and 6 males of 3 week old ICR mouse unit each replicate. The room for mouse experiment is controlled day/night period (16:8) and temperature (25°C, 80%RH). Each experiment, 3 concentrations of mangosteen pericarp extracts were used. The effect of the extracts will be recorded as acute dermal toxicity analysis, acute mouth injection toxicity analysis, eye irrigation analysis (Figure 19). For acute oral toxicity analysis, mouse in each replicate were drank 5ml mangosteen pericarp extract or 5 ml water (for control treatment). The mortalities of animals are assumed as toxicity to animal.

For determine the toxicity to mouse's dermal, each mouse was shave their hair (2 x 2 cm), and next 3 days 1 ml mangosteen pericarp extract or 1 ml water (for control treatment) were put on their skin for observe the inflammation which red skin color, bubble or swelling of animals are assumed as toxicity to animal. For determine the eye irrigation, 50 µl mangosteen pericarp extract and 50 µl water (for control treatment) were drop on their eyes (opposite side) for observe the eye

irrigation which red color - eye, swelling or blind of animals are assumed as toxicity to animal. The differences of mean toxicity from each treatment were analyzed by Analysis of Variance with CRD, 3 replicates using the SPSS (Statistical Package for the Social Sciences) for windows version 11.0 and Sigma plot 2000 program.

6.2 Effect of mangosteen pericarp extract on guppies.

Guppies, one of bio-indicator, were treated with crude mangosteen pericarp extract for determine toxicity to aquatic animal (Figure 20). The guppies experiment uses Completely Randomized Design with 5 replicates and 30 sampling 3 months old of both sex guppies in each replicate were treated with 3 concentrations of crude mangosteen pericarp extract and control treatments (distilled water) which were doing in the tank (30 x 30x 30 cm) including 3000 ml water. No operculum moving of guppies was assumed as mortality to guppies. The differences of mean mortality were analyzed by Analysis of Variance with CRD, 5 replicates. The $LC_{50,}$ statistic analysis was determined using the SPSS (Statistical Package for the Social Sciences) for windows version 11.0 and Sigma plot 2000 program.

6.3 Effect of mangosteen pericarp extract on honey bee.

For bee experiment, this experiment uses Completely Randomized Design with 5 replicates and 30 sampling bee in each replicate were treated with 3 concentrations of crude mangosteen pericarp extract and control treatments (distilled water). Bees were knocked in the refrigerator for 10 seconds then bees were put in the box (7 X 5x 4 cm) and were spray the extract on it using spreader (5 seconds). The character as no moving, no fly are assumed as mortality to bees. The differences of mean mortality were analyzed by Analysis of Variance with CRD, 5 replicates. The $LC_{50,}$ statistic analysis was determined using the SPSS (Statistical Package for the Social Sciences) for windows version 11.0 and Sigma plot 2000 program.

(a) (b) (c)

**Figure 19** Toxicity test method to ICR mouse: (a) eye irrigation (b) dermal inflammation (c) oral toxicity test

**Figure 20** Toxicity test to Guppy.

## 7. Molecular Analysis of potential resistance mechanisms.

### 7.1 Rearing insects treated with LC50 concentrations.

The F0 laboratory generation of brown planthopper was divided into three groups. The first group was reared without any insecticides pressure (non-selection) while another group was divided into two subgroups subject to selection pressure with imidacloprid and mangosteen pericarp extract. After selection progeny for each insecticide, each sub group was divided into two groups. One for observed the changing toxicity in term of LC50 using Completely Randomized Design with 5 replicates and 60 sampling of 3$^{rd}$ nymph brown planthopper unit each replicate. The mangosteen pericarp extract and imidacloprid were diluted into 5 concentrations with distilled water which contained 100 ml of the spreader. The 1 day old of 3$^{rd}$ nymph brown planthoppers were released from the cage, knock it by refrigerator (10 sec.) and put it on the grass plate then treated with each concentration by spreader (5 seconds). After that the brown planthoppers were transfer to the Rice plant (RD7, 10 days old) in the test tube (ǿ 1.5 cm, 15 cm) and were kept at 28 – 33°C and day/night period was adjusted about 16:8 hours. For the control treatment (absence insecticides), the 1 day old in each stage of brown planthoppers (60 samplings in each replicate) were sprayed with distilled water by spreader.

Another sub group was sprayed the mangosteen pericarp extract and imidacloprid on brown planthopper using LC50 value from another subgroup. The 1 day old of 3$^{rd}$ nymph brown planthoppers were released from the cage, knock it by refrigerator (10 sec.) and put it on the grass plate then treated with each concentration by spreader (5 seconds). After that the brown planthoppers were transfer to the Rice plant (RD7, 10 days old) were kept in the cage at 28 – 33°C and day/night period was adjusted about 16:8 hours. Surviving BPH were continued to be reared in the cage under the same conditions (at 28 – 33°C and day/night period about 16:8 hours) to

produce the next generation. The process was repeated up to 9 generations for monitoring trend to be resistant development in brown planthopper.

Each generation of both treatments, extract and imidacloprid were observed the detoxification mechanism for predict and compare trend to be resistant in each treatment. Moreover, F0 generation and F8 generation of extract treatment and imidacloprid treatment were obtained for analysis character of gene in brown planthopper in each treatment.

7.2 Detoxification enzyme mechanism analysis.

The procedure is same with the method in section 5, detoxification mechanisms of nymphs and adult *Nilaparvata lugens* (Stal.) with treated crude and purified pericarp of mangosteen fruit extract.

7.3 Total RNA extraction method.

In this experiment, RNA was isolated by Trizol ® reagent (guanidinium thiocyanate ($NH_2C$ (=NH) $NH_2$ · HSCN) -phenol-chloroform mixture). One $3^{rd}$ nymph brown planthopper was homogenized in 1ml Trizol ® reagent and let samples incubated for 5 minutes at room temperature then centrifuged at 12,000x g for 10 minutes at 4°C. Transfer supernatant into fresh tubes then add 0.2 ml BCP (1-bromo-3-chloropropane) and let samples stand for 15 minutes at room temperature after that centrifuged at 12,000g, 4°C 15 minutes. Transfer supernatant (colorless) into fresh tube and add 0.5 ml isopropanol, mix, incubated at room temperature for 10 minutes. Centrifuge at 12,000x g for 10 minutes at 4°C. Remove the supernatant, wash the RNA pellet once with at least 1 ml of 75% ethanol per ml Trizol® used. Vortex and centrifuged at 7,500x g at 4°C, 5 minutes. Air dry around 30 minutes. Elute RNA into final 50µl DNase, RNase Free Distilled water.

7.4 RT-PCR analysis.

7.4.1 Primer for the carboxylesterase. For this, amplified the carboxylesterase gene fragment in brown planthopper (*Nilaparvat lugens* Stal., the primers were designed by Genetyx program from 1644 base pair of carboxylesterase of brown planthopper (ACC: AF 302777) which accessed from Genbank (http://www.ncbi.nlm.nih.gov/). The method to design primer using the basically designed method such as melting temperature ($T_m$) of primer have to more than temperature of reverse transcription reaction, GC content more than 50%, avoid the secondary structure of primer and primer should have 18-30 nucleotide long. In this research, the 2 specific primers were the $138^{th}$ -$158^{th}$ region for the forward primer,

CarF: 5'-GACACCCAACCGCA CCATCGA-3', and at the 679$^{th}$ – 699$^{th}$ region for the reverse primer, CarR: 5'- GAG CCC TTG ACT TTG CGG CGA -3' (Figure 18). This region (562 base pair) is the conserve region compare with another insect in the same order.

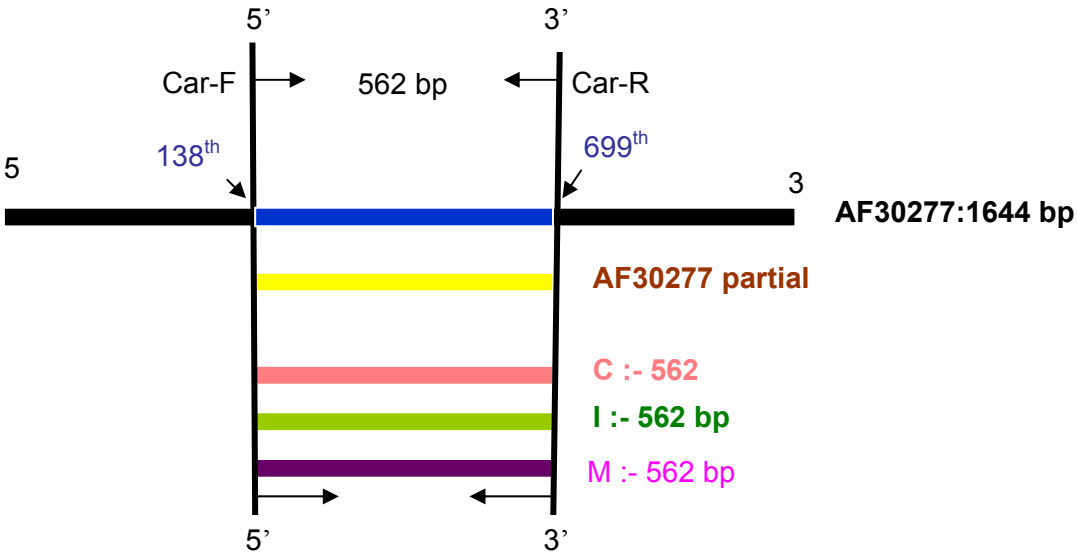



**Figure 21** Schematics of Carboxylesterase gene Structure from Genbank (acc: AF30277) and carboxylestrease PCR amplification. C is control group, I is Imidacloprid treatment, M= Mangosteen treatment, Car-F is forward primer and Car-R is reverse primer. The location and direction of PCR primers are indicated by arrow.

7.4.2 RT-PCR amplification. The carboxylesterase gene fragment (562 bp) of one brown planthopper per sample, 3 replicate was amplified using standard PCR reaction following commercial protocol from Invitrogen®. The first stand cDNA is synthesized with superscript III reverse transcriptase, one step (Invitrogen®). All reagents obtained from Invitrogen® except primers which were obtained from Operon®. The reaction was performed in total volumes of 50 µl including 2 µl total RNA, 10 µM of each primer, 0.5 mM dNTPs, 1.5 mM $MgCl_2$, 2U superscript III reverse transcriptase, one step and 2X PCR buffer. PCR was carried out by GeneAmp thermal cycler. The thermocylcler program conditions as described in table 1. PCR product was analyzed of DNA fragment by 0.8%w/v agarose gel in TAE buffer with electrophoresis at 135V for 25 minutes. Ethidium-bromide was used for staining for 15 minutes and de-stained using distilled water for 10 minutes PCR analysis was visualized and photograph by Gel Documentation. The RT-PCR products were determined by lamda DNA/ *Hind* III marker.

**Table 1** RT-PCR condition for amplified carboxylesterase gene

| Stage | Temperature (°C) | Time |
|---|---|---|
| Stage1: cDNA synthesis | 60 | 30.0 minutes |
| | 94 | 2.0 minutes |
| Stage2: PCR Cycle (35 cycles) | | |
| Denature | 94 | 0.30 minutes |
| Annealing | 62.0 | 0.30 minutes |
| Extension | 72 | 1.0 minutes |
| Stage3: Long Extension | 4 | Hold |

7.4.3 DNA sequencing analysis. PCR products were sent to BSU (Bioservice Unit, National Science and Technology Development Agency: NSTDA) for DNA sequencing, the PCR products were directly sequenced in both directions by sequencer (ABI PRISM, model 3000). After sequencing, the partial sequences were determine by compare with the Genbank database using BLAST program (http://www.ncbi.nlm.nih.gov/) to test identity and were used the Genetyx window program version 7.0 for sequence editing (5 replicated), sequence alignments, translated nucleotide sequences to amino acid sequences and alignment for nucleotide sequences and amino acid sequence analysis.

## 8. Effect of temperature to yield percentage of ethanolic mangosteen pericarp extract and their control efficiency to brown planthopper.

The crude mangosteen pericarp extract (4g) was dissolve in 20 ml Ethanol then were kept at 3 temperatures 28°C, 55°C, and 4°C for 3 months. The yield of the extract were check in every 0, 45, 90 days with 3 replicate. Moreover, every treatment was sent to Department of Chemistry, Faculty of Science, Srinakharinwirot University for analyzed the alpha- mangostin yield by HPLC (Hewlett Packard Series 1100) with column (Synergi 4u hydro-RP 80A, 150 x 4.06 mm 4 micron).

Moreover, this experiment was also analyzed the toxicity to brown planthopper which use the Completely Randomized Design with 5 replicates and 60 sampling of 3$^{rd}$ nymph brown planthopper unit each replicate. In the experiments, 3 concentrations of each treatment (28 °C, 55°C and 4°C) and control treatments (distilled water) were treated with 1 day old 3$^{rd}$ nymph brown planthopper. The

insects were released from the cage, knock it by refrigerator (10 sec.) and put it on the grass plate then treated with each concentration by spreader (5 seconds). After that the brown planthoppers were transfer to the Rice plant (RD7, 10 days old) in the test tube (ø 1.5 cm, 15 cm) and were kept at 28 – 33°C and day/night period was adjusted about 16:8 hours. For the control treatment (absence insecticides), the 1 day old in each stage of brown planthoppers (60 samplings in each replicate) were sprayed with distilled water by spreader. The insect which did not move and/ or lay down in the rice box is inferred as this insect is dead. The Mortality was recorded for each experiment in 5 replicates 24 and 48 hours after treatment. Raw bioassay data was analyzed using regression equation to obtain $LC_{50}$ by Sigma plot 2000 program.

**9. Place and duration**

Place: Department of Zoology, Faculty of Science Kasetsart University

Department of Chemistry, Faculty of Science, Mahidol University.

Department of Chemistry, Faculty of Science, Srinakharinwirot University

Pathumthani Rice Research Center, Krong 6, Pathumthani Province.

Samutsakhon Coastal Fisheries Research and Development Center, Samutsakhon Province.

Duration: May 2004 to February 2007.

# RESULTS

## Efficiency of mangosteen pericarp extract against brown planthopper

### 1. Quantitative and efficiency of extract in various solvent Analysis

1.1. Quantitative Result

Pericarps of mangosteen fruits were extracted by Soxhlet extraction with various solvents such as dichloromethane, acetone, hexane and ethanol. The yield of extract is shown in Table 2 and Figure 22. Each yield shows differently significant at 5% level using Duncan's multiple rang test which the ethanol extract shows highest yield compare when using another solvent. The hexane extract have character same with dichloromethane extract, yellow fine powder. Both extract have character contrast with ethanol and acetone extract which character is yellow-brown sticky extract.

**Table 2** % yield of pericarp of mangosteen fruit extract extracted with various solvent

| Solvent | No. Replicate | % w/w yield of extract [(1)] |
|---|---|---|
| 1. Hexane | 3 | $5.21 \pm 0.15^{a}$ |
| 2. Dichloromethane | 3 | $7.89 \pm 0.25^{b}$ |
| 3. Acetone | 3 | $20.67 \pm 1.77^{c}$ |
| 4. Ethanol | 3 | $29.46 \pm 0.95^{d}$ |

[(1)] Means ± SD followed by a common letter in the same column are not significantly at 5% level using Duncan's multiple rang test.

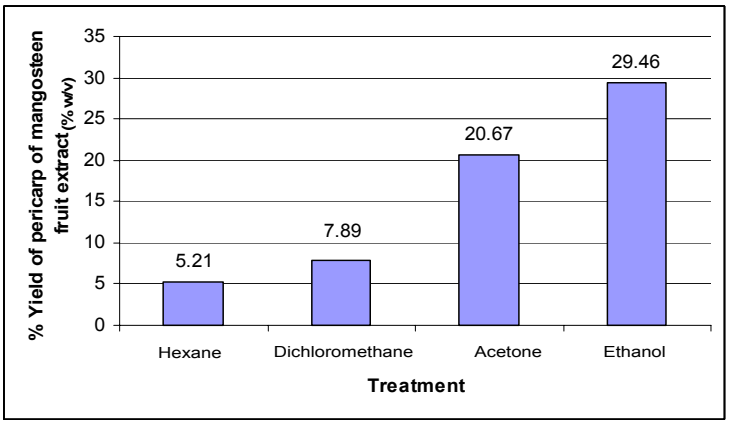



**Figure 22** % yield of pericarp of mangosteen fruit extract extracted with various

1.2. Efficiency of extracts of various solvent against BPH

1.2.1 Toxicity of pericarp of Mangosteen fruit extract with ethanol as solvent.

The mortality percentage at 24 hours of 3rd nymph *N. lugens* (Stal.) against ethanolic extract which re-dissolve in distilled water to make 5 concentrations, 0 (distilled water: Control), 2, 4, 6, 8% w/v are 0 ± 0, 35.57 ± 0.96, 50.00 ± 1.67, 62.67 ± 4.41 and 79.44 ± 4.19 respectively (Table3, Figure23). The simple linear regression of this result is Y = 8.334 + 9.25 X (X means percent concentrations of pericarp of mangosteen fruit extract and Y means mortality percentage of treated 3rd nymph of *N. lugens* Stal.) thus, $LC_{50}$ - values at 24 hours of *N. lugens* Stal after treated with ethanolic extract is 4.50 % w/v with correlation coefficient is 0.950 (Table7). The mortality percentage values differ among groups and increase with differently significant at 5% level using Duncan's Multiple Rang Test. After 48 hours after exposure, the mortality percentage dramatically increasing. The mortality percentage of 3rd nymph *N. lugens* (Stal.) against the same concentrations of ethanolic extract are 0 ± 0, 38.89 ± 3.85, 51.67 ± 1.67, 67.22 ± 6.74 and 83.89 ± 4.19 respectively (Table3). The simple linear regression of this result is Y = 9.113 + 9.805X (X means percent concentrations of pericarp of mangosteen fruit extract and Y means mortality percentage of treated 3rd nymph of *N. lugens* Stal.) thus, $LC_{50}$ - values at 48 hours of *N. lugens* Stal after treated with ethanolic extract is 4.17 %w/v with correlation coefficient is 0.974 (Table7). The mortality percentage values differ among groups and increase with differently significant at 5% level using Duncan's Multiple Rang Test.

**Table3** Mortality percentage(2) of BPH against crude extract of pericarp of mangosteen fruit with ethanol as solvent after 24 and 48 hr. under the laboratory condition.

| Concentration (% w/v) | Total treated | No. Replicate | % average mortality(2) | |
|---|---|---|---|---|
| | | | 24 hr. | 48 hr. |
| 0 (1) | 60 | 5 | 0.00 ± 0.00 a | 0.00 ± 0.00 a |
| 2 | 60 | 5 | 35.56 ± 0.96b | 38.89 ± 3.85b |
| 4 | 60 | 5 | 50.00 ± 1.67c | 51.67 ± 1.67c |
| 6 | 60 | 5 | 61.67 ± 4.41d | 67.22 ± 6.74d |
| 8 | 60 | 5 | 79.44 ± 4.19e | 83.89 ± 4.19 e |

(1) Control A = Distilled water.
(2) Means ± SD followed by a common letter in the same column are not significantly different at 5% level using Duncan's Multiple Range Test.

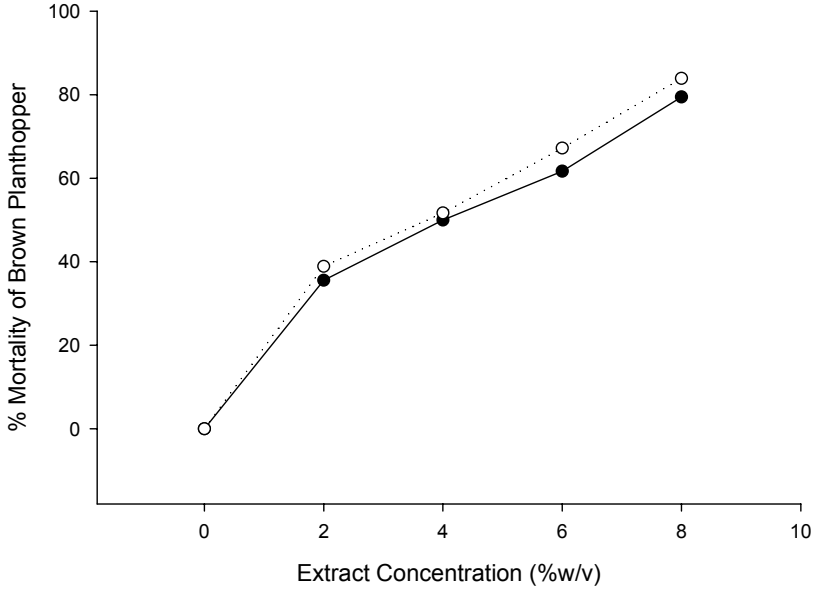



**Figure 23** Mortality percentage(2) of BPH against crude extract of pericarp of mangosteen fruit with ethanol as solvent after 24 (Line) and 48 hr. (Dot Line) under the laboratory condition.

2.2 Toxicity of pericarp of mangosteen fruit extracts with hexane as solvent.

The percentages at 24 hours of 3rjd nymph *N. lugens* (Stal.) against the crude extract which have hexane as solvent. This extract was re-dissolved in distilled water to make 5 concentrations, 0 (distilled water: Control), 20, 40, 60, 80% w/v, are 0 ± 0, 39.44 ± 4.19, 51.11 ± 2.55, 62.78 ± 8.22 and 70.00 ± 2.89 respectively (Table4, Figure 24). The simple linear regression of this result is Y = 12.00 + 0.82 X (X means percent concentrations of pericarp of mangosteen fruit extract and Y means mortality percentage of treated 3rd nymph of *N. lugens* Stal.) thus, $LC_{50}$ - values at 24 hours of *N. lugens* Stal after treated with ethanolic extract is 46.53 % w/v with correlation coefficient is 0.879 (Table7). The mortality percentage values differ among groups and increase with differently significant at 5% level using Duncan's Multiple Rang Test.

The mortality percentage at 48 hour of 3rd nymph *N. lugens* (Stal.) against the same concentrations of the extract with hexane as solvent are 0 ± 0, 47.78 ± 4.20, 53.33 ± 1.67, 77.22 ± 9.62 and 81.11 ± 8.55 respectively (Table4). The simple linear regression of this result is Y = 13.56 + 0.9583X (X means percent concentrations of pericarp of mangosteen fruit extract and Y means mortality percentage of treated 3rd nymph of *N. lugens* Stal.) thus, $LC_{50}$ - values at 48 hours of *N. lugens* Stal is 38.03 %w/v with correlation coefficient is 0.873 (Table7). The mortality percentage values differ among groups and increase with differently significant at 5% level using Duncan's Multiple Rang Test.

**Table 4** Mortality percentage[2] of BPH against crude extract of pericarp of mangosteen fruit with hexane as solvent after 24 and 48 hr. under the laboratory condition.

| Dose (% w/w) | Total treated | No. Replicate | % average mortality [2] | |
|---|---|---|---|---|
| | | | 24 hr. | 48 hr. |
| 0 [1] | 60 | 5 | 0.00 ± 0.00 [a] | 0.00 ± 0.00 [a] |
| 20 | 60 | 5 | 39.44 ± 4.19[b] | 47.78 ± 4.20[b] |
| 40 | 60 | 5 | 51.11 ± 2.55[c] | 53.33 ± 1.67[c] |
| 60 | 60 | 5 | 62.78 ± 8.22[d] | 77.22 ± 9.62[d] |
| 80 | 60 | 5 | 70.00 ± 2.89[e] | 81.11 ± 8.55[e] |

[1] Control A = Distilled water.
[2] Means ± SD followed by a common letter in the same column are not differently significant at 5% level using Duncan's Multiple Rang Test.

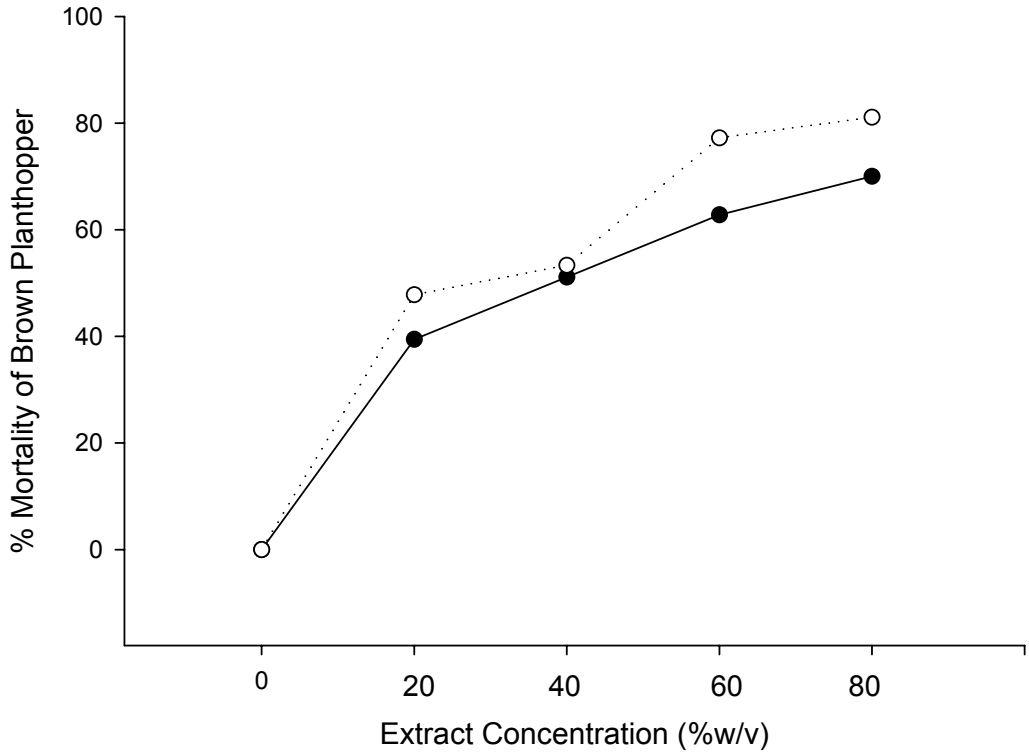



**Figure 24** Mortality percentage[2] of BPH against crude extract of pericarp of mangosteen fruit with hexane as solvent after 24 (Line) and 48 hr. (Dot Line) under the laboratory condition.

2.3 Toxicity of pericarp of mangosteen fruit extracts with dichloromethane as solvent.

The mortality percentages at 24 hours of 3rd nymph *N. lugens* (Stal.) against the crude extract which have dichloromethane as solvent. This extract was re-dissolved in distilled water to make 5 concentrations, 0 (distilled water: Control), 20, 40, 60, 80% w/v, are 0 ± 0, 36.67 ± 4.41, 51.67 ± 1.67, 56.11 ± 3.47 and 83.89 ± 8.54 respectively (Table5, Figure 25). The simple linear regression of this result is Y = 14.95 + 1.181 X (X means percent concentrations of pericarp of mangosteen fruit extract and Y means mortality percentage of treated 3rd nymph of *N. lugens* Stal.) thus, $LC_{50}$ - values at 24 hours of *N. lugens* Stal after treated with this extract is 29.68 % w/v with correlation coefficient is 0.858 (Table7). The mortality percentage values differ among groups and increase with differently significant at 5% level using Duncan's Multiple Rang Test.

The mortality percentage at 48 hour of 3rd nymph *N. lugens* (Stal.) against the same concentrations of the extract with dichloromethane as solvent are 0 ± 0, 39.44 ± 4.19, 53.33 ± 1.67, 64.45 ± 3.85 and 87.22 ± 3.47 respectively (Table5). The simple linear regression of this result is Y = 16.20 + 1.257X (X means percent concentrations of pericarp of mangosteen fruit extract and Y means mortality percentage of treated 3rd nymph of *N. lugens* Stal.) thus, $LC_{50}$ - values at 48 hours of *N. lugens* Stal is 26.89 %w/v with correlation coefficient is 0.870 (Table7). The mortality percentage values differ among groups and increase with differently significant at 5% level using Duncan's Multiple Rang Test.

**Table 5** Mortality percentage(2) of BPH against crude extract of pericarp of mangosteen fruit with dichloromethane as solvent after 24 and 48 hr. under the laboratory condition.

| Dose (% w/v) | Total treated | No. Replicate | % average mortality(2) | |
|---|---|---|---|---|
| | | | 24 hr. | 48 hr. |
| 0 (1) | 60 | 5 | 0.00 ± 0.00 a | 0.00 ± 0.00 a |
| 20 | 60 | 5 | 36.67 ± 4.41b | 39.44 ± 4.19b |
| 40 | 60 | 5 | 51.67 ± 1.66c | 53.33 ± 1.67c |
| 60 | 60 | 5 | 56.11 ± 3.47d | 64.44 ± 3.85d |
| 80 | 60 | 5 | 83.89 ± 2.54e | 87.22 ± 3.47e |

(1) Control A = Distilled water.
(2) Means ± SD followed by a common letter in the same column are not differently significant at 5% level using Duncan's Multiple Rang Test.

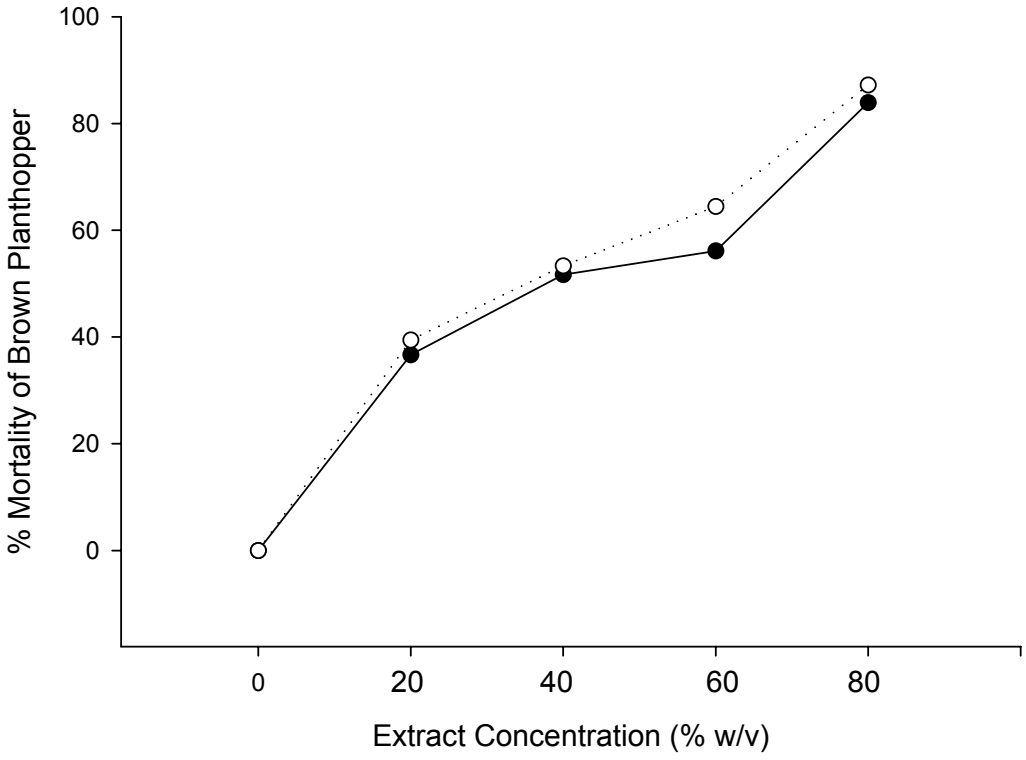



**Figure 25** Mortality percentage(2) of BPH against crude extract of pericarp of mangosteen fruit with dicholormethane as solvent after 24 (Line) and 48 hr. (Dot Line) under the laboratory condition.

2.4 Toxicity of pericarp of Mangosteen fruit extract with acetone as solvent.

The mortality percentages at 24 hours of 3rd nymph *N. lugens* (Stal.) against the crude extract which have dichloromethane as solvent. This extract was re-dissolved in distilled water to make 5 concentrations, 0 (distilled water: Control), 2, 4, 6, 8% w/v, are 0 ± 0, 35.00 ± 1.67, 45.00 ± 6.01, 51.11 ± 2.54 and 68.89 ± 2.55 respectively (Table6, Figure 23). The simple linear regression of this result is Y = 9.222 + 7.695X (X means percent concentrations of pericarp of mangosteen fruit extract and Y means mortality percentage of treated 3rd nymph of *N. lugens* Stal.) thus, $LC_{50}$ - values at 24 hours of *N. lugens* Stal after treated with this extract is 5.30 % w/v with correlation coefficient is 0.908 (Table7). The mortality percentage values differ among groups and increase with differently significant at 5% level using Duncan's Multiple Rang Test.

The mortality percentage at 48 hour of 3rd nymph *N. lugens* (Stal.) against the same concentrations of the extract with acetone as solvent are 0 ± 0, 38.33 ± 4.41, 52.19 ± 0.99, 61.89 ± 6.33 and 75.00 ± 5.77 respectively (Table6). The simple linear regression of this result is Y = 10.07 + 8.681X (X means percent concentrations of pericarp of mangosteen fruit extract and Y means mortality percentage of treated 3rd

nymph of *N. lugens* Stal.) thus, $LC_{50}$ - values at 48 hours of *N. lugens* Stal is 4.60 %w/v with correlation coefficient is 0.912 (Table7, Figure26). The mortality percentage values differ among groups and increase with differently significant at 5% level using Duncan's Multiple Rang Test.

**Table 6** Mortality percentage[2] of BPH against crude extract of pericarp of mangosteen fruit with acetone as solvent after 24 and 48 hr. under the laboratory condition.

| Dose (% w/w) | Total treated | No. Replicate | % average mortality[2] | |
|---|---|---|---|---|
| | | | 24 hr. | 48 hr. |
| 0 [1] | 60 | 5 | 0.00 ± 0.00 [a] | 0.00 ± 0.00 [a] |
| 2 | 60 | 5 | 35.00 ± 1.67[b] | 38.33 ± 4.40[b] |
| 4 | 60 | 5 | 45.00 ± 6.01[c] | 52.18 ± 0.99[c] |
| 6 | 60 | 5 | 51.11 ± 2.54[d] | 61.89 ± 6.33[d] |
| 8 | 60 | 5 | 68.89 ± 2.55[e] | 75.00 ± 5.77[e] |

[1] Control A = Distilled water.
[2] Means ± SD followed by a common letter in the same column with differently significant at 5% level using Duncan's Multiple Rang Test.

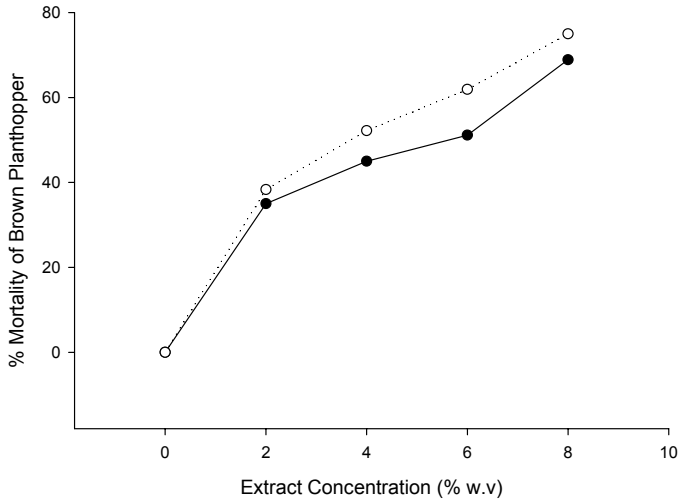



**Figure 26** Mortality percentage[2] of BPH against crude extract of pericarp of mangosteen fruit with acetone as solvent after 24 (Line) and 48 hr. (Dot Line) under the laboratory condition

**Table 7** Comparison $LC_{50}$ value (% w/v), Regression equation[(1)] and Correlation Coefficient ($r^2$) [(2)] of 3rd nymph brown planthopper affected by extracts of mangosteen pericarps with various solvent at 24 and 48 hours of exposure using the topical spray method.

| Type of solvent | Hours | Regression equation [(1)] | $LC_{50}$ | $r^2$ [(2)] |
|---|---|---|---|---|
| Ethanol | 24 | Y= 8. 334 + 9.25X | 4.50 | 0.950 |
| | 48 | Y= 9.113 + 9.805X | 4.17 | 0.974 |
| Acetone | 24 | Y= 9.222 + 7.695X | 5.30 | 0.908 |
| | 48 | Y= 10.07 + 8.681X | 4.60 | 0.912 |
| Dichloromethane | 24 | Y= 14.95 + 1.181X | 29.68 | 0.858 |
| | 48 | Y= 16.20 + 1.257X | 26.89 | 0.870 |
| Hexane | 24 | Y= 12.00 + 0.8167X | 46.53 | 0.879 |
| | 48 | Y= 13.56 + 0.9583X | 38.03 | 0.873 |

[(1)] Regression equation: Y= Mortality percentage and X= Extract concentration
[(2)] $r^2$ was a correlation determination between concentration and mortality. Soxhlet extraction methods were used for all experiments. The method was described in the text.

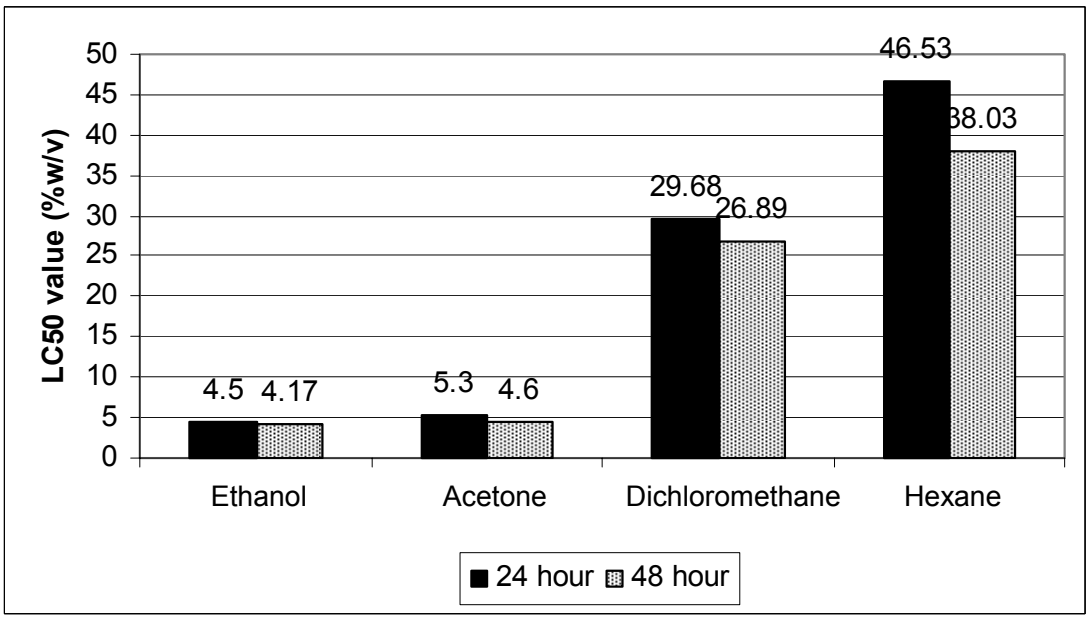



**Figure 27** Comparison $LC_{50}$ value (% w/v) of *N. lugens* affected by extracts of mangosteen pericarps with various solvent at 24 and 48 hours of exposure using the topical spray method**.**

**2. Comparison Toxicity result from Pericarp of Mangosteen Fruit Extract and imidacloprid Against various stages of BPH**

After test BPH with various solvent, the ethanol extract shows high efficiency for control BPH. Thus, in this process pericarp of mangosteen fruit extract which have ethanol as solvent was treated with various stages of nymph and adult brown planthopper. The Topical sprayer method was applied for this process. The toxicity in term of LC50 are shown in Table 8. 1st nymph gave the highest toxicity against extract compare with another stage. The correlation between concentration and mortality in most experiments indicated of $r^2$ as 0.74-0.95 showing that the effects of the extract on the mortality of brown planthopper were highly correlated (Table 8). The toxicity of commercial synthetic insecticide, imidacloprid, shows higher toxicity than pericarp of mangosteen fruit extract (Table 8). The correlation between concentration and mortality in most experiment indicated of $r^2$ as 0.92- 0.99 that means mortality of BPH were highly correlated.

**Table 8** Comparison LC50 value (%w/v) and Correlation coefficient ($r^2$)(1) of various stage of nymph and adult BPH(2) against pericarp of mangosteen fruit extract and imidacloprid at 24 and 48 hours after exposure using the topical spray method.

| Stage | Hour | $LC_{50}$ (imidacloprid) | $r^2$ | $LC_{50}$ (pericarp of Mangosteen fruit extract) | $r^2$ |
|---|---|---|---|---|---|
| N1 | 24 | 0.0018 | 0.96 | 1.60 | 0.792 |
| | 48 | 0.0017 | 0.94 | 1.41 | 0.742 |
| N2 | 24 | 0.0025 | 0.95 | 2.85 | 0.909 |
| | 48 | 0.0023 | 0.92 | 2.79 | 0.891 |
| N3 | 24 | 0.0042 | 0.99 | 4.50 | 0.950 |
| | 48 | 0.0039 | 0.99 | 4.17 | 0.974 |
| N4 | 24 | 0.0050 | 0.93 | 4.86 | 0.848 |
| | 48 | 0.0045 | 0.92 | 4.18 | 0.892 |
| N5 | 24 | 0.0046 | 0.95 | 4.51 | 0.807 |
| | 48 | 0.0042 | 0.93 | 3.98 | 0.821 |
| N6 | 24 | 0.0043 | 0.92 | 4.01 | 0.830 |
| | 48 | 0.0037 | 0.95 | 3.72 | 0.835 |

(1) $r^2$ was a correlation determination between concentration and mortality.
(2) N1 = 1st nymph brown planthopper, white color and 1 mm length, N2 = 2nd nymph brown planthopper, light yellowish color and 1.5 mm, N3 =3rd nymph brown planthopper, yellowish color and 2-2.5 mm length, N4= 4th nymph brown planthopper dark yellowish color and 3 mm length, 5th nymph light brown color, 3 mm length and N6= adult brown planthopper, brown color 3-3.5 mm length.

**Characterization of alpha-mangostin from Mangosteen Pericarp Extract Against Brown Planthopper**

**1. Elucidation of chemical structure of alpha mangostin.**

The dried of pericarp of mangosteen fruit which extracted with ethanol show the best control efficiency to brown planthopper. The solvent of this extract was evaporated in rotary evaporator to give a residue which was subjected to column chromatography on silica gel and checked purification with TLC (thin layer chromatography), give the compound ca. 2.956%w/w yield. After elucidation structure of this compound using NMR, IR, MS and comparison with the literature data from Ee *et al.* (2006).MS, this compound is alpha- mangostin.

This compound, alpha mangostin, is yellow coloring powder that classified as xanthone group. The structure contained two double bounds susceptible to hydrogenation, one methoxyl group and two hydroxyl groups as shown in Figure 28.

The data from MS show the molecular weight is 411.18 g/mol and the melting point is 180- 182 °. λmax: 215, 243, 317. IR υ max: 3422, 2922, 1642, 1610. 1H NMR and 13C NMR spectrum data were shown in Table 9-10.

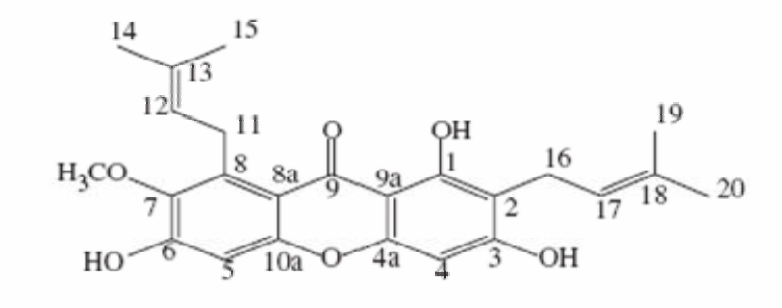



**Figure 28** Structure of alpha- mangostin from NMR chromatogram compare with report from EE *et al* (2006)

**Table 9** The 300MHz $^{1}$H NMR (Acetone-d6), spectrum data of alpha mangostin

| Chemical Shift (δ, ppm) | Assignment | Chemical Shift (δ, ppm) from reference (Ee *et al*, 2006) |
|---|---|---|
| 13.77 | singlet, 1H, OH-1 | 13.72 |
| 6.80 | singlet, 1H, H-5 | 6.72 |
| 6.38 | singlet, 1H, H-4 | 6.25 |
| 5.28 | triplet, 2H, H-12, H-17 | 5.26 |
| 4.10 | diplet, 2H, H-11 | 4.10 |
| 3.78 | singlet, 3H, 7-OMe | 3.78 |
| 3.33 | diplet, 2H, H-16 | 3.37 |
| 1.82 | singlet, 3H, H-20 | 1.83 |
| 1.81 | singlet, 3H, H-15 | 1.82 |
| 1.77 | singlet, 3H, H-14 | 1.71 |
| 1.63 | singlet, 3H, H-19 | 1.68 |

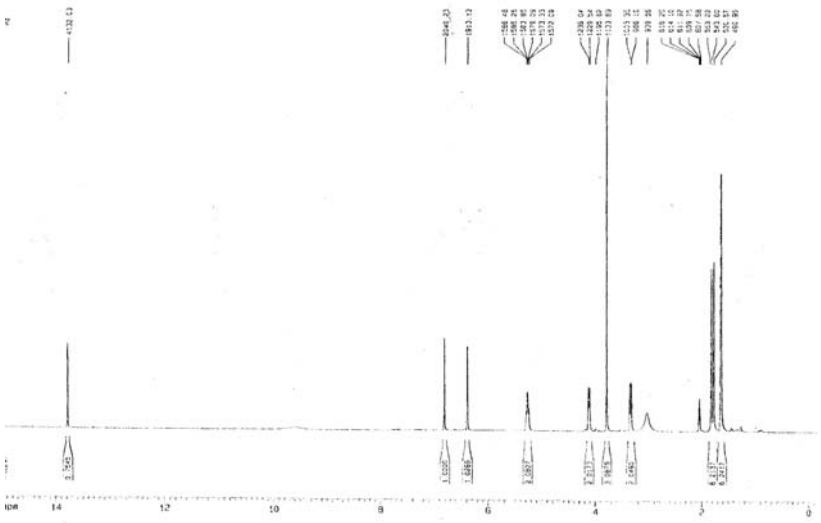
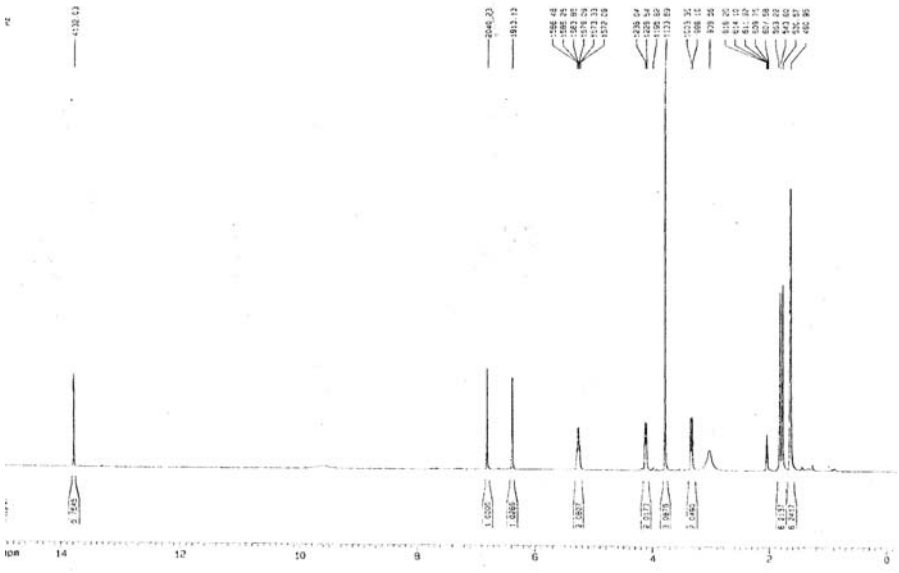


**Table 10** The 300MHz $^{13}C$ NMR (acetone-d6), spectrum data of alpha mangostin

| Chemical Shift (δ, ppm) | Assignment | Chemical Shift (δ, ppm) from reference (Ee *et al*, 2006) |
|---|---|---|
| 182.787 | C-9 | 181.8 |
| 162.901 | C-3 | 161.6 |
| 161.642 | C-1 | 160.2 |
| 157.333 | C-6 | 155.4 |
| 156.151 | C-10a | 155.2 |
| 155.650 | C-4a | 154.8 |
| 144.392 | C-7 | 142.7 |
| 138.053 | C-8 | 137.2 |
| 131.348 | C-13 | 131.7 |
| 124.728 | C-17 | 123.4 |
| 123.419 | C-12 | 122.1 |
| 111.904 | C-8a | 111.7 |
| 110.974 | C-2 | 109.7 |
| 103.548 | C-9a | 103.1 |
| 102.629 | C-5 | 101.6 |
| 93.098 | C-4 | 92.4 |
| 61.264 | 7-OMe | 61.2 |
| 26.824 | C-11 | 26.3 |
| 25.890 | C-14 | 25.7 |
| 25.850 | C-19 | 20.7 |
| 21.927 | C-16 | 21.3 |
| 18.241 | C-20 | 18.1 |
| 17.856 | C-15 | 17.7 |



**2. Toxicity of alpha manogstin against each stage of nymph and adult brown planthoppper.**

After purification the crude ethanol extracts by chromatography technique using dichloromethane: hexane as solvent. The crude extract gave yield of alpha mangostin *ca.* 2.956 %w/v.

After that treated alpha- mangostin with 1 day old various stage of nymph and adult of BPH by sprayer method. The toxicity of 1st, 2nd, 3rd, 4th and 5th stages of nymph and adult brown planthopper after treated with alpha mangostin in term of $LC_{50}$ *ca*. 1.39, 2.26, 5.44, 4.49, 4.03 and 3.84 % w/v at 24 hours exposure, respectively and *ca*. 1.24, 1.93, 4.45, 4.49, 4.03, 4.12, 4.25 %w/v at 48 hour exposure, respectively (Table 11). The correlation between concentration and mortality in most experiments indicated of $r^2$ of 0.73 – 0.90 showing that the effects of the extract on the mortality of *N. lugens* were highly correlated (Table 11, Figure 26). The longer exposure time show higher toxicity in term of LC50 value than the shorter time exposure.

**Table 11** $LC_{50}$ value (% w/v) Regression equation(1) and Correlation Coefficient ($r^2$)(2) of various stage of nymph and adult brown planthopper(3) against alpha- mangostin at 24and 48 hours of exposure using the Topical spray method.

| Stage | Hours | Regression Equation | $LC_{50}$ | $r^2$ |
|---|---|---|---|---|
| N1 | 24 | Y= 23.50 + 19.07X | 1.39 | 0.73 |
| | 48 | Y= 25.73 + 19.54X | 1.24 | 0.84 |
| N2 | 24 | Y= 22.27 + 12.29X | 2.26 | 0.78 |
| | 48 | Y= 24.56 + 13.16X | 1.93 | 0.76 |
| N3 | 24 | Y= -2.62 + 9.68X | 5.44 | 0.88 |
| | 48 | Y= 11.74 + 9.61X | 4.45 | 0.79 |
| N4 | 24 | Y= 11.67 + 8.53X | 4.49 | 0.90 |
| | 48 | Y= 13.00 + 9.17X | 4.03 | 0.89 |
| N5 | 24 | Y= 14.89 + 8.53X | 4.12 | 0.84 |
| | 48 | Y= 15.56+ 8.97X | 3.84 | 0.84 |
| N6 | 24 | Y= 14.90 + 8.25X | 4.25 | 0.83 |
| | 48 | Y= 16.22 + 8.64X | 3.91 | 0.81 |

(1) Regression equation: Y= Mortality percentage and X= mangostin concentration
(2) $r^2$ was a correlation determination between concentration and mortality.
(3) N1 = 1st nymph brown planthopper, white color and 1 mm length, N2 = 2nd nymph brown planthopper, light yellowish color and 1.5 mm, N3 =3rd nymph brown planthopper, yellowish color and 2-2.5 mm length, N4= 4th nymph brown planthopper dark yellowish color and 3 mm length, 5th nymph light brown color, 3 mm length and N6= adult brown planthopper, brown color 3-3.5 mm length.

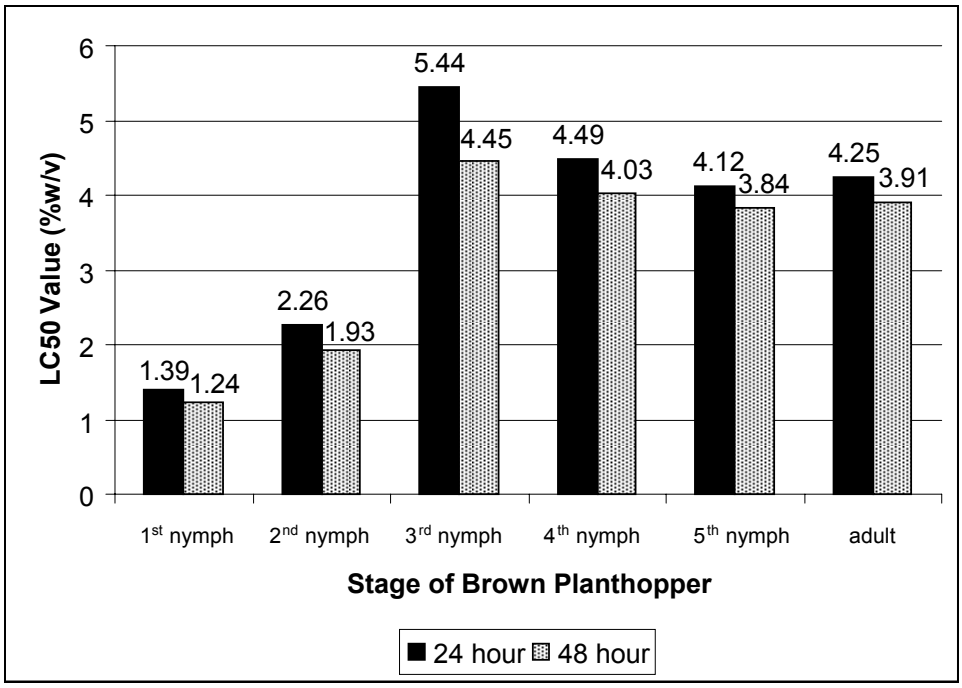



**Figure 29** $LC_{50}$ value (% w/v) of various stage of nymph and adult *N. lugens* against mangostin at 24 and 48 hours of exposure using the sprayer method.

## Characterization of Detoxification Mechanisms in Brown Planthoppers Against Crude Mangosteen Pericarp Extracts and Alpha mangostin

### 1. Optimization condition of buffer at various pH

1.1 Optimized pH for carboxylesterase activity in BPH

The method for detect activity of carboxylesterase was done by using UV-visible spectrophotometer with have paranitophenylacetate as substrate. When vary pH of buffer, phosphate buffer, the data show pH 8.0 is the best pH for detect activity of carboxylesterase of BPH as show in Figure 29.

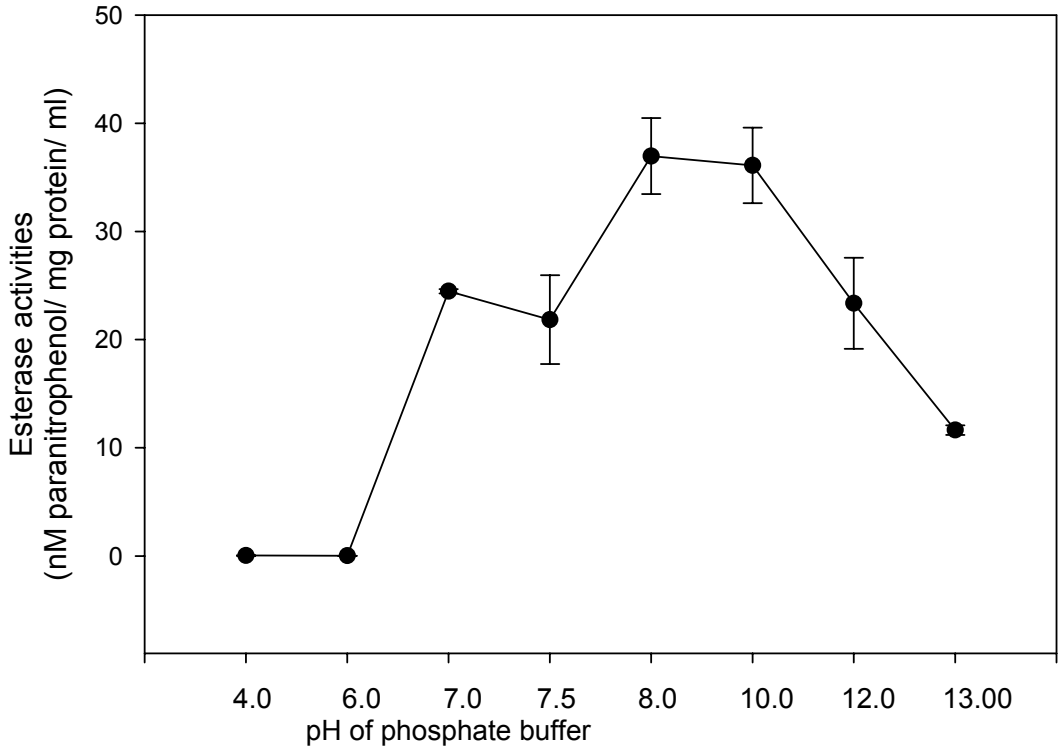



**Figure 30** Carboxylesterase activity in 3rd nymph brown planthopper in various pH of phosphate buffer.

1.2 Optimized pH for acetylcholinesterase activity in BPH

The method for detect activity of acetylcholinesterase was done by using UV- visible spectrophotometer with have acetylthiocholineiodide as substrate and observed at 412 nm. When vary pH of buffer, phosphate buffer, the data show pH 8.0 is the best pH for detect activity of acetylcholinesterase of BPH as show in Figure 31.

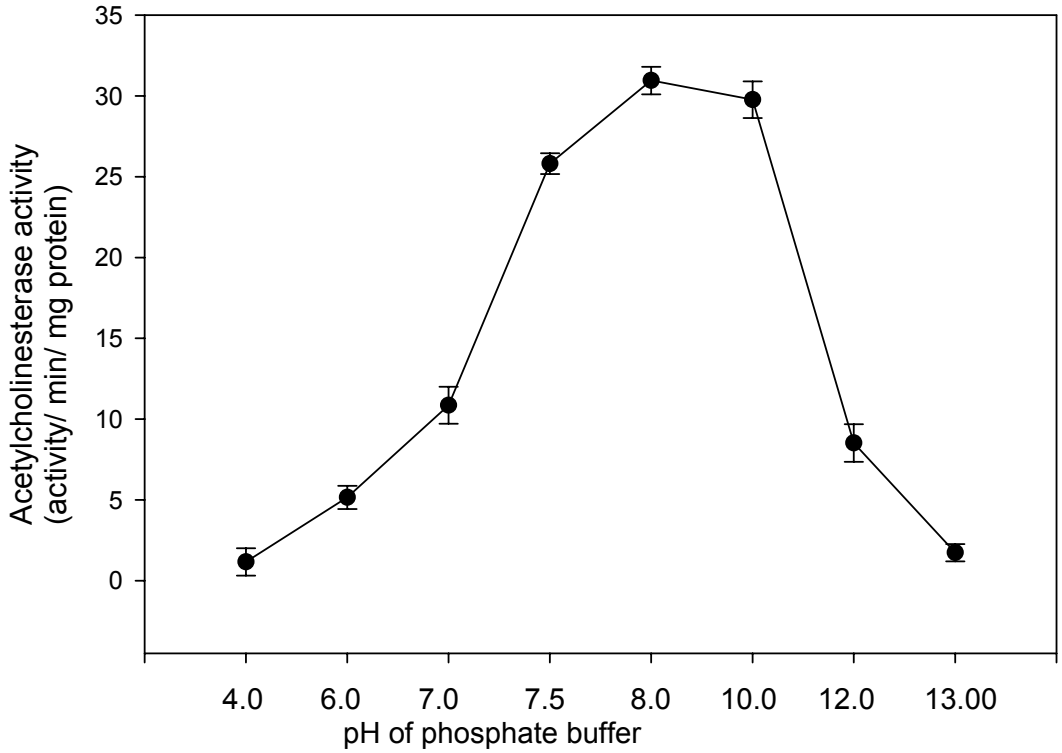



**Figure 31** Acetylcholinesterase activity in 3rd nymph brown planthopper against various pH of phosphate buffer.

1.3 Optimized pH for glutathione-S-transferase activity in BPH

The method for detect activity of glutathione-S-transferase was done by using UV- visible spectrophotometer. Anyway, in this procedure, I compared two substrates which almost use for detect activity of glutathione-S-transferase in animal using spectrophotometer. The first substrate was chlorodinitrobenzene (CDNB) which analyzed at 340 nm. Another substrate was dicholoromethane (DCNB) which can be detected at 344 nm. The activities of glutathione-S-transferase using DCNB as substrate at pH 8.0 was also the best pH for detect this enzyme same using CDNB as substrate (Figure 32-33). Hence, I preferred CDNB as the substrate because this substrate gave highest activity (table 12).

**Table 12** Comparison of glutathione-S-transferase activity (multiply with $10^{-3}$) (conjugated product/ mg protein/ ml) in brown planthopper at various pH of phosphate when using different substrate (CDNB and DCNB)

| pH of phosphate buffer | CDNB (340 nm) [(1)] | DCNB (344 nm) [(1)] |
|---|---|---|
| 4.0 | 0.0040 ± 0.0017 [a] | 0.0002 ± 0.00009 [a] |
| 6.0 | 0.0098 ± 0.0021 [b] | 0.0004 ± 0.00010 [b] |
| 7.0 | 0.0034 ± 0.0006 [c] | 0.0007 ± 0.00007 [c] |
| 7.5 | 0.0077 ± 0.0002 [d] | 0.0006 ± 0.00007 [c] |
| 8.0 | 0.0190 ± 0.0006 [e] | 0.0014 ± 0.00011 [d] |
| 10.0 | 0.0023 ± 0.0013 [f] | 0.0004 ± 0.00013 [b] |
| 12.0 | 0.0018 ± 0.0002 [f] | 0.0002 ± 0.00005 [a] |
| 13.0 | 0.0032 ± 0.0002 [c] | 0.0001 ± 0.00003 [e] |

[(1)] Different letters within the same column are not differently significant at 5% level using Duncan's Multiple Rang Test.

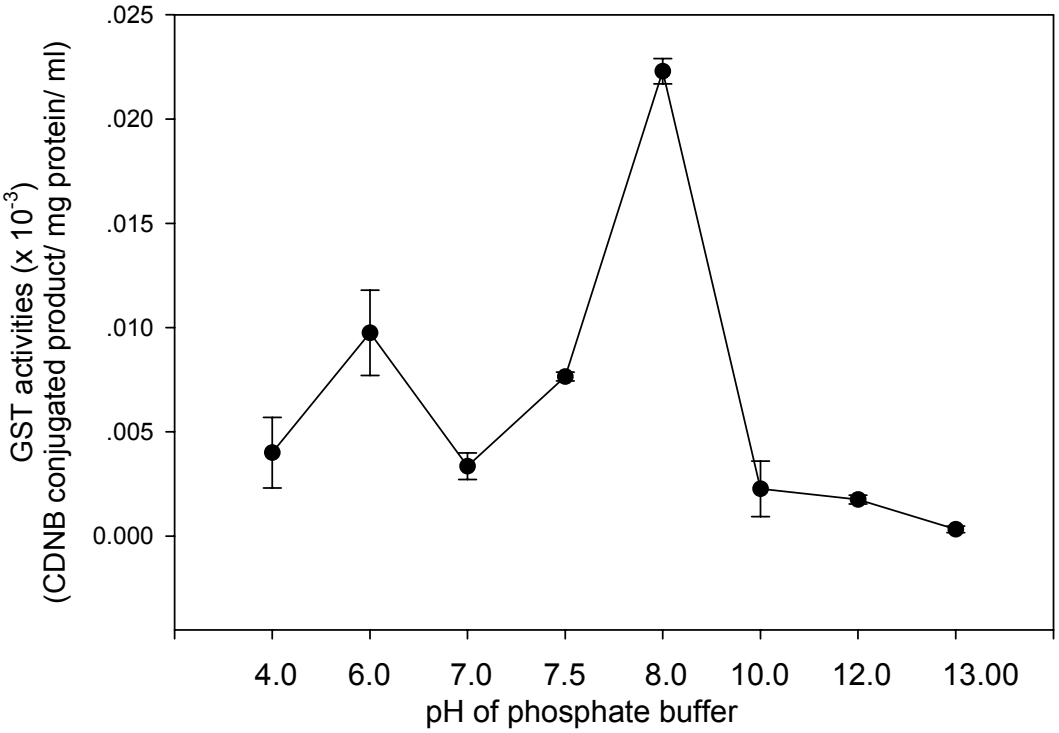



**Figure 32** Glutathione-S-transferase activity in BPH against various pH using CDNB as substrate

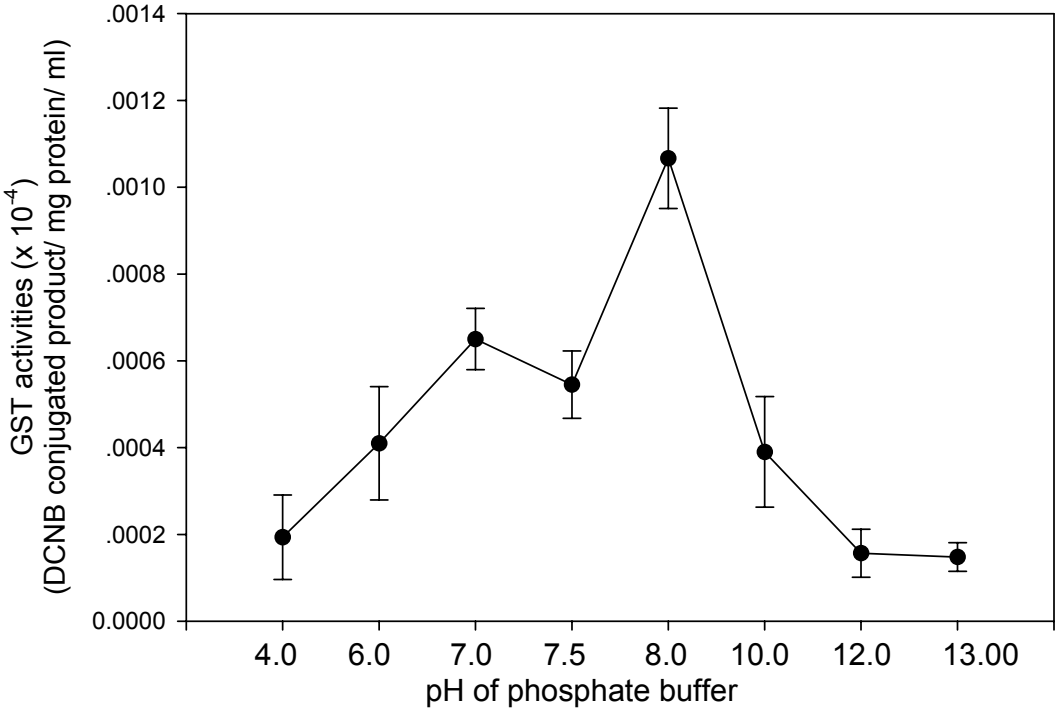



**Figure 33** Glutathione-S-transferase activity in BPH in various pH using DCNB as substrate

**2. Detoxification enzyme mechanisms of BPH After treated Pericarp of Mangosteen Fruit Extract Which Extract from various solvents.**

2.1 Carboxylesterase activity characteristic after treated with pericarp of mangosteen fruit extract.

After treated with extract, carboxylesterase activity of BPH trend to be decreased at high concentration of every extracts except the extract which used acetone as solvent. All extract show no differently significant at 5% level using Duncan's Multiple Rang Test of protein concentration (Table 13). The correction factor when compare between control and each concentrations showed all of the extract can inhibit enzyme activity between 1.09 to 6.00 fold (Table 13). Anyway, the ethanol seemed to be more efficiency (Table 13). However, the extract using acetone as solvent trend to be increased enzyme activity after treated the extract to 3rd nymph brown planthopper with no differently significant at 5% level using Duncan's Multiple Rang Test of protein concentration (Figure 34).

**Table 13** Carboxylesterase activity[(1)] (nM paranitrophenol/ mg protein/min) and CF[(2)]of 3rd nymph brown planthopper after treated with pericarp of mangosteen fruit extract with various solvents at 24 hour under the laboratory condition.

| Concentration[(3)] | Control | Conc.1 | Conc. 2 | Conc.3 | Conc.4 |
|---|---|---|---|---|---|
| 1. Hexane | | | | | |
| Protein Conc. | 56.11±0.20$^{a}$ | 55.99±0.19$^{a}$ | 56.04±0.15$^{a}$ | 56.35±0.11$^{a}$ | 56.17±0.30$^{a}$ |
| Enzyme activity | 16.91±0.72$^{a}$ | 15.50±0.72$^{b}$ | 13.07±0.45$^{c}$ | 10.49±0.76$^{d}$ | 7.68 ± 1.28$^{e}$ |
| CF | - | 1.09 | 1.29 | 1.61 | 2.20 |
| 2.Dichlolro - methane | 56.11±0.20$^{a}$ | 56.19±0.20$^{a}$ | 56.11±0.15$^{a}$ | 54.17±0.13$^{a}$ | 55.11±0.20$^{a}$ |
| Protein Conc. | 16.91±0.72$^{a}$ | 11.49±0.70$^{b}$ | 6.62±0.77$^{c}$ | 4.46± 1.20$^{d}$ | 2.82± 0.60$^{e}$ |
| Enzyme activity | | | | | |
| CF | - | 1.47 | 2.55 | 3.79 | 6.00 |
| 3. Ethanol | | | | | |
| Protein Conc. | 56.11±0.20$^{a}$ | 56.21±0.12$^{a}$ | 55.91±0.20$^{a}$ | 56.13±0.20$^{a}$ | 56.17±0.16$^{a}$ |
| Enzyme activity | 16.91±0.72$^{a}$ | 10.70±1.81$^{b}$ | 9.74±0.46$^{c}$ | 8.92±0.98$^{d}$ | 6.43±0.09$^{e}$ |
| CF | - | 1.58 | 1.74 | 1.90 | 2.63 |
| 4. Acetone | | | | | |
| Protein Conc. | 56.11±0.20$^{a}$ | 55.89±0.21$^{a}$ | 56.31±0.18$^{a}$ | 55.91±0.20$^{a}$ | 55.59±0.17$^{a}$ |
| Enzyme activity | 16.91±0.72$^{a}$ | 7.07±1.65$^{b}$ | 8.50± 1.28$^{c}$ | 10.16±1.02$^{d}$ | 14.78±0.40$^{e}$ |
| CF | - | 2.39 | 1.99 | 1.66 | 1.14 |

(1) Means ± SD, 5 replicates, n = 60 adults were employed, 24 hours check per batch for all experiments followed by different letters within the same row is differently significant at 5% level using Duncan's Multiple Rang Test

(2) CF is a correction factor = (enzyme activity of control)/ (enzyme activity of treatment).

(3) Hexane and Dichloromethane: conc. 1= 20%w/v, conc. = 40%w/v, conc. 3 = 60%w/v and conc.4 = 80%w/v. Ethanol and Acetone: conc. 1= 2%w/v, conc. = 4%w/v, conc. 3 = 6%w/v and conc.4 = 8%w/v.

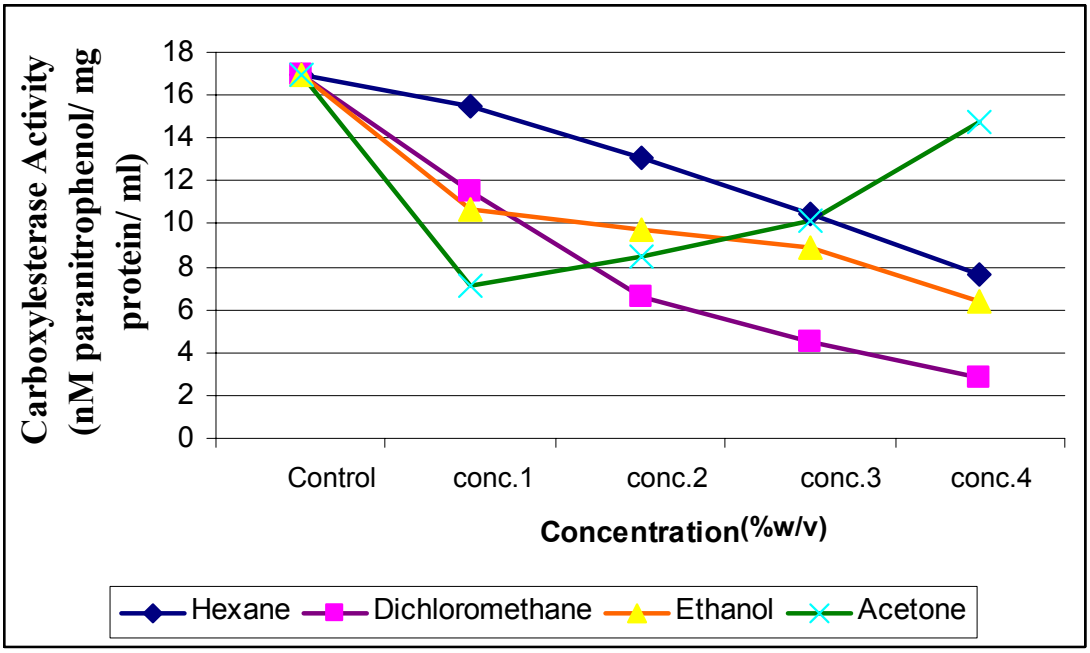



**Figure 34** Carboxylesterase activity (nM paranitrophenol/ mg protein/min) of BPH after treated with pericarp of mangosteen fruit extracts with various solvent at 24 hour under the laboratory condition.

**Note:** Hexane and Dichloromethane: conc. 1= 20%w/v, conc. = 40%w/v, conc. 3 = 60%w/v and conc.4 = 80%w/v. Ethanol and Acetone: conc. 1= 2%w/v, conc. = 4%w/v, conc. 3 = 6%w/v and conc.4 = 8%w/v.

2.2 Acetylcholinesterase activity characteristic after treated with pericarp of mangosteen fruit extract.

After treated with extract, acetylcholinesterase activity of BPH trend to be decreased at high concentration of every extracts except the extract which used acetone as solvent. All extract show no differently significant at 5% level using Duncan's Multiple Rang Test of protein concentration (Table 14). The correction factor when compared between control and each concentration showed all of the extract can inhibit enzyme activity between 0.70 to 5.88 fold (Table 14). Anyway, the ethanol seemed to be more efficiency than another which showed inhibitor efficiency (Table 14). However, the extract using acetone as solvent trend to be increase enzyme activity after treated the acetone extract to 3$^{rd}$ nymph brown planthopper with no significant different using Duncan's Multiple Rang Test of protein concentration.

**Table 14** Acetylcholinesterase activity[(1)] (acetylcholinesterase activity/ mg protein/min) and CF[(2)] of BPH after treated with mangosteen pericarp extract with various solvents at 24 hour under the laboratory condition.

| Concentration[3] | Control | Conc.1 | Conc. 2 | Conc.3 | Conc.4 |
|---|---|---|---|---|---|
| 1. Hexane | | | | | |
| Protein Conc. | 56.11±0.20[a] | 55.99±0.19[a] | 56.04±0.15[a] | 56.35±0.11[a] | 56.17±0.30[a] |
| Enzyme activity | 2.53 ± 0.04[a] | 3.59±0.12 [b] | 2.69±0.45 [c] | 1.29±0.15 [d] | 0.68 ± 0.37[e] |
| CF | - | 0.70 | 0.94 | 1.96 | 3.72 |
| 2.Dichlolromethane | | | | | |
| Protein Conc. | 56.11±0.20[a] | 56.19±0.20[a] | 56.11±0.15[a] | 54.17±0.13[a] | 55.11±0.20[a] |
| Enzyme activity | 2.53±0.04 [a] | 2.45±0.10 [b] | 1.68±0.10 [c] | 1.53± 0.29 [d] | 1.28± 0.10 [e] |
| CF | - | 1.03 | 1.51 | 1.65 | 1.98 |
| 3. Ethanol | | | | | |
| Protein Conc. | 56.11±0.20[a] | 56.21±0.12[a] | 55.91±0.20[a] | 56.13±0.20[a] | 56.17±0.16[a] |
| Enzyme activity | 2.53 ± 0.04[a] | 1.83±0.04 [b] | 1.63±0.39 [c] | 1.26±0.98 [d] | 0.43±0.09 [e] |
| CF | - | 1.38 | 1.55 | 2.01 | 5.88 |
| 4. Acetone | | | | | |
| Protein Conc. | 56.11±0.20[a] | 55.89±0.21[a] | 56.31±0.18[a] | 55.91±0.20[a] | 55.59±0.17[a] |
| Enzyme activity | 2.53 ± 0.04[a] | 1.47±0.12 [b] | 1.76± 0.36 [c] | 1.88±0.36 [d] | 1.98±0.30 [e] |
| CF | - | 1.72 | 1.44 | 1.35 | 1.28 |

(1) Means ± SD, 5 replicates, n = 60 adults were employed, 24 hours check per batch for all experiments followed by different letters within the same row are significantly different at P<0.05, DMRT.
(2) CF is a correction factor = (enzyme activity of control)/ (enzyme activity of treatment).
(3) Hexane and Dichloromethane: conc. 1= 20%w/v, conc. = 40%w/v, conc. 3 = 60%w/v and conc.4 = 80%w/v. Ethanol and Acetone: conc. 1= 2%w/v, conc. = 4%w/v, conc. 3 = 6%w/v and conc.4 %w/v.

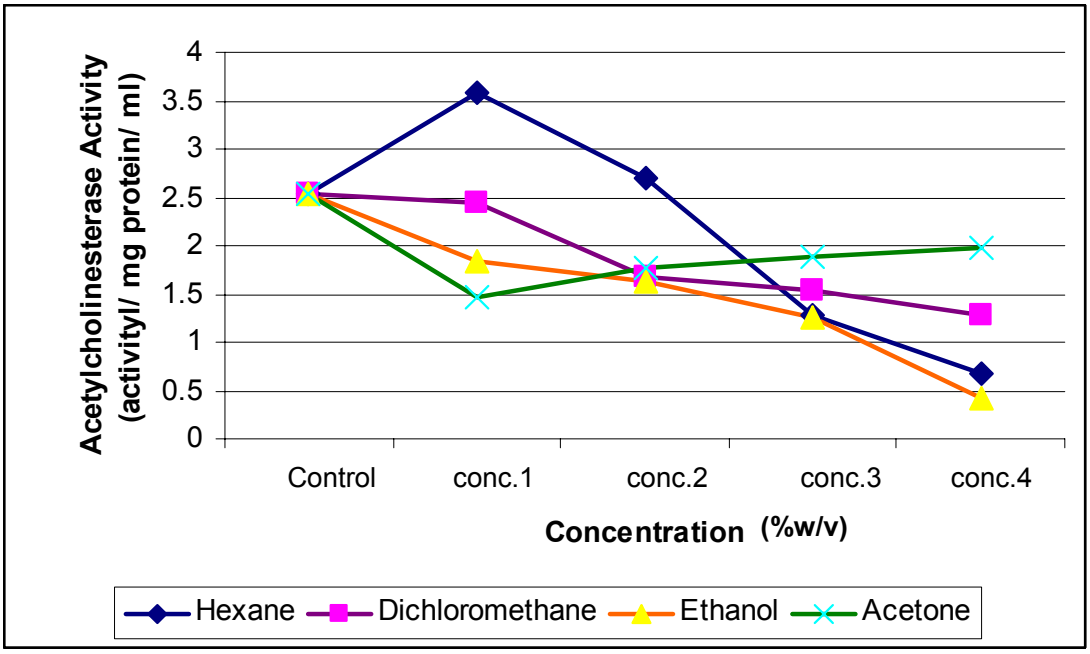



**Figure 35** Acetylcholinesterase activity (activity/ mg protein/min) of BPH after treated with pericarp of mangosteen fruit extract with various solvents at 24 hour under the laboratory condition.

**Note:** Hexane and Dichloromethane: conc. 1= 20%w/v, conc. = 40%w/v, conc. 3 = 60%w/v and conc.4 = 80%w/v. Ethanol and Acetone: conc. 1= 2%w/v, conc. = 4%w/v, conc. 3 = 6%w/v and conc.4 = 8%w/v.

2.3 Glutathione-S-transferase (GST) activity characteristic after treated with pericarp of mangosteen fruit extract.

After treated with extract, GST activity of BPH trend to be decreased at high concentration of every extracts except the extract which used acetone as solvent. All extract show no differently significant at 5% level using Duncan's Multiple Rang Test of protein concentration (Table 15). The correction factor when compared between control and each concentrations showed all every of extracts can inhibit enzyme activity between 0.70 to 5.88 fold (Table 15). Anyway, the ethanol seems to be more efficiency than another which shows inhibitor efficiency (Table 15).

However, the extract using acetone as solvent trend to be induced enzyme activity after treated to BPH with no significant different at 5% using Duncan's Multiple Rang Test of protein concentration. Thus, BPH may adapt to resistant to this type of extract in the future.

**Table 15** Glutathione-S-trasferase activity[(1)] (CDNB conjugated product/ mg protein/min) and CF[(2)]of BPH after treated with pericarp of mangosteen fruit extract with various solvent at 24 hour under the laboratory condition.

| Concentration[3] | Control | Conc.1 | Conc. 2 | Conc.3 | Conc.4 |
|---|---|---|---|---|---|
| 1. Hexane | | | | | |
| Protein Conc. | 56.11±0.20[a] | 55.99±0.19[a] | 56.04±0.15[a] | 56.35±0.11[a] | 56.17±0.30[a] |
| Enzyme activity | 9.38 ± 1.28[a] | 6.21±1.35 [b] | 4.21±0.73 [c] | 3.92±0.84 [d] | 1.21 ± 0.09[e] |
| CF | - | 1.51 | 2.22 | 2.39 | 7.75 |
| 2.Dichloro-methane | | | | | |
| Protein Conc. | 56.11±0.20[a] | 56.19±0.20[a] | 56.11±0.15[a] | 54.17±0.13[a] | 55.11±0.20[a] |
| Enzyme activity | 9.38±1.28 [a] | 7.43±0.01 [b] | 6.44±1.35 [c] | 4.98± 1.27 [d] | 2.21± 0.35 [e] |
| CF | - | 1.26 | 1.46 | 1.88 | 4.24 |
| 3. Ethanol | | | | | |
| Protein Conc. | 56.11±0.20[a] | 56.21±0.12[a] | 55.91±0.20[a] | 56.13±0.20[a] | 56.17±0.16[a] |
| Enzyme activity | 9.38 ± 1.28[a] | 7.68±0.73 [b] | 6.44±1.35 [c] | 5.99±1.27 [d] | 3.92±1.27 [e] |
| CF | - | 1.22 | 1.46 | 1.57 | 2.39 |
| 4. Acetone | | | | | |
| Protein Conc. | 56.11±0.20[a] | 55.89±0.21[a] | 56.31±0.18[a] | 55.91±0.20[a] | 55.59±0.17[a] |
| Enzyme activity | 9.38 ± 1.28[a] | 2.92±0.84 [b] | 2.65± 0.57 [c] | 4.20±0.79 [d] | 4.90±0.47 [e] |
| CF | - | 3.21 | 3.54 | 2.23 | 1.90 |

[(1)] Means ± SD, 5 replicates, n = 60 adults were employed, 24 hours check per batch for all experiments followed by different letters within the same row are significantly different at $P<0.05$, DMRT. (Each activity multiply with $10^{-6}$)
[(2)] CF is a correction factor = (enzyme activity of control)/ (enzyme activity of treatment).
[(3)] Hexane and Dichloromethane: conc. 1= 20%w/v, conc. = 40%w/v, conc. 3 = 60%w/v and conc.4 = 80%w/v. Ethanol and Acetone: conc. 1= 2%w/v, conc. = 4%w/v, conc. 3 = 6%w/v and conc.4 = 8%w/v.

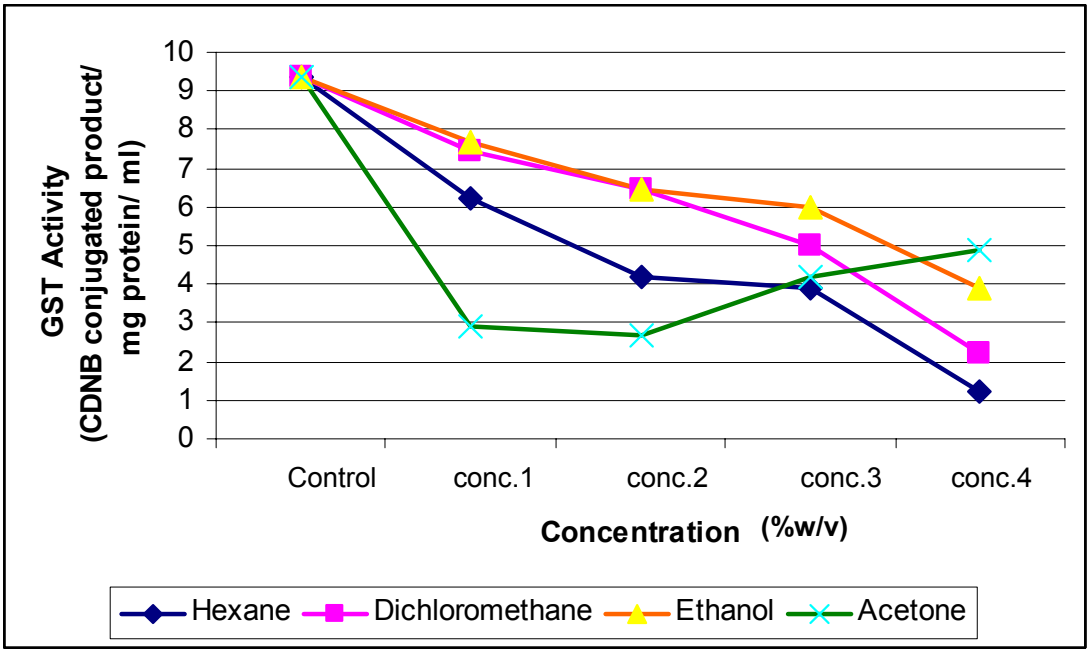



**Figure 36** Glutathione-S-trasferase activity (CDNB conjugated product/ mg protein/min) of BPH after treated with pericarp of mangosteen fruit extract with various solvent at 24 hour under the laboratory condition.

**Note:** Hexane and Dichloromethane: conc. 1= 20%w/v, conc. = 40%w/v, conc. 3 = 60%w/v and conc.4 = 80%w/v. Ethanol and Acetone: conc. 1= 2%w/v, conc. = 4%w/v, conc. 3 = 6%w/v and conc.4 = 8%w/v.

**3. Comparison of Detoxification enzyme mechanisms in various stages of brown planthopper after treated Crude Ethanolic Pericarp of Mangosteen Fruit Extract and imidacloprid.**

3.1 Carboxylesterase Activity

Carboxylesterase activity of brown planthopper was inhibited after treated with pericarp of mangosteen fruit extract with no significant different at 5% level using Duncan's Multiple Rang Test of protein concentration (data not shown). The correction factor when compare between control and each concentrations shows ethanolic extract can inhibit enzyme activity of each stage of nymph and adult brown planthopperbetween 1.18 to 1.57 fold (Table 16). The inhibition was increased when concentration of extract increased. For Imidacloprid, it show inhibit efficiency to carboxylesterase with no significant different at 5% level using Duncan's Multiple Rang Test of protein concentration (data not shown). The correction factor when compared between control and each concentration showed imidaclprid can inhibit enzyme activity between 1.13 to 2.48 fold (Table 16).

**Table 16** Carboxylesterase activity[(1)] (nM paranitrophenol/ mg protein/min) and CF[(2)]of each stage of nymph and adult brown planthopper[(3)] after treated with ethanolic mangosteen pericarp extract and Imidacloprid at LC50 dose

| Stage | Control | Ethanolic Extract [CF] | Imidaclorid [CF] |
|---|---|---|---|
| N1 | 27.46±2.8 [a] | 23.36±1.01[b] [1.18] | 20.46±2.81[c] [1.32] |
| N2 | 28.45±0.81[a] | 14.47±1.20[b] [1.57] | 11.46±0.97[c] [2.48] |
| N3 | 16.91±0.97[a] | 9.74±0.46 [b] [1.74] | 12.46±1.01[c] [1.36] |
| N4 | 16.62±0.77[a] | 13.07±0.93[b] [1.27] | 13.61±1.81[c] [1.22] |
| N5 | 17.39±0.62[a] | 11.80±0.26[b] [1.47] | 12.39±2.81[c] [1.40] |
| N6 | 23.21±0.35[a] | 19.21±2.84[b] [1.21] | 20.46±0.08[c] [1.13] |

(1) Means ± SD, 5 replicates and different letters within the same row are significant different at 5% level using Duncan's Multiple Rang Test.
(2) CF is a correction factor = (enzyme activity of control)/ (enzyme activity of treatment).
(3) N1 = 1st nymph brown planthopper, white color and 1 mm length, N2 = 2nd nymph brown planthopper, light yellowish color and 1.5 mm, N3 =3rd nymph brown planthopper, yellowish color and 2-2.5 mm length, N4= 4th nymph brown planthopper dark yellowish color and 3 mm length, 5th nymph light brown color, 3 mm length and N6= adult brown planthopper, brown color 3-3.5 mm length.

3.2 Acetylcholinesterase Activity

Acetylcholinesterase activity of each stage of nymph and adult brown planthopper were inhibited after treated with pericarp of mangosteen fruit extract with no significant different at 5% level using Duncan's Multiple level Rang Test of protein concentration (data not shown). The correction factor when compare between control and each concentrations shows ethanolic extract can inhibit enzyme activity of each stage of nymph and adult brown planthopperbetween 1.77 to 4.65 fold (Table 17). The inhibition was increased when concentration of extract increased.

For Imidacloprid, it show inhibit efficiency to acetylcholinesterase with no significant different at 5% level using Duncan's Multiple rang Test of protein concentration (data not shown). The correction factor when compared between control and each concentration showed imidacloprid can inhibit enzyme activity between 4.22 to 9.74 fold (Table 17).

**Table 17** Acetylcholinesterase activity[(1)] (activity/ mg protein/min) and CF[(2)]of various stage nymph and adult brown planthopper[(3)] after treated with mangosteen pericarp extract and Imidacloprid at LC50 does.

| Stage | Control | Ethanolic Extract [CF] | Imidaclorid [CF] |
|---|---|---|---|
| N1 | 5.45±2.65 [a] | 1.56±1.32 [b] [3.49] | 1.29±1.94 [c] [4.22] |
| N2 | 4.75±0.36[a] | 2.69±0.29 [b] [1.77] | 0.53±0.10 [c] [8.96] |
| N3 | 2.53±0.04 [a] | 0.544±0.10[b] [4.65] | 0.52±0.04 [c] [4.87] |
| N4 | 4.75±0.39[a] | 2.16±0.30[b] [2.20] | 0.88±0.24[c] [5.40] |
| N5 | 3.64±0.29[a] | 1.30±0.15[b] [2.80] | 0.53±0.36[c] [6.87] |
| N6 | 4.58±0.04[a] | 2.16±0.10[b] [2.12] | 0.47±0.12[c] [9.74] |

(1) Means ± SD, 5 replicates and different letters within the same row are significant different at 5% level using Duncan's Multiple Rang Test.
(2) CF is a correction factor = (enzyme activity of control)/ (enzyme activity of treatment).
(3) N1 = 1st nymph brown planthopper, white color and 1 mm length, N2 = 2nd nymph brown planthopper, light yellowish color and 1.5 mm, N3 =3rd nymph brown planthopper, yellowish color and 2-2.5 mm length, N4= 4th nymph brown planthopper dark yellowish color and 3 mm length, 5th nymph light brown color, 3 mm length and N6= adult brown planthopper, brown color 3-3.5 mm length.

### 3.3 Glutathione-S-transferase Activity

Glutathione-s-transferase activity of every stage of nymph and adult brown planthopper was inhibit after treated with pericarp of mangosteen fruit extract with no significant different at 5% level using Duncan's Multiple Rang Test of protein concentration (data not shown). The correction factor when compare between control and each concentrations shows ethanolic extract can inhibit enzyme activity of each stage of nymph and adult brown planthopper between 1.36 to 2.97 fold (Table 18). The inhibition was increased when concentration of extract increased.

For Imidacloprid, it show inhibit efficiency to glutathione-s-transferase with no significant different at 5% level using Duncan's Multiple Rang Test of protein concentration (data not shown). The correction factor when compared between control and each concentration showed imidacloprid can inhibit enzyme activity between 1.37 to 5.26 fold (Table 18).

**Table 18** GST activity[(1)] (CDNB conjugated product/ mg protein/min) and CF[(2)] of various stage nymph and adult brown planthopper[(3)] after treated with ethanolic mangosteen pericarp extract and Imidacloprid at LC50 dose.

| Stage | Control | Ethanolic Extract [CF] | Imidaclorid [CF] |
|---|---|---|---|
| N1 | 1.67±0.006[a] | 1.09±0.001[b] [1.53] | 1.03±0.002[c] [1.62] |
| N2 | 2.69±0.06[a] | 1.41±0.003[b] [1.91] | 1.08±0.005[c] [2.49] |
| N3 | 9.38±1.28[a] | 6.44±1.35[b] [1.46] | 2.13±0.001[c] [4.40] |
| N4 | 1.57±0.002[a] | 1.03±0.003[b] [1.52] | 0.81±0.002[c] [1.94] |
| N5 | 1.23±0.002[a] | 1.09±0.001[b] [1.36] | 0.90±0.003[c] [1.37] |
| N6 | 3.21±0.73[a] | 1.08±0.005[b] [2.97] | 0.61±0.001[c] [5.26] |

(1) Means ± SD, 5 replicates and different letters within the same row are significant different at 5% level using Duncan's Multiple Rang Test.
(2) CF is a correction factor = (enzyme activity of control)/ (enzyme activity of treatment).
(3) N1 = 1st nymph brown planthopper, white color and 1 mm length, N2 = 2nd nymph brown planthopper, light yellowish color and 1.5 mm, N3 =3rd nymph brown planthopper, yellowish color and 2-2.5 mm length, N4= 4th nymph brown planthopper dark yellowish color and 3 mm length, 5th nymph light brown color, 3 mm length and N6= adult brown planthopper, brown color 3-3.5 mm length.

**4. Detoxification enzyme mechanisms of various stage brown planthopper after treated with alpha mangostin**

The *in vitro* studies showed that mangostin reduced carboxylesterase (CarE), acetylcholinesterase (AchE) and glutathione-S-transferase (GST) activities upto 1.21-2.05 fold, 1.24-2.50 fold and 1.01-3.34 fold, respectively (Table 19). The high activities of both esterase (carboxylesterase and acetylcholinesterase) shows they may play a major role in detoxification of alpha mangostin. However, in this experiment showed no changing of protein level.

**Table19** Detoxification enzymes activities[1, 2] and Correction factor (CF)[3] of Various stage of brown planthopper (4) against Mangostin at LC50 does at 24 hours exposure under laboratory condition.

| | N1 | N2 | N3 | N4 | N5 | N6 |
|---|---|---|---|---|---|---|
| Carboxylesterase (CarE) | | | | | | |
| Control | 17.57±1.66[a] | 18.45±0.81[a] | 13.57±1.66[a] | 16.62±0.77[a] | 17.39±0.62[a] | 3.21±0.35[a] |
| Treatment | 12.04± 1.42[b] | 8.98±2.48[b] | 11.24±2.14[b] | 13.50±0.70[b] | 13.77±0.91[b] | 18.54±1.35[b] |
| | [1.49] | [2.05] | [1.21] | [1.23] | [1.26] | [1.25] |
| Acetylcholinesterase (AchE) | | | | | | |
| Control | 0.91±0.31[a] | 0.45±0.81[a] | 0.53±0.31[a] | 0.62±0.77[a] | 1.39±0.62[a] | 1.21±0.35[a] |
| Treatment | 0.53±0.07[b] | 0.18±0.48[b] | 0.20±0.02[b] | 0.50±0.70[b] | 0.77±0.91[b] | 0.54±1.35[b] |
| | [1.72] | [2.50] | [2.50] | [1.24] | [1.81] | [2.24] |
| Gluthathione-S-transferase (GST) | | | | | | |
| Control | 7.46±2.81[a] | 6.45±0.81[a] | 9.38±1.27[a] | 6.62±0.77[a] | 7.39±0.62[a] | 9.21±0.35[a] |
| Treatment | 5.35±3.34[b] | 2.98±2.48[b] | 2.81±1.73[b] | 6.55±1.49[b] | 3.77±0.91[b] | 6.54±1.35[b] |
| | [1.39] | [2.18] | [3.34] | [1.01] | [1.96] | [1.41] |

(1) Means ± SD, 5 replicates, n = 60 adults were employed, 24 hours check per batch for each experiments followed by different letters within the same column are significant different at 5% level using Duncan's Multiple Rang Test.
(2) Enzyme assays were followed Hemingway (2000) and Visetson et al.(2003) the unit of CarE is nM paranitrophenol product/ min/ mg protein. The unit of GST are nM CDNB conjugated product/ min/ mg protein (all activities x 10-6). The unit of AchE are nM product/ min/ mg protein.
(3) CF is a correction factor = (enzyme activity of control)/ (enzyme activity of treatment).
(3) N1 = 1st nymph brown planthopper, white color and 1 mm length, N2 = 2nd nymph brown planthopper, light yellowish color and 1.5 mm, N3 =3rd nymph brown planthopper, yellowish color and 2-2.5 mm length, N4= 4th nymph brown planthopper dark yellowish color and 3 mm length, 5th nymph light brown color, 3 mm length and N6= adult brown planthopper, brown color 3-3.5 mm length.

## 5. Inhibitor type studies of Detoxification enzyme mechanisms of 3rd nymph brown planthopper ater treated with pericarp of mangosteen fruit extract.

Because of detoxification enzymes in terms of carboxylesrase, acetylcholinesterase and glutathione-s-transferase from 3rd nmph brown planthopper showed inhibition against pericarp of mangosteen fruit extract. Thus, Km and Vmax would be the values to classify type of inhibition occur to the system against the extract. The method was done for enzyme activities with plant extract mixing with varying substrate concentration against enzyme concentration and looking for the Km and Vmax values.

5.1 Inhibitor type of extract to Carboxylesterase of 3rd nymph brown planthopper.

This experiment used 5%w/v pericarp of mangosteen fruit extract which were the LC50 for these BPH against extract. The enzyme activity after treated with the extract showed Km ca. 0.2040 ± 0.03M and Vmax ca. 1.7545 ±0.10 nM paranitrophenol/ mg protein/ ml compare with control, Km ca. 0.0012 ±0.10 M and Vmax ca. 0.5081 ±0.08 nM paranitrophenol/ mg protein/ ml. Thus, this type of activity is uncompetitive inhibition (Table 20, Figure37-39).

5.2 Inhibitor type of extract to Acetylcholinesterase of 3rd nymph brown planthopper

This experiment used 5%w/v pericarp of mangosteen fruit extract which were the LC50 for these BPH against extract. The enzyme activity after treated with the extract showed Km ca. 0.033 ±0.10 M and Vmax ca. 2.099 ±0.04 acetylcholinesterase activity/ mg protein/ ml compare with control, Km ca. 0.019 ±0.05 M and Vmax ca. 0.0106 ±0.01 acetylcholinesterase activity/ mg protein/ ml. Thus, this type of activity is uncompetitive inhibition (Table 20, Figure 40-42).

5.3 Inhibitor type of Glutathione-S-transferase of 3rd nymph brown planthopper

This experiment used 5%w/v pericarp of mangosteen fruit extract which were the LC50 for these BPH against extract. The enzyme activity after treated with the extract showed Km ca. 0.0307 ±0.05 M and Vmax ca. 0.0067 ±0.020 nM CDNB conjugated product/ mg protein/ ml compare with control, Km ca. 0.0514 ±0.040 M and Vmax ca. 0.0992 ±0.10 CDNB conjugated product/ mg protein/ ml. Thus, this type of activity is uncompetitive inhibition (Table 20, Figure43-45).

**Table 20** Km and Vmax values(1) of detoxification enzymes in BPH after treated with pericarp of mangosteen fruit extract under the laboratory condition.

| Type of enzymes(3) | Crude extract | Km values (M) | Vmax values |
|---|---|---|---|
| Carboxylesterase | - | $0.0012 \pm 0.10^{a}$ | $0.0581 \pm 0.08^{a}$ |
| | + | $0.2040 \pm 0.03^{b}$ | $1.7545 \pm 0.10^{b}$ |
| Acetylcholinesterase | - | $0.0190 \pm 0.05^{c}$ | $0.0106 \pm 0.01^{c}$ |
| | + | $0.0330 \pm 0.10^{d}$ | $2.0999 \pm 0.04^{d}$ |
| Glutathione-s-transferase(4) | - | $0.0514 \pm 0.04^{e}$ | $0.0992 \pm 0.10^{e}$ |
| | + | $0.0307 \pm 0.05^{f}$ | $0.0067 \pm 0.02^{f}$ |

(1) means ± SD from 3 replication 60 individual/replicate followed by the different letters within the same column are significantly different at $P<0.05$

(2) Treated at LC50 concentration of pericarp of mangosteen fruit extract against 3rd nymph brown planthopper

(3) The unit of carboxylesterase is nM paranitrophenol/ mg protein/ min. The unit of acetylcholinesterase is activity/ mg protein/ min and the unit of glutathione-s-transferase is CDNB conjugated product/ mg protein/ min.

(4) Activity of glutathione-s-transferase = activity * $10^{-6}$

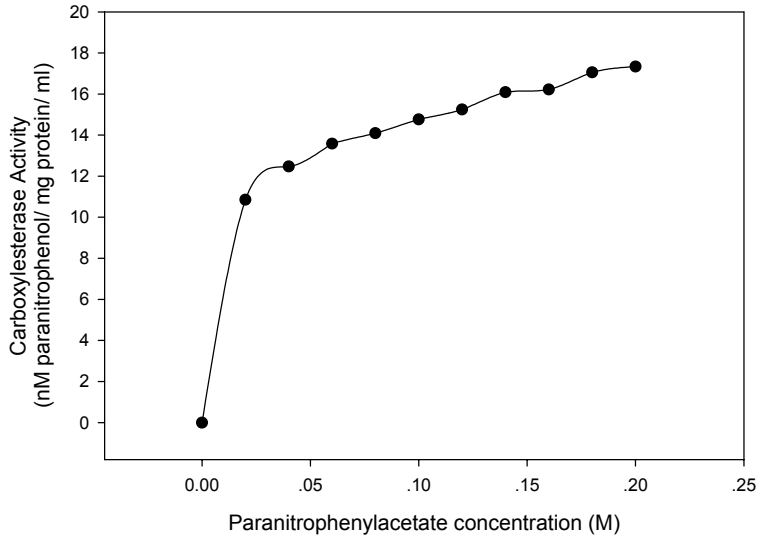



**Figure** 37 Average carboxylesterase activity of BPH before treated with pericarp of mangosteen fruit extract (control group) using various concentration paranitrophenylacetate as substrate under laboratory condition

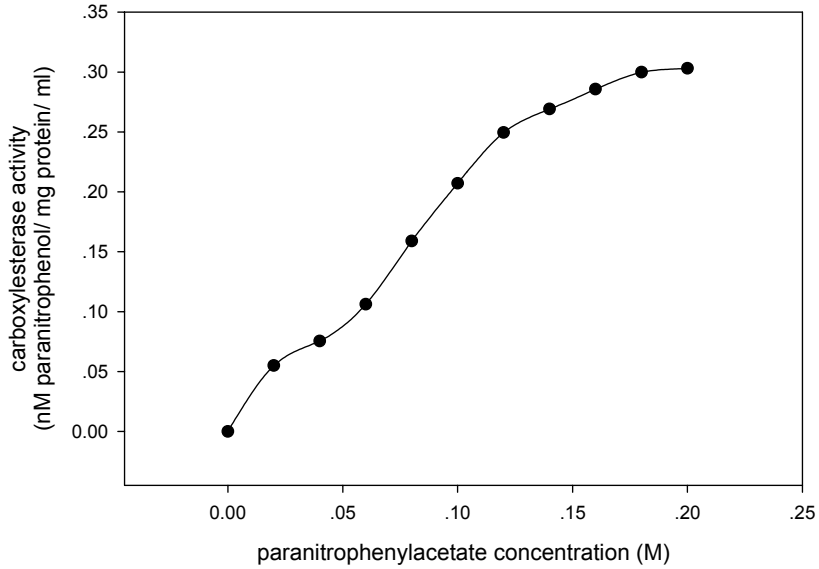
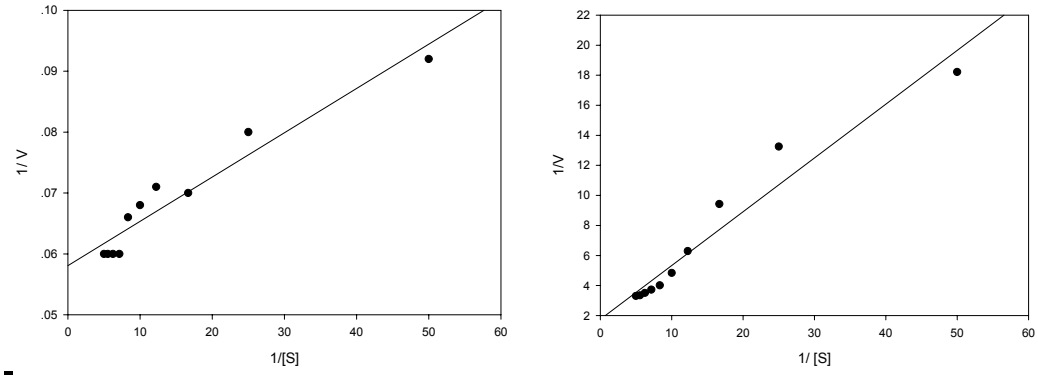



**Figure** 38 Average carboxylesterase activity of BPH after treated with pericarp of mangosteen fruit extract (treated group) using various concentration paranitrophenylacetate as substrate under laboratory condition




(A) (B)

**Figure** 39 Double reciprocal plot of average carboxylesterase activity of BPH (A) before treated by pericarpof mangosteen fruit extract (B) after treated with pericarp of mangosteen fruit extract using paranitrophenylacetate as substrate under laboratory condition

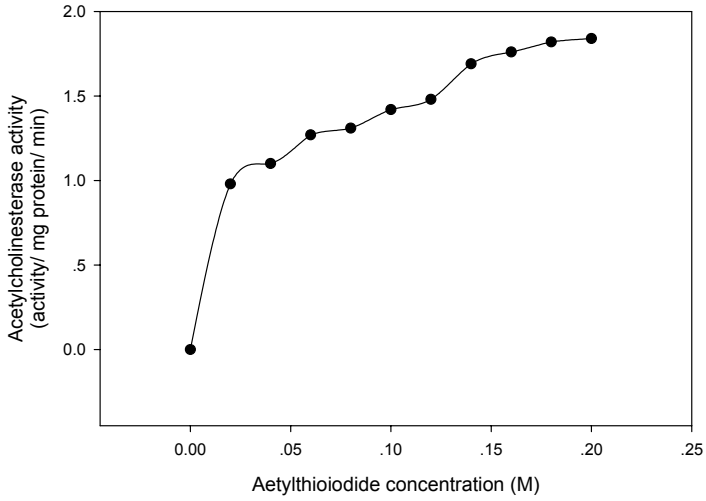



**Figure 40** Average acetylcholinesterase activity of BPH before treated with pericarp of mangosteen fruit extract (control group) using various concentration paranitrophenylacetate as substrate under laboratory condition.

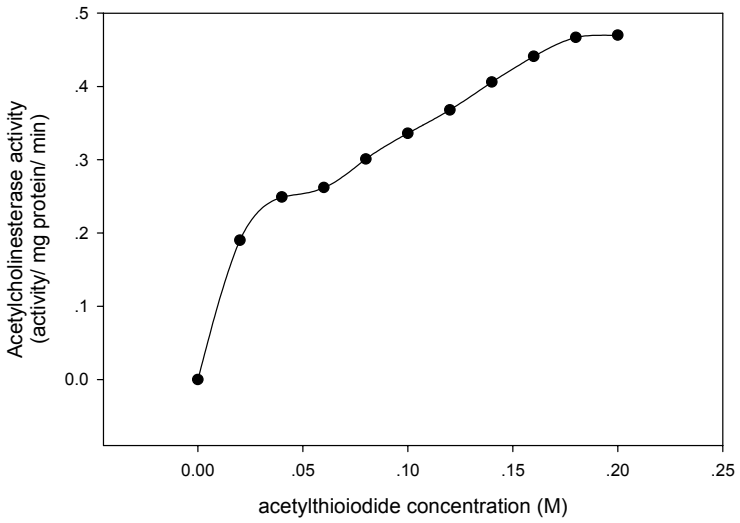
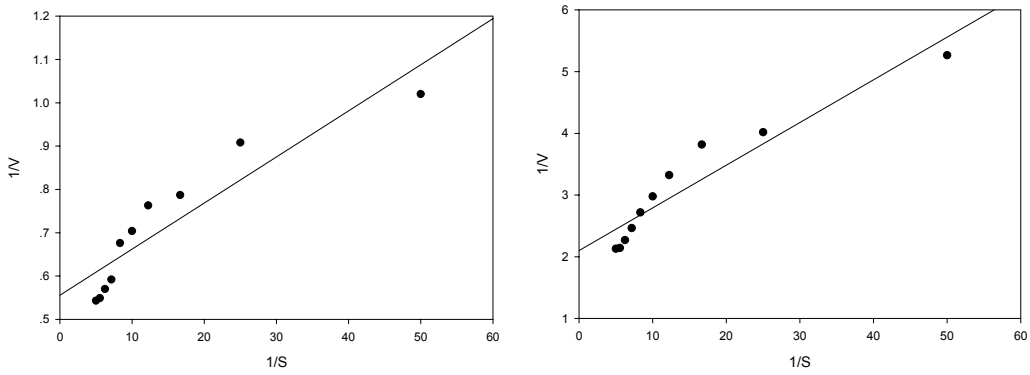



**Figure 41** Average acetylcholinesterase activity of BPH after treated with pericarp of mangosteen fruit extract (treated group) using various concentration paranitrophenylacetate as substrate under laboratory condition.




(A) (B)

**Figure 42** Double reciprocal plot of average acetylcholinesterase activity of BPH (A) before treated by pericarp of mangosteen fruit extract (B) after treated with pericarp of mangosteen fruit extract using acetylthioiodide as substrate under laboratory condition

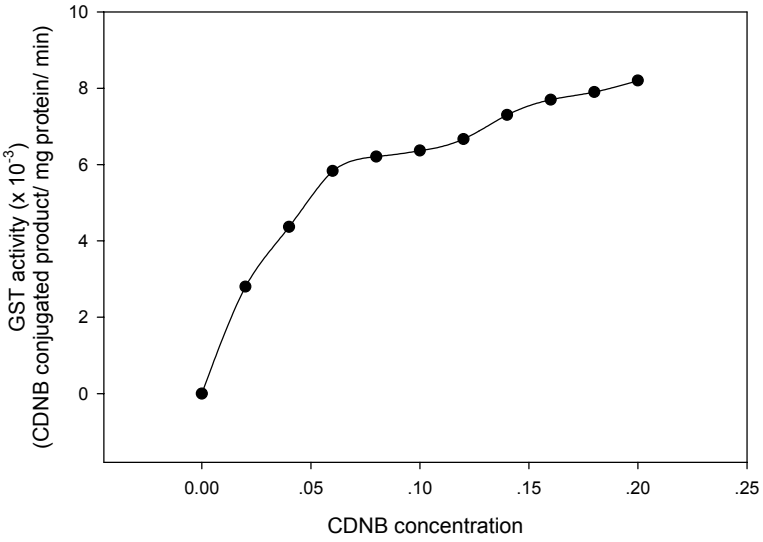



**Figure** 43 Average GST activity of BPH before treated with pericarp of mangosteen Fruit extract (control group) using various concentration of CDNB as substrate under laboratory condition

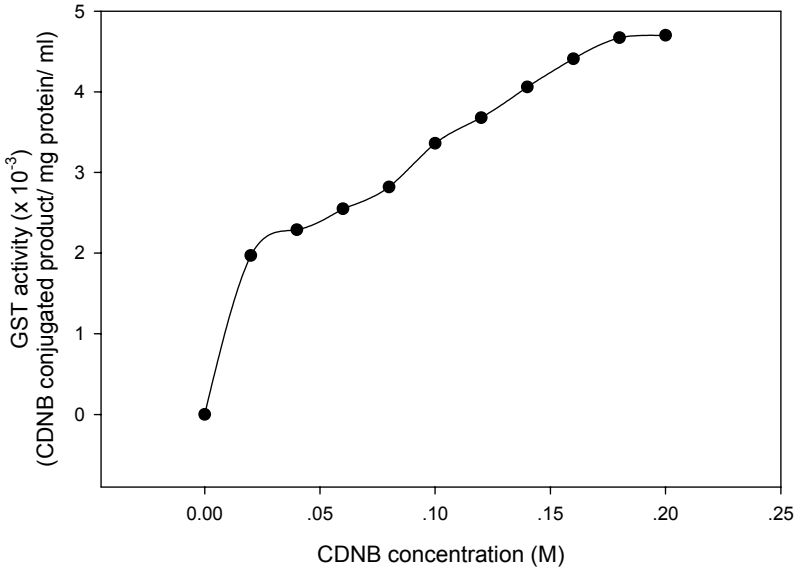
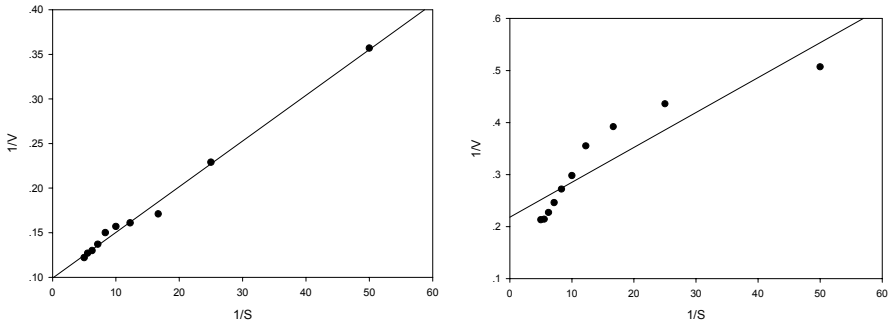



**Figure** 44 Average GST activity of BPH after treated with pericarp of mangosteen Fruit extract (treated group) using various concentration of CDNB as substrate under laboratory condition



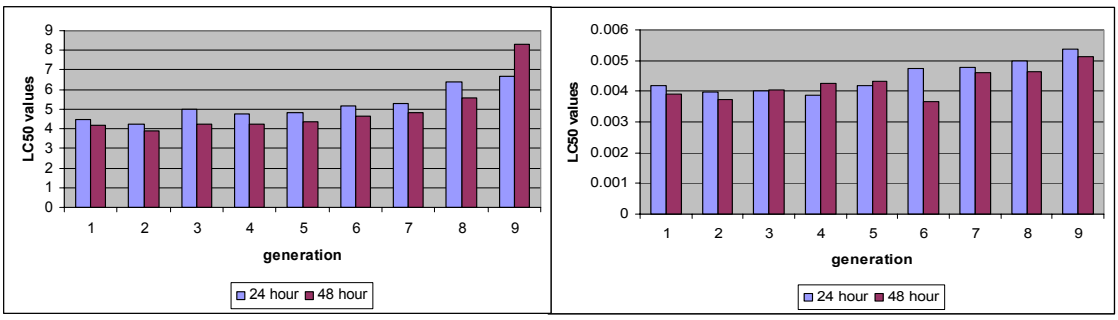

(A) (B)

**Figure** 45 Double reciprocal plot of average glutathione-s-transferase activity of BPH (A) before treated by pericarp of mangosteen fruit extract (B) after treated with pericarp of mangosteen fruit extract using acetylthioiodide as substrate under laboratory condition

## Effects of Pericarp from Mangosteen Fruit Extracts and imidacloprid after sequential spraying on Brown Planthopper

### 1. Toxicity of ethanolic mangosteen pericarp extracts and imidacloprid after sequential spraying on 3rd nymph brown planthopper

Topical application of the ethanolic extracts against BPH (F0- F8), each generation, LC50 values were higher than for previous generations (Table 21, Figure44). Extract concentrations applied each generation were highly correlated with mortality with $r^2$ values of 0.90 – 0.99. The longer exposure time of 48 hours show lower $LC_{50}$ values than those at 24 hours. Anyway, topical application every generation of the synthetic insecticide, imidacloprid to BPH (F0- F8), each generation in which $LC_{50}$ values were higher than for previous generations (Table 21). Extract concentrations applied each generation were highly correlated with mortality with $r^2$ values of 0.90 – 0.99. The longer exposure time of 48 hours gave lower $LC_{50}$ values than those at 24 hours.




(a) (b)

**Figure 46** LC50 value (%w/v)at 24 hour of 3rd nymph brown planthopper (F0-F8) treated with pericarp of mangosteen fruit extract (a) and imidacloprid (b)

**Table 21** Comparison $LC_{50}$ value (% w/v) Regression equation[(1)] and Correlation Coefficient ($r^2$)[(2)] of *N. lugens* after sequential spraying by extracts of pericarp mangosteen fruit and imidacloprid at 24 and 48 hour exposure.

| | Pericarp mangosteen fruit extract | | | Imidacloprid | | |
|---|---|---|---|---|---|---|
| | Regression equation | $LC_{50}$ | $R^2$ | Regression Equation | $LC_{50}$[(3)] | $r^2$ |
| F0 | 24Hr.:Y=8.33+9.25X | 4.50 | 0.95 | Y=-1.68+12330.6X | 4.19 | 0.99 |
| | 48Hr.:Y=9.11+9.81X | 4.17 | 0.97 | Y=-0.23+12845.5X | 3.91 | 0.99 |
| F1 | 24Hr.:Y=8.33+9.89X | 4.22 | 0.94 | Y=6.81+10829.70X | 3.99 | 0.98 |
| | 48Hr.:Y=9.66+10.4X | 3.88 | 0.92 | Y=7.88+11316.04X | 3.72 | 0.99 |
| F2 | 24Hr.:Y=8.31+8.32X | 5.01 | 0.93 | Y=-0.23+12512.6X | 4.01 | 0.97 |
| | 48Hr.:Y=12.4+8.89X | 4.23 | 0.89 | Y=-0.24+11141.2X | 4.30 | 0.97 |
| F3 | 24Hr.:Y=10.6+8.24X | 4.76 | 0.90 | Y=-0.14+12993.2X | 3.86 | 0.89 |
| | 48Hr.:Y=13.5+8.59X | 4.25 | 0.85 | Y=-0.18+11783.3X | 4.26 | 0.83 |
| F4 | 24Hr.:Y=10.8+8.18X | 4.80 | 0.89 | Y=-0.99+1223.84X | 4.17 | 0.94 |
| | 48Hr.:Y=14.3+8.22X | 4.34 | 0.83 | Y=-0.46+11635.1X | 4.34 | 0.92 |
| F5 | 24Hr.:Y=11.0+7.58X | 5.14 | 0.88 | Y=0.10+10488.2X | 4.76 | 0.89 |
| | 48Hr.:Y=12.9+8.0X | 4.62 | 0.85 | Y=0.33+10860.7X | 3.65 | 0.89 |
| F6 | 24Hr.:Y=8.66+7.83X | 5.28 | 0.93 | Y=0.59+10335.80X | 4.78 | 0.97 |
| | 48Hr.:Y=10.6+8.14X | 4.84 | 0.90 | Y=0.82+10708.29X | 4.59 | 0.96 |
| F7 | 24Hr.:Y=2.20+7.50X | 6.37 | 0.99 | Y=4.69+9055.30X | 5.00 | 0.95 |
| | 48Hr.:Y=3.78+8.33X | 5.55 | 0.98 | Y=5.94+9482.68X | 4.65 | 0.93 |
| F8 | 24Hr.:Y=1.44+7.28X | 6.67 | 0.99 | Y=4.03+8573.59X | 5.36 | 0.97 |
| | 48Hr.:Y=1.78+8.28X | 8.28 | 0.98 | Y=4.36+8915.61X | 5.12 | 0.97 |

[(1)] Regression equation: Y= Mortality percentage and X= Extract concentration
[(2)] $r^2$ was a correlation determination between concentration and mortality. Soxhlet extraction methods were used for all experiments. The method was described in the text.
[(3)] Each $LC_{50}$ value multiple with $10^{-3}$

**2. Effect on Detoxification mechanism after sequential spray the extract and Imidacloprid on 3rd nymph brown planthopper.**

2.1 *In vitro* studies for detoxification enzyme in BPH

2.1.1. Carboxylesterase

*In vitro* studies showed that the extract and imidacloprid inhibited carboxylesterase. The inhibit efficiency which showed as the correlation factor (CF) (enzyme activity of control per enzyme activity of treatment) in each generation show different except generation4 and 5 for mangosteen extract and 5 -6 of imidacloprid (Table 22).

For ethanolic mangosteen pericarp experiment, Probability values between enzyme activities of F0 generation and F8 generation using paired t-test are 0.506 for control and 0.049 for treatment.

For imidacloprid experiment, Probability values between enzyme activities of F0 generation and F8 generation using paired t-test are 0.506 for control and 0.002 for treatment. The correlation factor ca. 1.06 to 9.08 fold and 1.07 to 2.23 fold for imidacloprid and pericarp mangosteen fruit extract, respectively.

Anyway, the high activity of esterase indicates that they may play a major role in detoxification of mangostin, the biologically active compound that has been identified from mangosteen pericarp. There was also no change in protein level (data not shown). However, the correlation factor trend to be increasing after treated with imidacloprid (Table 22).

2.1.2. Acetylcholinesterase

*In vitro* studies showed that the extract and imidacloprid inhibited acetylcholinesterase. The inhibit efficiency which showed as the correlation factor (CF) (enzyme activity of control per enzyme activity of treatment) in each generation show different significant (Table 23). The correlation factor ca. 2.49 to 4.02 fold and 1.71 to 4.02 fold for imidacloprid and pericarp mangosteen fruit extract, respectively.

For ethanolic mangosteen pericarp experiment, Probability values between enzyme activities of F0 generation and F8 generation using paired t-test are 0.871 for control and 0.886 for treatment.

For imidacloprid experiment, Probability values between enzyme activities of F0 generation and F8 generation using paired t-test are 0.871 for control and 0.816 for treatment. In this experiment, there was no change in protein level (data not shown). However, the enzyme activity as correlation factor trend to be increasing in generation 6-8 after treated with imidacloprid (Table 23).

### 2.1.3. Glutathione-S-transferase

*In vitro* studies showed that the extract and imidacloprid inhibited glutathione-S-transferase. The inhibit efficiency in terms of correlation factor (CF) (enzyme activity of control per enzyme activity of treatment) in each generation showed *ca*. 1.15 to 3.30 fold and 1.02 to 6.47 fold for imidacloprid and pericarp mangosteen fruit extract, respectively.

For ethanolic mangosteen pericarp experiment, Probability values between enzyme activities of F0 generation and F8 generation using paired t-test are 0.000 for control and 0.003 for treatment.

For imidacloprid experiment, Probability values between enzyme activities of F0 generation and F8 generation using paired t-test are 0.000 for control and 0.001 for treatment. In this experiment, there was no change in protein level (data not shown).

However, the enzyme activity as correlation factor trend to be increasing in generation 6-8 after treated with imidacloprid (Table 24). Glutathione-s-transferase was less activity than carboxylesterase and acetylcholinesterase. It means BPH almost use both esterases for detoxify pericarp of mangosteen fruit extract and imidacloprid than glutathione-s-transferase.

**Table 22** Comparison of carboxylesterase activity (nM paranitrophenol/ mg protein/ min) and Correlation factor (CF) of *N. lugens* after sequential spraying by extracts of pericarp mangosteen fruit and imidacloprid at 24 hour exposure.

| Generation | Pericarp mangosteen fruit extract | | | Imidacloprid | | |
|---|---|---|---|---|---|---|
| | Protein conc. | Enzyme activity | CF[(1)] | Protein conc. | Enzyme activity | CF[(1)] |
| F0: control | 56.11±0.18 | 16.91±0.97 | 1.74 | 56.11±0.18 | 16.91±0.97 | 3.03 |
| Treatment[2] | 56.29±0.15 | 9.74±0.46 | | 55.89±0.20 | 5.58±0.24 | |
| F1: control | 56.31±0.20 | 22.68±0.75 | 2.23 | 56.31±0.20 | 22.68±0.75 | 9.08 |
| Treatment[2] | 55.89±0.25 | 10.28±1.26 | | 56.29±0.17 | 9.77±1.92 | |
| F2: control | 56.21±0.21 | 21.07±3.61 | 1.46 | 56.21±0.21 | 21.07±3.61 | 2.05 |
| Treatment[2] | 56.19±0.17 | 14.49±2.35 | | 55.89±0.17 | 10.28±0.46 | |
| F3: control | 56.81±0.20 | 18.75±0.21 | 1.50 | 56.81±0.20 | 18.75±0.21 | 1.68 |
| Treatment[2] | 57.09±0.15 | 12.52±1.92 | | 56.98±0.21 | 11.16±0.92 | |
| F4: control | 56.71±0.20 | 18.18±3.05 | 1.07 | 56.71±0.20 | 18.18±3.05 | 1.17 |
| Treatment[2] | 56.89±0.25 | 17.02±1.33 | | 57.09±0.15 | 15.49±1.91 | |
| F5: control | 56.31±0.12 | 20.66±2.66 | 1.07 | 56.31±0.12 | 20.66±2.66 | 1.19 |
| Treatment[2] | 56.19±0.15 | 18.77±3.19 | | 56.79±0.18 | 17.73±2.35 | |
| F6: control | 56.31±0.18 | 20.67±1.53 | 1.16 | 56.31±0.18 | 20.67±1.53 | 1.19 |
| Treatment[2] | 55.89±0.20 | 17.73±1.91 | | 55.69±0.22 | 17.30±0.52 | |
| F7: control | 57.13±0.13 | 24.39±4.12 | 1.38 | 57.13±0.13 | 24.39±4.12 | 1.09 |
| Treatment[2] | 56.89±0.15 | 17.73±5.29 | | 57.19±0.17 | 22.45±3.33 | |
| F8: control | 56.31±0.19 | 21.90±11.8 | 1.34 | 56.31±0.19 | 21.90±11.85 | 1.06 |
| Treatment[2] | 56.19±0.15 | 16.36±4.08 | | 56.59±0.23 | 20.75±3.12 | |

[(1)] CF is a correction factor = (enzyme activity of control)/ (enzyme activity of treatment).
[(2)] Treatment = extract concentration at $LC_{50}$ value.

**Table 23** Comparison of acetylcholinesterase activity (activity/ mg protein/ min) and Correlation factor (CF) of 3rd nymph brown planthopper *N. lugens* after sequential spraying by ethanolic extracts of pericarp mangosteen fruit and imidacloprid at 24 hour exposure.

| Generation | Pericarp mangosteen fruit extract | | | Imidacloprid | | |
|---|---|---|---|---|---|---|
| | Protein conc. | Enzyme activity | CF[(1)] | Protein conc. | Enzyme activity | CF[(1)] |
| F0: control | 56.11±0.18 | 4.53±0.04 | 1.78 | 56.11±0.18 | 4.53±0.04 | 3.19 |
| Treatment[2] | 56.29±0.15 | 2.54±0.10 | | 55.89±0.20 | 1.42±0.34 | |
| F1: control | 56.31±0.20 | 4.65±0.43 | 2.16 | 56.31±0.20 | 4.65±0.43 | 2.49 |
| Treatment[2] | 55.89±0.25 | 2.16±0.30 | | 56.29±0.17 | 1.87±0.21 | |
| F2: control | 56.21±0.21 | 4.75±0.40 | 1.80 | 56.21±0.21 | 4.75±0.40 | 2.71 |
| Treatment[2] | 56.19±0.17 | 2.64±1.26 | | 55.89±0.17 | 1.75±0.07 | |
| F3: control | 56.81±0.20 | 4.68±0.28 | 1.64 | 56.81±0.20 | 4.68±0.28 | 2.85 |
| Treatment[2] | 57.09±0.15 | 2.86±0.29 | | 56.98±0.21 | 1.64±0.21 | |
| F4: control | 56.71±0.20 | 4.88±0.24 | 1.77 | 56.71±0.20 | 4.88±0.24 | 3.11 |
| Treatment[2] | 56.89±0.25 | 2.75±0.21 | | 57.09±0.15 | 1.57±0.24 | |
| F5: control | 56.31±0.12 | 4.66±0.32 | 1.71 | 56.31±0.12 | 4.66±0.32 | 4.02 |
| Treatment[2] | 56.19±0.15 | 2.72±0.07 | | 56.79±0.18 | 1.16±0.10 | |
| F6: control | 56.31±0.18 | 4.68±0.31 | 1.78 | 56.31±0.18 | 4.68±0.31 | 2.54 |
| Treatment[2] | 55.89±0.20 | 2.64±0.40 | | 55.69±0.22 | 1.84±1.42 | |
| F7: control | 57.13±0.13 | 4.69±1.17 | 1.88 | 57.13±0.13 | 4.69±1.17 | 3.21 |
| Treatment[2] | 56.89±0.15 | 2.50±0.28 | | 57.19±0.17 | 1.46±0.31 | |
| F8: control | 56.31±0.19 | 4.83±3.01 | 2.05 | 56.31±0.19 | 4.83±3.01 | 3.66 |
| Treatment[2] | 56.19±0.15 | 2.35±2.16 | | 56.59±0.23 | 1.32±0.61 | |

[(1)] CF is a correction factor = (enzyme activity of control)/ (enzyme activity of treatment).
[(2)] Treatment = extract concentration at $LC_{50}$ value.

**Table 24** Comparison of glutathione-s-transferase activity (CDNB conjugated product/ mg protein/ min) and Correlation factor (CF) of 3rd nymph brown planthopper after sequential spraying by ethanolic extracts of pericarp mangosteen fruit and imidacloprid at 24 hour exposure.

| Generation | Pericarp mangosteen fruit extract | | | Imidacloprid | | |
|---|---|---|---|---|---|---|
| | Protein conc. | Enzyme activity | CF(1) | Protein conc. | Enzyme activity | CF(1) |
| F0: control | 56.11±0.18 | 9.38±1.28 | 1.46 | 56.11±0.18 | 9.38±1.28 | 1.56 |
| Treatment(2) | 56.29±0.15 | 6.44±1.35 | | 55.89±0.20 | 6.03±1.03 | |
| F1: control | 56.31±0.20 | 3.46±0.03 | 6.47 | 56.31±0.20 | 3.46±0.03 | 2.42 |
| Treatment(2) | 55.89±0.25 | 0.53±0.04 | | 56.29±0.17 | 1.43±0.02 | |
| F2: control | 56.21±0.21 | 5.17±0.76 | 3.60 | 56.21±0.21 | 5.17±0.76 | 1.94 |
| Treatment(2) | 56.19±0.17 | 1.43±0.02 | | 55.89±0.17 | 2.67±0.45 | |
| F3: control | 56.81±0.20 | 7.03±0.23 | 3.04 | 56.81±0.20 | 7.03±0.23 | 3.30 |
| Treatment(2) | 57.09±0.15 | 2.33±0.57 | | 56.98±0.21 | 2.13±0.36 | |
| F4: control | 56.71±0.20 | 6.60±1.97 | 1.41 | 56.71±0.20 | 6.60±1.97 | 1.25 |
| Treatment(2) | 56.89±0.25 | 4.67±3.79 | | 57.09±0.15 | 5.27±0.51 | |
| F5: control | 56.31±0.12 | 5.02± 0.35 | 1.29 | 56.31±0.12 | 5.02± 0.35 | 2.39 |
| Treatment(2) | 56.19±0.15 | 3.86±0.36 | | 56.79±0.18 | 2.10±0.41 | |
| F6: control | 56.31±0.18 | 6.90±2.95 | 1.58 | 56.31±0.18 | 6.90±2.95 | 1.79 |
| Treatment(2) | 55.89±0.20 | 4.37±2.33 | | 55.69±0.22 | 3.86±0.03 | |
| F7: control | 57.13±0.13 | 7.43±0.05 | 1.02 | 57.13±0.13 | 7.43±0.05 | 1.15 |
| Treatment(2) | 56.89±0.15 | 7.30±0.03 | | 57.19±0.17 | 6.45±1.05 | |
| F8: control | 56.31±0.19 | 1.20±0.52 | 1.30 | 56.31±0.19 | 1.20±0.52 | 2.26 |
| Treatment(2) | 56.19±0.15 | 0.92±0.45 | | 56.59±0.23 | 0.53±0.40 | |

(1) CF is a correction factor = (enzyme activity of control)/ (enzyme activity of treatment).
(2) Treatment = extract concentration at $LC_{50}$ value.

2.2 Molecular Analysis

2.2.1 PCR Amplification

From this study, one pair of CarF and CarR primers was used to amplify DNA fragment from carboxylesterase gene of control treatment (F0), F8 of imidacloprid treatment and F8 of ethanolic mangosteen pericarp extract treatment of brown planthopper using RT-PCR technique. A single PCR product of 562 bp of carboxylesterase gene was successful amplified from control treatment (F0, C), F8 of imidacloprid treatment (I) and F8 of ethanolic mangosteen pericarp extract treatment (M), respectively as show in figure 47.

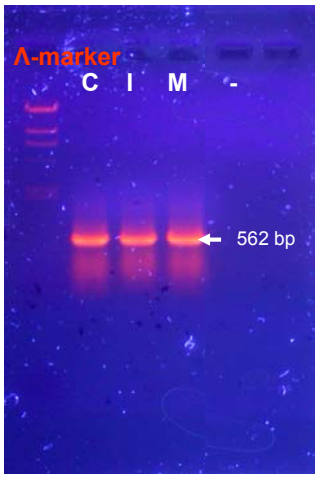



**Figure 47** The PCR product of 562 bp carboxylesterase gene from brown planthopper; control treatment (C), F8 of imidacloprid treatment (I) and F8 of pericarp mangosteen fruit extract (M).

2.2.2 Sequence alignment

The results of sequences alignment from each treatment of brown planthopper was shown in figure 48. Compare with control (C) and sequence from GenBank (AF30277) (A), Imidacloprid (I) treatment and pericarp mangosteen fruit extract (M) show 100% identical homology with each other. Moreover, the amino acid sequence also identical homology in every treatment.

```
A: gacacccaaccgcaccatcgaggcttacctcggcattccttacgcgcaaccgcccatcgg   60
    T  P  N  R  T  I  E  A  Y  L  G  I  P  Y  A  Q  P  P  I  G
C: ............................................................   60
    T  P  N  R  T  I  E  A  Y  L  G  I  P  Y  A  Q  P  P  I  G
I: ............................................................   60
    T  P  N  R  T  I  E  A  Y  L  G  I  P  Y  A  Q  P  P  I  G
M: ............................................................   60
    T  P  N  R  T  I  E  A  Y  L  G  I  P  Y  A  Q  P  P  I  G
A: ctctcgcaggttcaaggatccggagccatttggcaaatggttggggactttcaatggaac  120
    S  R  R  F  K  D  P  E  P  F  G  K  W  L  G  T  F  N  G  T
C: ............................................................  120
    S  R  R  F  K  D  P  E  P  F  G  K  W  L  G  T  F  N  G  T
I: ............................................................  120
    S  R  R  F  K  D  P  E  P  F  G  K  W  L  G  T  F  N  G  T
M: ............................................................  120
    S  R  R  F  K  D  P  E  P  F  G  K  W  L  G  T  F  N  G  T
A: caaagagcccaccaagtgtttgcaagtgaatggatttttgcccggaaaaccagtcgaggg  180
    K  E  P  T  K  C  L  Q  V  N  G  F  L  P  G  K  P  V  E  G
C: ............................................................  180
    K  E  P  T  K  C  L  Q  V  N  G  F  L  P  G  K  P  V  E  G
I: ............................................................  180
    K  E  P  T  K  C  L  Q  V  N  G  F  L  P  G  K  P  V  E  G
M: ............................................................  180
    K  E  P  T  K  C  L  Q  V  N  G  F  L  P  G  K  P  V  E  G
A: ctctgaggactgtttgtatttgaatgtttacacgccgtcacgcaatggtgtagggtaccc  240
    S  E  D  C  L  Y  L  N  V  Y  T  P  S  R  N  G  V  G  Y  P
C: ............................................................  240
    S  E  D  C  L  Y  L  N  V  Y  T  P  S  R  N  G  V  G  Y  P
I: ............................................................  240
    S  E  D  C  L  Y  L  N  V  Y  T  P  S  R  N  G  V  G  Y  P
M: ............................................................  240
    S  E  D  C  L  Y  L  N  V  Y  T  P  S  R  N  G  V  G  Y  P
A: tgtcatggtttttatacatggcgggggttttgttgatggagatggaaccagtggcttcta  300
    V  M  V  F  I  H  G  G  G  F  V  D  G  D  G  T  S  G  F  Y
C: ............................................................  300
    V  M  V  F  I  H  G  G  G  F  V  D  G  D  G  T  S  G  F  Y
I: ............................................................  300
    V  M  V  F  I  H  G  G  G  F  V  D  G  D  G  T  S  G  F  Y
M: ............................................................  300
    V  M  V  F  I  H  G  G  G  F  V  D  G  D  G  T  S  G  F  Y
A: cggaccggataaattgttgctcaccaaggacattattctagttacaatccattatcgact  360
    G  P  D  K  L  L  L  T  K  D  I  I  L  V  T  I  H  Y  R  L
C: ............................................................  360
    G  P  D  K  L  L  L  T  K  D  I  I  L  V  T  I  H  Y  R  L
I: ............................................................  360
    G  P  D  K  L  L  L  T  K  D  I  I  L  V  T  I  H  Y  R  L
M: ............................................................  360
    G  P  D  K  L  L  L  T  K  D  I  I  L  V  T  I  H  Y  R  L
```

**Figure 48** Alignment of an open reading frame in carboxylesterse gene and deduced amino acid sequence (one letter code) of AF30277 (partial) (A), control (C), imidacloprid treatment (I) and pericarp mangosteen fruit extract treatment (M). Nucleotides identical to AF30277 are marked as a dot

```
A: aggattcctcgggttcgcaagtttggatgatggagactttgctggaaactatggcctgaa   420
    G  F  L  G  F  A  S  L  D  D  G  D  F  A  G  N  Y  G  L  K
C: ..........................................................   420
    G  F  L  G  F  A  S  L  D  D  G  D  F  A  G  N  Y  G  L  K
I: ..........................................................   420
    G  F  L  G  F  A  S  L  D  D  G  D  F  A  G  N  Y  G  L  K
M: ..........................................................   420
    G  F  L  G  F  A  S  L  D  D  G  D  F  A  G  N  Y  G  L  K
A: agatcagtcgttggcgctgaagtgggtgaaggagaacattgctaaattcggaggagatgg   480
    D  Q  S  L  A  L  K  W  V  K  E  N  I  A  K  F  G  G  D  G
C: ..........................................................   480
    D  Q  S  L  A  L  K  W  V  K  E  N  I  A  K  F  G  G  D  G
I: ..........................................................   480
    D  Q  S  L  A  L  K  W  V  K  E  N  I  A  K  F  G  G  D  G
M: ..........................................................   480
    D  Q  S  L  A  L  K  W  V  K  E  N  I  A  K  F  G  G  D  G
A: cgacaaggtgactgtggtgggggagagtgcgggcgccgccagtgctcacttccacattct   540
    D  K  V  T  V  V  G  E  S  A  G  A  A  S  A  H  F  H  I  L
C: ..........................................................   540
    D  K  V  T  V  V  G  E  S  A  G  A  A  S  A  H  F  H  I  L
I: ..........................................................   540
    D  K  V  T  V  V  G  E  S  A  G  A  A  S  A  H  F  H  I  L
M: ..........................................................   540
    D  K  V  T  V  V  G  E  S  A  G  A  A  S  A  H  F  H  I  L
A: atcgccgcaaagtcaagggctc                                      562
    S  P  Q  S  Q  G  L
C: .......................                                     562
    S  P  Q  S  Q  G  L
I: .......................                                     562
    S  P  Q  S  Q  G  L
M: .......................                                     562
    S  P  Q  S  Q  G  L
```

**Figure 48** Alignment of an open reading frame in carboxylesterse gene and deduced amino acid sequence (one letter code) of AF30277 (partial) (A), control (C), imidacloprid treatment (I) and pericarp mangosteen fruit extract treatment (M). Nucleotides identical to AF30277 are marked as a dot (continued)

**Toxicity of Pericarp of Mangosteen Fruit Extract to non-target organisms.**

**1. Toxicity of crude ethanolic mangosteen pericarp extract against guppies.**

The toxicity in term of $LC_{50}$ *ca*. 2.53 ppm in female and 10.91 ppm in male guppies at 24 hours exposure (Table 25). The correlation between concentration and mortality in most experiments indicated of $r^2$ of 0.94 – 0.97 showing that the effects of the extract on the mortality of guppies were highly correlated (Table25). The longer exposure time to 48 hours give significantly different at $P<0.5$ and the high concentration gave high toxicity than less extract concentration. However, toxicity of guppies shows toxicity also depend on sex which female show in toxicity in terms of LC50 higher than male.

**Table25** Mortality percentage[(2)] of guppies against crude ethanolic extract of pericarp of mangosteen fruit after 24 and 48 hr. under the laboratory condition.

| Treatment | Female | | Male | |
|---|---|---|---|---|
| | 24 hour | 48 hour | 24 hour | 48 hour |
| 0 | 0 ± 0 [a] | 0 ± 0 [a] | 0 ± 0 [a] | 0 ± 0 [a] |
| 1 | 22.22 ± 1.92 [b] | 39.99 ± 5.77 [b] | 39.99 ± 1.92 [b] | 54.44 ± 11.71 [b] |
| 2 | 56.66 ± 5.77 [c] | 62.22 ± 7.70 [c] | 54.44 ± 5.77 [c] | 73.33 ± 11.55 [c] |
| 3 | 94.45 ± 6.93 [d] | 98.89 ± 1.92 [d] | 90.00 ± 5.77 [d] | 97.78 ± 1.92 [d] |
| Regression equation | Y= 8.48+ 16.41X | Y= 19.86+ 10.79X | Y= 3.43+ 4.27X | Y= 9.55+ 4.68X |
| $LC_{50}$ | 2.53 | 2.79 | 10.91 | 8.64 |
| $R^2$ | 0.97 | 0.95 | 0.97 | 0.94 |

(1) Control A = Distilled water.
(2) Means ± SD followed by a common letter in the same column are not significantly different at 5% level using Duncan's Multiple Rang Test.
(3) Dose for female is 0, 0.167, 3.33 and 5 ppm and for male is 0, 6.67, 13.33 and 20 ppm, respectively.

**2. Toxicity of Ethanolic Mangosteen Pericarp Extract Against ICR Mouse.**

Ethanolic mangosteen pericarp extract was applied to 4 weeks old ICR mouse for observed toxicity 3 ways such as acute oral, acute eye irrigation and acute dermal inflammation. The drinking experiment showed no acute toxicity to ICR mouse although it takes at dose 100% w/v, 5 ml. The different in sex showed no different in toxicity (Table 26). Moreover, this extract not showed dermal inflammation (no red skin, skin no burn and no swelling) in both sex of ICR mouse (Table 26). However, in eye irrigation experiment, this extract make eye of ICR mouse of both sex irritated (red eye color) but it can become to be normal in 3 days (Figure 49, Table 27). Thus, this extract is safe for mammal which showed less acute toxic via oral, dermal and eye application methods.

**Table 26** Actue oral toxicity and acute dermal inflammation toxicity of ethanolic mangosteen pericarp extract against ICR mouse after 24 hour exposure. (n = 3 replicates).

| Concentration | Acute oral toxicity | | No. Acute dermal inflammation [1] | |
|---|---|---|---|---|
| | No. dead Female | No. dead Male | No. inflamed Female | No. inflamed Male |
| Control | 0 | 0 | 0 | 0 |
| 30 %w/v | 0 | 0 | 0 | 0 |
| 50%w/v | 0 | 0 | 0 | 0 |
| 70 %w/v | 0 | 0 | 0 | 0 |
| 100%w/v | 0 | 0 | 0 | 0 |

[1] Symptom for inflammation is red skin color, skin is swelling and/or burn.

**Table 27** eye irritation toxicity [1] of ethanolic mangosteen pericarp extract against ICR mouse after 24 hour exposure (n = 3 replicates).

| | 0 hour | | 24 hour | | 48 hour | | 72 hour | |
|---|---|---|---|---|---|---|---|---|
| | No. Female | No. Male | No. Female | No. Male | No. Female | No. Male | No. Female | No. male |
| Control | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 0.1%w/v | 0 | 0 | 3 | 3 | 3 | 3 | 0 | 0 |
| 2% w/v | 0 | 0 | 3 | 3 | 3 | 3 | 0 | 0 |
| 6% w/v | 0 | 0 | 3 | 3 | 3 | 3 | 0 | 0 |
| 10%w/v | 0 | 0 | 3 | 3 | 3 | 3 | 0 | 0 |

[1] Symptom for eye irritation is red color eye and/or swelling.

**(a)** **(b)**

**Figure 49** Eye irritation of ICR mouse after treated with pericarp of mangosteen fruit extract. (a) Compare between normal eye (left side) and treated eye (right side). (b) Treated eye showed eye irritation.

### 3. Toxicity of Pericap of Mangosteen Fruit Extract against Bees.

The toxicity in term of $LC_{50}$ values *ca*. 4.38 % w/v at 24 hours exposure (Table 28). The correlation between concentration and mortality in most experiments indicated of $r^2$ of 0.73 – 0.90. It is indicated that the effects of the extract on the mortality of bees were highly correlated (Table 28). The longer exposure time to 48 hours give significantly different at 5% level using Duncan's Multiple Rang Test and the high concentration gave high toxicity than less extract concentration.

**Table28** Mortality percentage[(2)] of bee against crude extract of pericarp of mangosteen fruit after 24 and 48 hr. under the laboratory condition.

| Dose (%v/v) | 24 hour | 48 hour |
|---|---|---|
| 0[(1)] | 0 ± 0 [a] | 0 ± 0 [a] |
| 2 | 34.47 ± 0.57 [b] | 41.37 ± 1.92 [b] |
| 4 | 43.33 ± 1.92 [c] | 51.72 ± 1.77 [c] |
| 6 | 65.51 ± 11.94 [d] | 79.31 ± 2.80 [d] |
| Regression equation | Y = 5.02 + 10.27X | Y = 5.86 + 12.41X |
| $LC_{50}$ | 4.38 | 3.56 |
| $r^2$ | 0.95 | 0.95 |

[(1)] Distilled water.
[(2)] Means ± SD followed by a common letter in the same column are not significantly different at 5% level using Duncan's Multiple Range Test.

## Effect of temperature to yield percentage of ethanolic mangosteen pericarp extract and their control efficiency to brown planthopper

### 1. Effect of temperature to yield of ethanolic mangosteen pericarp extract

The extract was kept with different temperature refrigerator and incubator as 4 °C, 25°C and 55°C for 3 months for observe weight changing of crude extract and active ingredient compound, alpha- mangostin using HPLC. The yield of crude showed no changing in any time contrast with % mangostin which showed significant different when keep at higher temperature (Table 29, Figure 50). Probability values between 0 days and 90 days using pair t-test is 0.215, 0.015 and 0.139 for 4°C, 25°C and 55°C, respectively.

**Table 29** Amount of pericarp of mangosteen fruit extract which keep in different temperature.

| Time (days) | 4°C | | 25°C | | 55°C | |
|---|---|---|---|---|---|---|
| | % crude | % mangostin | % crude | %mangostin | % crude | %mangostin |
| 0 | 20 | 1.1010±0.003 | 20 | 1.1010±0.003 | 20 | 1.1010±0.003 |
| 45 | 20 | 1.0950±0.012 | 20 | 1.0907±0.029 | 20 | 1.0887±0.041 |
| 90 | 20 | 1.0930±0.010 | 20 | 1.0893±0.004 | 20 | 1.0700±0.029 |

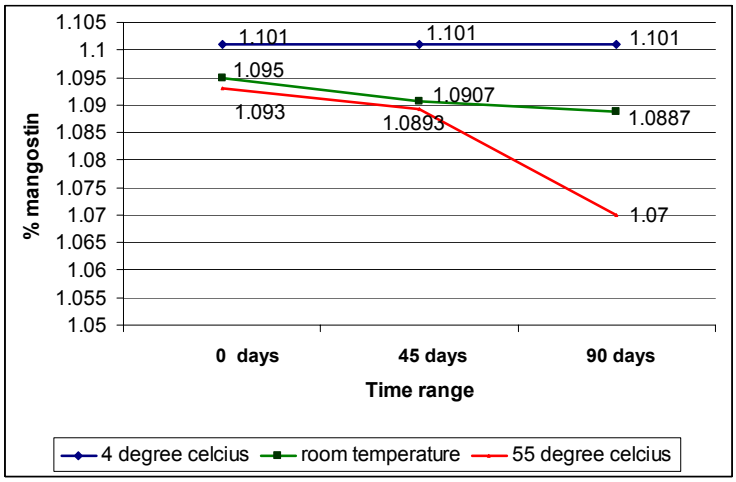



**Table 50** Amount of pericarp of mangosteen fruit extract which keep in different temperature.

**2. Effect of temperature to brown planthopper control efficiency of ethanolic mangosteen pericarp extract.**

The efficiencies of crude ethanolic extracts as toxicity value were checked. There are no quite different in LC50 value in each treatment except 55 Celsius experiment which trend to increase the LC50 values as shown in Table 30 and Figure 52. This result means temperature can affected brown planhopper control efficiency.

**Table 30** Toxicity of pericarp of mangosteen fruit extract which keep in different temperature against 3rd BPH.

| Time (days) | 4°C | | Room temperature | | 55°C | |
|---|---|---|---|---|---|---|
| | LC50 | $R^2$ | LC50 | $r^2$ | LC50 | $r^2$ |
| 0 | 4.50 | 0.95 | 4.50 | 0.95 | 4.50 | 0.95 |
| 45 | 4.52 | 0.83 | 4.53 | 0.90 | 4.80 | 0.85 |
| 90 | 4.53 | 0.85 | 4.55 | 4.89 | 5.01 | 0.98 |

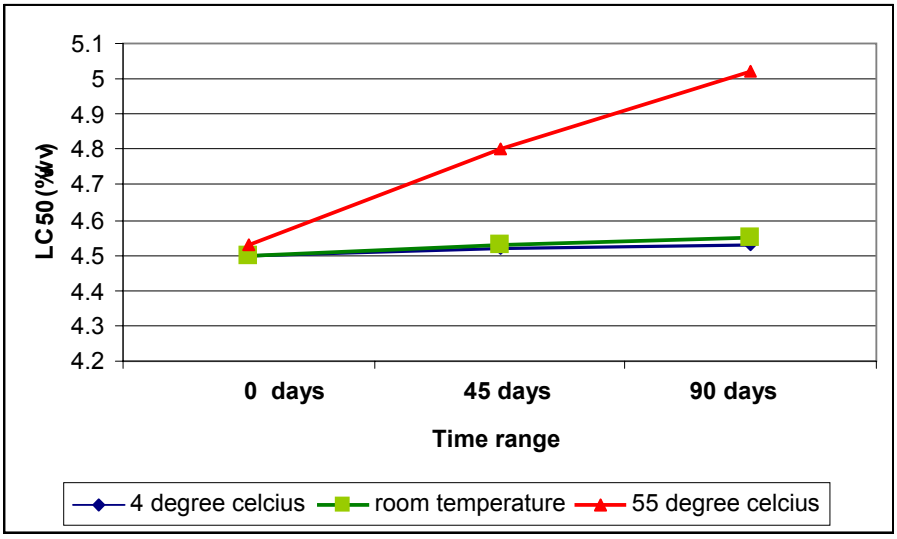



**Figure 51** Toxicity of pericarp of mangosteen fruit extract which keep in different temperature against 3rd BPH.

# DISCUSSION

The successful researches on plant extracts against some key agricultural pests have been developing by many scientists. A lot of insecticidal plants have revealed good tendency for insect control namely, Chili (*Capsicum frutescans* L.) for the control of *Sitophilus zeamais* Motschulsky (Bullangpoti *et al.,* 2002), sweet apple (*Annona squamosa* L.) for control *Nephotettix virecens* (Distant) (Srisaard, 2005), nudsage (*Cyperus rotandus* L.) for the control of *Pomacea canaliculata* L. (Ruamthum, 2002) and *Aedes aegypti* (Srikong, 2006), The fruit rind of mangosteen (*Garcina mangostana* L.), rambutan's seed (*Nephilium lappaceum* L.) and kaffir lime's leave (*Citrus hystix* DC.) for the control of *Sitophyllus oryzae* L. (Bullangpoti, 2004a), seed jam bean (*Pachyrhizus erosua* (Urb) and Ya-Knong-Chang (*Heliotropium indicum* L.) for control *Aedes aegypti* (Srikong, 2006), neem (*Azadirachta indica*) for control *Damalinia limbata* (Annette *et al.*, 2007), *Anopheles stephensi* Liston (Lucantoni *et al.*, 2006) and *Spodoptera litura* Fabricius (Senthil Nathan and Kalaivani, 2006).

After trying to measure efficacy of insecticidal plants for many years, I found that the pericarp of mangosteen fruit (*Garcina mangostana* L.) extract has been revealed promising efficiency on insect control under laboratory condition. Therefore in this research I studied the effect of pericarp of mangosteen fruit extract and alpha-mangostin from this extract for controlling brown planthopper in terms of $LC_{50}$ and the mechanisms of detoxification enzyme activities namely, carboxylesterase, acetylcholinesterase and glutathione-S-transferase, were trailed using enzyme-substrate assays with spectrophotometer and molecular analysis technique. I also studied the toxicity of such extracts to non-target organisms such as fishes, bees and mouse in order to determine the safety commercial standard for the product. All of result can divide as bellows;

## 1. Efficiency of Pericarp Mangosteen Fruit Extract Against Brown Planthopper

### 1.1 Quantitative and efficiency of extract in various solvent analysis.

The pericarp of mangosteen fruit was extracted by Soxhlet's extraction method with various solvent; hexane, dichloromethane, actone and ethanol. The yield of crude extract showed 5.21% w/w, 7.89%w/w, 20.67%w/w and 29.46%w/w, respectively (Table2). In this result, the ethanol extract can give yield more than using other solvents. Different yield can be different because using different solvents which have different polarity. However the amount of ethanol pericarp mangosteen fruit extract higher than yield from nutgrass extract (12.81%w/w) (Ruamthum, 2002) and seed kernel extract (20.12%w/w), (Visetson, 2001) but less than Seed yam bean extract (34.32%w/w) (Srikong, 2005). The different yield can be happened. It is depend on kind of plant type, growing sources, plant composition, plant part, solvent, temperatures and extract method (Visetson, *et al*. 2001). Thus, before extract the plant, we should concern about what we need or what we want such as if we need

citronellal from kaffir lime leaf, we should extract by water distillation method because this method is suitable for extract volatile oil substance than other method. Moreover, we should concern about place to keep plant and plant part because it can affect to yield of active ingredient compound (Visetson *et al*. 2005). For this research I found that the ethanol is the best solvent for extract the pericarp of mangosteen which can give the highest yield.

Not only yield of the extract, this research also focus toxicity different as shown in table 3-7 and figure 20-24. The toxicity in each solvent extract show toxicity increasing when use high concentration. The ethanol extract have highest toxicity than other extract (LC50 at 24 hour = 4.50 %w/v) though acetone extract is 5.30%w/v, dichloromethane extract is 29.68%w/v and hexane extract is 46.53%w/v. Thus, this result can show different solvent not only give different in yield but also give different in toxicity value. As table 7, the different time exposure (24 and 48 hour) can give different mortality data, thus we can conclude that time exposure is affected to toxicity to organisms. Moreover, ethanol extract show different toxicity when compare with *S. orzae* L. which showed LC50 5.25 %w/w (Bullangpoti *et al.*, 2004). The different to control insect pest can be occurred because different organism have different detoxification mechanism and efficiency to release or avoid toxic substance out from their body. The toxicity to control insect pest is different from another report such as Saisongkhroh who use seed of sugar apple or chili extract to control *Spodoptera litura* L. which shows LC50 1.642 %w/v and 4.880 %w/v, respectively and the report of EE et al. (2006) which shows control efficiency of ground stem bark of mangosteen which extract with hexane, ethylacetate and ethanol to *Aedes aegypti* as LC50 value as 0.01807, 0.0301, 0.1881 %w/v.

Thus, before develop botanical insecticides to commercial product, there are many data to be concerned such as plant type, growing place, geography, organism type, time exposures, plant composition and plant part etc.

1.2 Comparison toxicity results from ethanolic mangosteen pericarp extract and imidacloprid against various stage of brown planthopper.

Using topical sprayer method of the best control efficiency, ethanol extract, with various stages of nymph and adult brown planthopper, LC50 values at 24 hour after exposure were 1.60%w/v, 2.85%w/v, 4.50 %w/v, 4.86%w/v, 4.51%w/v and 4.01%w/v for 1$^{st}$ nymph, 2$^{nd}$ nymph, 3$^{rd}$ nymph, 4$^{th}$ nymph, 5$^{th}$ nymph and adult brown planthopper, respectively (Table 8 and Figure 25). The toxicity data is different compare with toxicity data of Bullangpoti (2004) who tested this extract on adult *Sitophilus oryzae* that showed LC50 value ca. 5.25%w/v. As result in table 8 and figure 25, the mortality data (LC50 value) is depend on stage of brown planthopper. The early stage of this insect such as 1$^{st}$ nymph stage to 2$^{nd}$ nymph stage has less efficiency to fight and/or protect to this extract though the higher stages of nymph can be successful to protect themselves to this extract. However at adult stage, LC50 seems to be decrease or this extract can control this stage better than 3$^{rd}$ to 5$^{th}$ nymph brown planthopper. It is because this stage they may spend energy and focus to develop egg for next generation more than to protect themselves as show in evolution

and Animal-Plant interaction basic, some animal prefer to produce high population of next generation for protect their population from other affected event. Thus, brown planthopper may use this way to protect their population, causing no energy for produce or protect themselves from xenobiotics. Moreover, I recommended that we should use this extract to control brown planthopper when the stage is less than 3rd nymph stage.

However, when compare control efficiency of imidacloprid to brown planthopper, the toxicity value of imidacloprid is higher than ethanolic pericarp of mangosteen fruit extract 829.41 – 1213.04 time. Thus, imidacloprid show control efficiency to brown planthopper better than using mangosteen pericarp extract. The LC50 value of imidacloprid in each stage of brown planthopper at 24 hour is 0.0018 %w/v, 0.0025 %w/v, 0.0042%w/v, 0.0050 %w/v, 0.0046 %w/v and 0.0043%w/v (table8 and figure25). Thus, we should increase the efficiency of the product before develop the mangosteen pericarp extract to be a commercial product such as add some synergists. However, the toxicity result is same with result of pericarp mangosteen fruit extract that age stage is affected to toxicity.

The correlation between concentration and mortality in most experiment indicated $r^2$ 0.742 to 0.974 for pericarp mangosteen fruit extract experiment and 0.92 to 0.99 for imidacloprid experiment showing that the effect of the both substance on the mortality of brown planthopper were highly correlated (Table 8). Moreover, as show in table 8, the exposure time as 24 and 48 hour did not show different significant toxicity at 5% level using Duncan's Multiple Range Test. However, if we observed the longer time exposure more than 48 hour or use synergist, the mortality value may different as the report of Visetson (2001) who used TPP and PB in neem seed kernels extract in control of *Callosobruchus* or Bullangpoti (2004) who increase efficiency of pericarp mangosteen fruit extract with TPP, DEM and PB.

**2. Characterization of active ingredient of pericarp of mangosteen fruit extract against brown planthopper**

Alpha- mangostin, the active ingredient compound was isolated by column chromatography method from dried crude of pericarp of mangosteen fruit which extracted by ethanol as solvent. After process in this research, ethanol extract give alpha mangostin ca. 2.956%w/w. This yield is higher than other active ingredient compound from another plant extracts such as citronellal from lemon grass (0.12%w/w), selinnadiene from nut grass (0.11%w/w), Capsicin from chili (0.24%w/w) (Visetson *et al.*, 2005) and alpha mangostin (1%w/w) (Ee *et al.*, 2007).

This compound is yellow coloring powder (figure 14) and can be classified as xanthone group. The structure of alpha manngostin contain two double bonds susceptible to hydrogenation, one methoxyl group and two hydroxyl group as show in figure 27. This structure can be say that alpha mangostin have more polarity position, thus the best extraction to get this extract should be suitable to polarity compound and use solvent such as ethanol that have more polarity.

After that treated mangostin with various stage of nymph and adult of BPH by topical sprayer method. The toxicity of 1st, 2nd, 3rd, 4th and 5th stages of nymph and adult BPH after treated with alpha-mangostin in term of $LC_{50}$ *ca*. 1.39, 2.26, 5.44, 4.49, 4.03 and 3.84 % w/v at 24 hours exposure, respectively and *ca*. 1.24, 1.93, 4.45, 4.49, 4.03, 4.12, 4.25 %w/v at 48 hour exposure, respectively (Table 9). The correlation between concentration and mortality in most experiments indicated of $r^2$ of 0.73 – 0.90 showing that the effects of the extract on the mortality of *N. lugens* were highly correlated (Table 9, Figure 26). The longer exposure time to 48 hours give significantly different and the high concentration gave high toxicity than less extract concentration. The LC50 value for alpha mangostin to brown planthopper is quite similar to crude ethanolic extract. Thus, alpha mangostin is the main active ingredient for control brown planthopper.

The mortality values as LC50 value of alpha mangostin to control brown planthopper is less than control efficiency to *Aedea aegypti* which shows LC50 0.0194%w/v (Ee et al., 2006). However, as the result from table 9, we can conclude that alpha mangostin is bioactive toward brown planthopper and the control efficiency still depends on stage of brown planthopper.

**3. Characterization of detoxification mechanism of brown planthoper against crude and active ingredient of pericarp of mangosteen fruit extract**

3.1 pH suitable of detoxification enzyme mechanism of brown planthopper.

In this research, I focus three important detoxification enzymes, carboxylesterase, acetylcholinesterase and glutathione-s-transferase. Both esterase group is in phase I reaction in detoxification enzyme mechanism which carboxylesterase play important role of many insect pest to be resistant to many insecticides such as report of Yang et al. (2004) though acetylcholinesterase play important to studied as nervous affected such as some nicotinic insecticide as imidacloprid as report of Endo et al. (2001) or Liu (2005). Before study enzyme, we should be investigated the pH suitable for each detoxification enzyme in brown planthopper because different organism and different body part have different pH optimum for enzyme activity.

The optimum pH of every detoxification enzymes which be studied in this research, carboxylesterase, acetylcholinesterase and glutathione-s- transferase, is 8.0 as shown in figure 28 -31. This pH is different in other insect which pH is 7.5 such as *Stiophilus oryzae* (Bullangpoti, 2004), *Nephotettix virescens* (Srisaard, 2005), *Aedes aegypti* (Srikong, 2005) and *Spodoptera litura* (F.) (Saisongkhroh, 2005). This result shows types of organisms can be affect to pH optimum of enzyme because enzymes have character as isozyme. The pH optimum in different organisms could occur. Thus, before study about enzyme we should investigate the pH optimum first. However, for glutathione-S-transferase activity detection, this research use two substrate for detect enzyme activity as using 1-chloro-2,4-dinitrobenzene (CDNB) and 1,2- dichloro-4-nitro-benzne (DTNB), the activity that suitable to detect glutathione-s-transferase activity in brown planthopper is CDNB as shown in table 12 and figure

30-31. This result is different from Saisongkhoh (2005) who uses DCNB as substrate which suitable for detect glutathione-s-transferase activity than uses CDNB.

3.2 Detoxification enzyme mechanism of brown planthopper after treated pericarp of mangosteen fruit extract which extracted by various solvents.

After treated with extract which extracted with various solvent about 24 hour, the live insects were used to investigate the detoxification enzyme mechanism for observe trend to be resistant in the future. Some insect or animal use this mechanism for protect themselves from toxic substance. The mechanism is various which depend on age, sex, toxic substance, organisms' type and dose (Visetson *et al.,* 2003). In this research, each solvent show different in another as shown in table 13-15.

Carboxylesterase activity of brown planthopper trends to be decreased in every solvent except extract which extract with acetone. Thus, if use acetone extract for control brown planthopper at a long time, the brown planthopper can adapt itself to be resistant to this extract using the detoxification enzyme mechanism to change their compound to be more polarity which make rapidly excretion out from their body. The correction factor when compare with control and each concentration shows every type of extract can inhibit enzyme activity between 1.09 to 6.00 fold (Table 13). Moreover, the extract which extract with ethanol seems to be more efficiency than another which shows inhibitor efficiency than another (Table 13). The extract which has acetone as solvent for extract different from another treatment, the carboxylestearse trends to be increase.

After treated with extract, Acetylcholinesterase activity of brown planthopper trend to be decrease in every solvents except extract which extracted with acetone as solvent although the protein concentration did not different in each treatment (Table 4). The correction factor (CF) shows every type of extract can inhibit this enzyme though acetone extract seems to be increase when use the high concentration of the extract. It can be conclude that brown planthopper can develop itself to be resistant the acetone extract if use it at the long time.

Glutathione-s-transferase is detoxification enzyme in phase II reaction. After treated with various extract, this enzyme trend to be inhibits in every solvents except acetone extract. The correction factor to inhibit this enzyme is around 0.70 to 5.88 fold (table 15). Anyway, ethanol extract seems to be best inhibitor to this enzyme than another extract. About acetone extract, the activity of glutathione-s-transferase seems to be increased. Thus, brown planthopper may adapt to resistant to this extract in the future.

As the result, every extract seems to have characterized as inhibitor except acetone extract and the ethanol shows the best inhibit than other extract. When compare with the toxicity data in table 7, the ethanol extract shows the best extract which can control brown planthopper than other extract. Thus, I prefer to focus the efficiency to control brown planthopper, identify the active ingredient compound,

formulated it and looking effect to non-target organisms for get a safety data for this extract.

The detoxification enzymes which were investigated in this research is carboxylesterase, acetylcholinesterase and glutathione-s-transferase. The caroboxylesterase is seems to have the role that brown planthopper use to protect itself to this extract as compare activity in table 13-15 because the treated brown planthopper show the highest carboxylesterase activity compare with other enzyme activities. Thus, carboxylesterase is the major detoxification enzyme which brown planthopper use for decrease the toxicity of the ethanolic extract. This result same with small and Hemingway (2000) who say that carboxylesterase has important role of rice insect pest to be resistant to many insecticides. The detoxification enzyme mechanism in each organism is different. It is depend on type of organisms or toxic substance. This result is same with another paper such as Srikhong (2006) and Saisongkhon (2006) who study carboxylesterase and glutathione-s-transferase in *Aedes agypti* L. and *Spodoptera litura* L., respectively after treated insect with some botanical insecticides, the carboxylesterase have important role to be resistant as shows higher activity than glutathione-s-transferase although research of Saisongkhon use the DCNB as substrate but they can gave the final product as conjugated product same using CDNB as substrate. However, glutathione-s-transferse have important role to fight of some disease in mammal such as cancer because this enzyme have efficienct to protect oxidative stress in the cell (Lin and Yang.2007)

3.3 Comparison of detoxification enzyme mechanism of various stage of brown planthopper after treated crude ethanolic pericarp of mangosteen fruit extract and imidacloprid.

Carboxylesterase activity of brown planthopper was inhibited after treated with pericarp of mangosteen fruit extract. The correction factor when compare between control and each concentrations shows every type of extract can inhibit enzyme activity between 1.18 to 1.57 fold (Table 16). The inhibition is increase when concentration of extract increasing. About imidacloprid, this chemical have efficiency same with the extract that it act as inhibitor of carboxylesterase. The correction factor when compare between control and each concentrations shows every type of extract can inhibit enzyme activity between 1.13 to 2.48 fold (Table 16). The inhibition is increase when concentration of extract increasing.

Acetylcholinesterase activity of BPH was inhibit after treated with pericarp of mangosteen fruit. The correction factor when compare between control and each concentrations shows every type of extract can inhibit enzyme activity between 1.77 to 4.65 fold (Table 17). The inhibition is increase when concentration of extract increasing. About imidacloprid, this chemical have efficiency same with the extract that it act as inhibitor of acetylcholinesterase. The correction factor when compare between control and each concentrations shows every type of extract can inhibit enzyme activity between 4.22 to 9.74 fold (Table 17). The inhibition is increase when concentration of extract increasing.

Glutathione-S-transferase activity of brown planthopper was inhibited after treated with pericarp of mangosteen fruit extract with no different significant of 5% DMRT of protein concentration (Table 18). The correction factor when compare between control and each concentrations shows every type of extract can inhibit enzyme activity between 1.36 to 2.97 fold (Table 18). The inhibition is increase when concentration of extract increasing. About imidacloprid, this chemical have efficiency same with the extract that it act as inhibitor of carboxylesterase. The correction factor when compare between control and each concentrations shows every type of extract can inhibit enzyme activity between 1.37 to 5.26 fold (Table 18). The inhibition is increase when concentration of extract increasing. Anyway, this enzymes show activity less than carboxylestearase and acetylcholinesterase that means BPH using both esterase for decrease toxicity than glutathione-S-transferase.

As the result, brown planthopper have carboxylesterase as important enzyme to be resistant which shows higher activity than other enzymes. Pericarp mangosteen fruit extract shows activity as inhibitor to all type of detoxification enzymes; however, the efficiency as inhibitor is lower than imidacloprid (table 16-18). From table 8, the result shows imidacloprid have control efficiency than the extract. Thus, imidacloprid have efficiency than pericarp mangosteen fruit extract. Although the pericarp mangosteen fruit extract have efficiency to control brown planthopper less than imidacloprid, this extract have environmental friendly than synthetic insecticide which it have biodegradable as shown in the report of Visetson (2005) who said water have important role for hydrolysis of many botanical insecticides such as salinadiene from nutgrass tuber which degradation more than 80% after go to natural environment such as river at 12 hour. Thus, this ethanolic mangosteen pericarp extract will not accumulation in environment as imidacloprid or other synthetic insecticides.

3.4 Detoxification enzyme mechanism of various stage brown planthopper after treated with mangostin and inhibitor type studies of detoxification enzyme mechanism of brown planthopper after treated with pericarp of mangosteen fruit extract.

From the result of table 19, the *in vitro* studies showed than mangostin reduced carboxylesterase, acetylcholinesterase and glutathione-s-transferase activity up to 1.21 to 2.05 fold, 1.24-2.50 fold and 1.01 to 3.34 fold, respectively. However, glutathione-s-transferase activity did not show high activity compare with carboxylesterase and acetylcholinesterase. Thus, mangostin is the one of active ingredient compound for control population of brown planthopper and carboxylesterase activity shows highest activity (table 19). The result indicated of complete metabolism of mangostin in phase I reaction possible hydrolysis by carboxylestearse.

Because of detoxification enzymes in terms of carboxylesrase, acetylcholinesterase and glutathione-s-transferase from BPH showed inhibition against pericarp of mangosteen fruit extract. Thus, Km and Vmax would be the values to classify type of inhibition occur to the system against the extract. The method was

done for enzyme activities with plant extract mixing with varying substrate concentration against enzyme concentration and looking for the Km and Vmax values.

This experiment used 4.5%w/v pericarp of mangosteen fruit extract which were the LC50 for these brown planthopper against extract. The carboxylesterase activity after treated with the extract showed Km ca. 0.2040 ± 0.03M and Vmax ca. 1.7545 ±0.10 nM paranitrophenol/ mg protein/ ml compare with control, Km ca. 0.0012 ±0.10 M and Vmax ca. 0.5081 ±0.08 nM paranitrophenol/ mg protein/ ml. Thus, the type of inhibitor is uncompetitive inhibition (Table 20, Figure35-37). Same with acetylcholinesterase activity after treated with the extract showed Km ca. 0.033 ±0.10 M and Vmax ca. 2.099 ±0.04 acetylcholinesterase activity/ mg protein/ ml compare with control, Km ca. 0.019 ±0.05 M and Vmax ca. 0.0106 ±0.01 acetylcholinesterase activity/ mg protein/ ml. Thus, this type of inhibitor to acetylcholinesterase is uncompetitive inhibition (Table 20, Figure 38-40). About glutathione-s-transferase activity after treated with the extract, it is showed Km ca. 0.0307 ±0.05 M and Vmax ca. 0.0067 ±0.020 CDNB conjugated product/ mg protein/ ml compare with control, Km ca. 0.0514 ±0.040 M and Vmax ca. 0.0992 ±0.10 CDNB conjugated product/ mg protein/ ml. Thus, this type of this activity is uncompetitive inhibition (Table 20, Figure41-43).

Thus, all of inhibitor characterization are uncompetitive inhibition which the ethanolic mangosteen extract is inhibit in enzyme-substrate complex causing it can not break to producr their product. This reult is different with Yu (1984) who report that many plant extracts can inhibit detoxification enzymes as nonspecific noncompetitive inhibition. The inhibition type of this extract is similar to other botanical insecticides which have been reported by the numbers of workers such as Ruamthum (2002), Bullangpoti (2004) and Srikhong (2006). Anyway, this research use crude of mangosteen pericarp extract, thus, the inhibitor activity may from mixer of many compounds in the extract. I recommended we make sure the inhibitor activity using the purified extract in the future.

**4. Effect of pericarp of mangosteen fruit extract and imidacloprid after sequential spraying on 3$^{rd}$ nymph brown planthopper**

4.1 Toxicity of extract of mangosteen fruit pericarp and imidacloprid after sequential spray on brown planthopper

Topical application every generation of the extract to 3$^{rd}$ nymph brown planthopper (F0- F8) showed differences in $LC_{50}$ at both 24 and 48 hours. The $LC_{50}$ values were higher than for previous generations (Table 21, Figure44). Extract concentrations applied each generation were highly correlated with mortality with $r^2$ values of 0.90 – 0.99. The longer exposure time of 48 hours gave significantly lower $LC_{50}$ values than those at 24 hours.

Anyway, topical application every generation of the synthetic insecticide, imidacloprid to 3$^{rd}$ nymph brown planthopper (F0- F8) showed differences in $LC_{50}$ at

both 24 and 48 hours and the $LC_{50}$ values were higher than for previous generations (Table 21). Extract concentrations applied each generation were highly correlated with mortality with $r^2$ values of 0.90 – 0.99. The longer exposure time of 48 hours gave significantly lower $LC_{50}$ values than those at 24 hours.

The result indicated that brown planthopper can adapt itself to be resistant if we uses this extract at the long time same with using imidacloprid; however, brown planthopper seems to be resistant when sequential use imidacloprid faster than use pericarp mangosteen fruit extract (table 21). Thus, this extract may the good choice for control of brown planthopper in rice field than imidacloprid. The develop to be resistant of brown planthopper can be happened if sequential use at the long time as shown in many reports such as Kimura et al. (1973), (Nagata and Moriya, 1969) and Budhasamai (1990). That's why this insect stills the primary insect pest of rice field till the present.

4.2 Effect on detoxification mechanism after sequential spray the extract and imidacloprid on brown planthopper.

4.2.1 in vitro studies

*In vitro* studies showed that the crude ethanolic mangosteen pericarp extract and imidacloprid inhibited carboxylesterase, acetylcholinesterase and glutathione-s-transferase. The inhibit efficiency which showed as the correlation factor (CF) (enzyme activity of control per enzyme activity of treatment) in each generation show for carboxylesterse is 1.06 to 9.08 fold and 1.07 to 2.23 fold for imidacloprid and ethanolic pericarp mangosteen fruit etract, respectively. The correlation factor for acetylcholinesterase is 2.49 to 4.02 fold and 1.71 to 4.02 fold for imidacloprid and pericarp mangosteen fruit extract, respectively. The correlation factor of glutathione-s-transferase is 1.15 to 3.30 fold and 1.02 to 6.47 fold for imidacloprid and pericarp mangosteen fruit extract, respectively. In this experiment, there was no change in protein level (Table 21-24). However, the enzyme activity as correlation factor trend to be increasing after treated with imidacloprid (Table 21-24).

As the result, glutathione-s-transferase activity is lower than carboxylesterase and acetylcholinesterase activity. The high activities of these both esterases indicate that they may play a major role in detoxification of mangostin, the biologically active compound that has been identified from mangosteen pericarp and imidacloprid than glutathione-s-transferase.

Moreover, this result also indicated that the resistant mechanism of brown planthopper may come from detoxification enzyme mechanisms, especially carboxylesterase. The result similar to the suggestion from many scientists who believed that the resistance of the insect pests against the insecticides is comes from the detoxification enzyme mechanism that insect produce after exposure to insecticides for a long period of time such as report of Chen and Sun (1994), Small and Hemingway (2000), Vontas et al. (2001) or Visetson (2005).

4.2.2 Molecular analysis

From this study, one pair of CarF and CarR promers were used to amplify DNA fragment from carboxylesterase gene, the main detoxification enzyme of brown planthopper to be resistant. The RNA from this gene was isolated from each treatment of brown planthopper using Trizol reagent and amplified using RT-PCR technique. A single PCR product of 562 bp was successful amplified as show in figure 45.

The sequencing result in each treatment; control, imidacloprid and pericarp mangosteen fruit treatment were aligned using Gentyx program for window version 7.0 which shown in figure 46. The edited sequences in each treatment were compare sequence form Genbank (acc: AF30277) which show 100% homology in each treatment about nucleotide sequence and also amino acid sequence (figure 47). This result is same with the result of Small and Hemingway (2000) who say NI-EST1 from five individual insecticide susceptible, low esterase activity strain Sri Lankan-S adults were identical in sequence to Sri Lanka-R esterase although Sri Lanka-R was 8.50 fold more resistant to malathion than Sri Lanka-S at the LT50 level. Moreover, the inferred amino acid sequence of each treatment shows 100% identity to each other.

Thus, although the toxicity data in term of LC50 value is increasing which means this insect try to resistant to this botanical insecticide and imidacloprid, the nucleotide changing in gene still no different as shown in table 22 and figure 47. However, this result focus only conserves region of carboxylesterase gene in brown planhopper and no long time sequential use (only F8 generation). The changing in this region may need longer time than this as evolution's law.

In the alphid, *Myzus persicae,* resistant is also due to the amplification of an identical gene to the insecticide-susceptible insects (Devonshire, 1977). However, the aphids primarily reproduce asexuality, unlike brown planhthopper. In contrast, small *et al.* (1998) described that in the sexually reproducing mosquito *Culex quinquefasciatus* the amplified esterase differ from, and code for, protein which have a higher affinity for organophosphates than their susceptible equivalents

**5. Toxicity of pericarp of mangosteen fruit extract to non target organisms**

5.1 Toxicity of guppies against crude of mangosteen fruit extract

This research use guppy, *Poecilia reticulata* as the representative for observed the toxicity to aquatic organisms. This kind of fish is can be potential bioindicator for urban metal pollution, especially their (1) spatial distribution over sites of all pollution region and (2) variation in metal accumulation levels deflecting the degree of pollution (Widianarko et al., 2000). Anyway, in this research we focus on sex of guppy because not only concentration of the extract, sex also affected to

toxicity data to organisms (Visetson, 2005). However, the body size of fish did not influence on the metal flux from sediment to water (Widianarko et al., 2000).

After treated pericarp of mangosteen fruit extract with guppies, the toxicity in term of $LC_{50}$ *ca*. 2.53 ppm in female and 4.27 ppm in male at 24 hours exposure (Table 25, Figure 49). The correlation between concentration and mortality in most experiments indicated of $r^2$ of 0.94 – 0.97 showing that the effects of the extract on the mortality of guppies were highly correlated (Table25). The longer exposure time to 48 hours give no significantly different at 5% level using Duncan's Multile Range Test and the high concentration gave high toxicity than less extract concentration. However, toxicity of guppies shows toxicity also depend on sex which female show toxicity in terms of LC50 higher than male.

The toxicity from this extract is higher than another insecticide such as imidacloprid which have acutely toxic to adult fish at relative concentration (over 80 ppm) with juvenile fish being considerably more susceptible (no fish kind show). However, imidacloprid is extremely toxic at low concentration of imidacloprid to some species of aquatic animals including freshwater crustacean *Hyalella aztecaia* and the estuary crustacean *Mysidopsis behi* (Buffin, 2003). Moreover, the toxicity of ethanolic mangosteen pericarp extract is different from result of Saisongkhorh (2006) who test toxicity of yam bean extract and seed of sweet apple extract to *Poecilia latipiana*, the LC50 is 0.157 ppm and 0.147 ppm, respectively. Thus, pericarp of mangosteen fruit extract seems to be friendly to fish more then other botanical insecticides however I recommened that we should use the ethanolic mangosteen pericarp extract in the area where no fish or aquatic organisms such as apply this extract to use in different control way to another insect pests. Moreover, Visetson et al. (2005) found that water have important role for hydrolysis of many botanical insecticdes such as salinadiene from nutgrass tuber which will degradation more than 80% after go to natural environment as river at the time 12 hour.

5.2 Toxicity of ICR mouse against crude of mangosteen fruit extract

The toxicity to mammal is also need to focus the toxicity data before develop insecticides commercial product. The process is pericarp of mangosteen fruit extract was treated with 4 weeks ICR mouse for observed toxicity 3 ways such as drinking, eye irrigation and dermal inflammation. The drinking experiment showed no acute toxicity to ICR mouse although it takes at dose 100% w/v, 5 ml. The different in sex showed no different in toxicity with 5% level using Duncan's Multiple Range Test. Moreover, this extract not showed dermal inflammation in both sex of ICR mouse. However, in eye irritation experiment, this extract make eye of ICR mouse of both sex irrigatted but it can become to be normal in 3 days (Figure 50). Thus, this extract is very safe to mammal which showed less acute toxicity as drinking, dermal inflammation and eye irrigation.

In contrast with imidacloprid, the reseach of Buffin (2003) show this insecticide have acute toxicity to mouse at LD50 131 mg/ kg as oral dose and the 24

hour dermal LD50 in rat is > 5,000 mg/kg. However, this chemical is considered non-irritating to eye and skin from test on rabbits.

Compare with other botanical insecticide, the result is similar to the result from Ruamthum (2002) show that oral test to ICR mouse with tuber nutgrass extract which no acute toxicity. The botanical insecticides seem to be friendly to mammal than synthetic insecticides. However, this research did not investigate organ of ICR mouse after treated the extract and also did not investigate the detoxification enzyme in this animal for be a standard as toxicity data to human.

5.3 Toxicity of bee against crude of mangosteen fruit extract.

Bee is also focus to determine toxicity to benefit animal. The process is treated pericarp of mangosteen fruit extract with bees at the toxicity values in term of $LC_{50}$ *ca*. 4.38 % w/v at 24 hours exposure (Table 26, Figure 51). The correlation between concentration and mortality in most experiments indicated of $r^2$ of 0.95 showing that the effects of the extract on the mortality of bee were highly correlated (Table 26). The longer exposure time to 48 hours give no significantly different at 5% level using Duncan's Multiple Range Test and the high concentration gave high toxicity than less extract concentration.

Buffin (2003) said the application of imidacloprid by foliar spraying, is highly toxic to honey bees (data not shown). When compare with other botanical insecticides such as the result from Srikhong (2006) who test seed of yam bean extract against bee, LC50 is 2.17 %w/v at 16 hour after exposure moreover; she also test toxicity of nutgrass tuber extract to bee which did not show any toxicity to this organism. The results indicate that pericarp mangosteen fruit extract have toxicity to bee however, its toxicity is less than synthetic insecticide as imidacloprid and some botanical insecticides such as seed of yam bean extract.

From many result about toxicity of pericarp mangosteen fruit extract to non target organisms such as mouse, bee and guppy, this extract is friendly to mouse more than synthetic insecticide such as imidacloprid. However, this ethanolic mangosteen pericarp extract show the high toxicity to guppies and bees although it show the toxicity less than other botanical insecticide. Thus, for apply to use this ethanol mangosteen pericarp extract, we should avoid to uses in the area which have aquatic organisms or may apply use this extract to another insect pests. Anyway, the studied on efficiency of this extract and immigration and accumulation of this extract should be concerned and study in the future.

6. Effect of temperature to yield percentage of ethanolic mangosteen pericarp extract and their control efficiency to brown planthopper

The ethanol extract showed the best efficiency to control brown planthopper. The ethanolic mangosteen pericarp extracts were kept at different temperature using refrigerator and incubator for upto 3 months. The alpha mangostin was analyzed by HPLC at the end of each experiment.

The extract was kept with different temperature at 4 °C, room temperature (25 °C) and 55°C and was kept up to 3 months. The yield of crude showed no changing in any time contrast with % mangostin which showed different when keep at higher temperature (Table 27, Figure 52). The result indicated that temperature can affected to yield of alpha- mangostin, the active ingredient compound which may degradation at the highest temperature. The efficiencies of extracts as toxicity value were checked. There are no different in LC50 value in each treatment except 55 Celsius experiment as shown in Table 28 and Figure 53. This result means temperature can affect to yield of mangostin and brown planhopper control efficiency which the compound may not stable in bond at the high temperature.

# CONCLUSION AND RECOMMENDATION

## Conclusion

The pericarp of mangosteen fruit extract which extracted with ethanol solvent gave the highest yield (29.46 %w/w) and showed the best control of 3rd nymph brown planthopper, LC50 4.5%w/v ($r^2$=0.95) with 3rd nymph brown planthopper when compared with other solvents; hexane, acetone and dichloromethane. The active ingredient compound, mangostin (2.956%w/w) showed LC50 5.44%w/v ($r^2$=0.88). This extract was less toxic than Imidacloprid showing LC50 0.0042%w/v ($r^2$=0.99).

The toxicity data in term of LC50 values showed significant different in each generation same with imidacloprid that showed trend to be resistant. The LC50 values in each generation except 7th -8th generation ca. 4.5 ± 1.3 %w/v after sequential spray this extract on 3rd nymph brown planthopper. The *in vitro* detoxification enzyme activity such as carboxylesterase, acetylcholinesterase and glutathione-s-transferase from BPH after 24 hours exposure was also observed which carboxylesterase showed the role to detoxify than another enzyme. Compare with the control treatment and sequence from Genbank, the carboxylesterase gene expression of imidacloprid treatment showed homology in each treatment.

This extract showed toxicity to guppies (LC50 = 2.58, 4.27 ppm for female and male, respectively ($r^2$ =0.97), bee (LC50 = 4.38%w/v, $r^2$ =0.95) and mouse (no acute toxicity via oral, dermal application but showed temporary eye irritation to the mouse.

After formulated extracts were kept under different temperature (room temperature, 55 °C and 4°C) for 3 months, the amount of alpha-mangostin at 55 °C quite different with another temperature; moreover, the toxicity data in term of LC50 also showed which LC50 of 55°C different with another temperature.

## Recommendation

1. The investigate toxicity in environmental fate should be done in the future research and should concern about economic problems as it should be developed into the commercial product or not.

2. We should be study efficiency of sequential uses of this extract longer. Because of the final result may exhibit the molecular trends clearly.

3. Trials for new method to extract which can give the higher yield of crude and give higher active ingredient.

4. Further Investigated how different of plant chemical composition of mangosteen fruit from various places.

5. Apply and develop method for formulate the extract which can give high control efficiency and easy to use before develop as commercial product.

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# APPENDIX A

# ENZYME ACTIVITY ANALYZED METHOD

# APPENDIX A

## Enzyme Activity Analyzed Method

This research using modified method of Yang et al. (2004) and Visetson and Milne (2001) that's described below;

**Apendix A1** The reagent for analyzed

1. Buffer : Potassium phosphate buffer ($KH_2PO_4$)
   Potassium dihydrogenthiophosphate (M.W = 136.09)

   0.1 M = M.W / L
   = 13.609 g / 1000 ml distilled water
   Adjust pH with pH meter

2. 1mM EDTA
   0.45224 g/ 1000 ml distilled water

3. 1L 0.1 M Phosphate buffer + 1 ml 1mM EDTA

4. 10 mM GSH
   0.15g GSH reduced form in 50 ml 0.1 M Phosphate buffer

5. PVPP: 50%w/w material

**Appendix A2** Enzyme Isolation Method

Homogenized material with 2000 µl 0.1 M PPB + EDTA
1000 µl 10 mM GSH
50% w/w PVPP
Centrifuged at 18,000 rpm, 4ºC 5 min in refrigerated centrifuge

**Appendix A3** pNPA assay (Modified from Visetson (2001) and Visetson et al. (2003, 2004). This protocol is for analyzed carboxylesterase activity using spectrophotometer at absorbance 400 nm (3 min)

Stock solution:
- 1 ml 1mM EDTA + 0.1 M Phosphate buffer
- 10 mM Glutathione reduced form (0.15 g GSH / 50 ml buffer)
- Substrate: 0.12 M paranitrophenylacetate( pNPA) (0.1 g / 5 ml ethanol)

**Appendix Table A1** pNPA assay protocol

| Solution reference | Blank | Sample |
|---|---|---|
| 0.1 M Phosphate buffer | 2900 µl | 2900 µl |
| Substrate | 50 µl | 50 µl |
| 0.1 M Phosphate buffer + EDTA (Homogenized buffer) | 50 µl | - |
| Supernatant (enzyme) | - | 50 µl |

Enzyme activity (nM paranitrophenol product/ mg protein/ml)
= O.D x 58.8235 x (total volume)

**Appendix A4** CDNB assay protocol (Modified from Visetson et al. (2002, 2003) and Bullangpoti (2002). This protocol is for analyzed glutathione-s-transferase activity using spectrophotometer at absorbance 340 nm (3 min)

Stock solution:
- 1 ml 1mM EDTA + 0.1 M Phosphate buffer
- 10 mM Glutathione reduced form (0.15 g GSH / 50 ml buffer)
- Substrate: 150 mM CDNB (0.152 g / 5 ml ethanol)

**Appendix Table Table A2** CDNB assay protocol

| Solution reference | Blank | Sample |
|---|---|---|
| 0.1 M Phosphate buffer | 1150 µl | 1150 µl |
| 0.1 M Phosphate buffer + GSH | 20 µl | - |
| Supernatant (enzyme) | - | 20 µl |
| *Equilibrate 35 °C for ca. 3 min…..* | | |
| Substrate | 10 µl | 10 µl |

Enzyme activity (CDNB conjugated product/ mg protein/ ml)
=(O.D x 1.316) / (9.6 x 1000)

**Appendix A5** DCNB assay (Modified from Visetson et al. (2002, 2003) and Bullangpoti (2002). This protocol is for analyzed glutathione-s-transferase activity using spectrophotometer at absorbance 344 nm (3 min)

Stock solution:

- 1 ml 1mM EDTA + 0.1 M Phosphate buffer
- 10 mM Glutathione reduced form (0.15 g GSH / 50 ml buffer)
- Substrate: 150 mM DCNB (0.144 g / 5 ml ethanol)

**Appendix Table Table A3** DCNB assay protocol

| Solution reference | Blank | Sample |
|---|---|---|
| 0.1 M Phosphate buffer +GSH | 1100 µl | 1100 µl |
| 0.1 M Phosphate buffer | 200 µl | - |
| Supernatant (enzyme) | - | 200 µl |
| *Equilibrate 35 °C for ca. 3 min…..* | | |
| Substrate | 10 µl | 10 µl |

Enzyme activity (mM conjugated product/ mg protein/ ml)
= (O.D x 1.316) / (10 x 1000)

**Appendix A6** Acetylthioiodide assay (Modified from Visetson et al. (2001). This protocol is for analyzed cholinesterase activity using spectrophotometer at absorbance 412 nm (3 min)

Stock solution:

- 0.1mM Phosphate buffer pH7.5
- Substrate: 0.1 M acetylthiocholine iodide (ATCL) in 0.1 M phosphate buffer (0.1446 g/ 5 ml PPB) : (F.w. 289.2)
- 0.01 M DTNB (5,5'-dithiobis (2-nitrobenzoic acid)) in phosphate buffer (0.0198 g/ 5ml PPB)

**Appendix Table Table A4** ACTh assay protocol

| Solution reference | Blank | Sample |
|---|---|---|
| 0.1 M Phosphate buffer | 2765 µl | 2765 µl |
| 0.01 M DTNB | 75 µl | 75 µl |
| 0.1 M Phosphate buffer + EDTA (Homogenized buffer) | 100 µl | - |
| Supernatant (enzyme) | - | 100 µl |
| Substrate (ATCL) | 60 µl | 60 µl |

Enzyme activity (activity/ mg protein/ ml)
= O.D x 73.529 x total volume (ml)

# APPENDIX B

# BRADFORD METHOD

# APPENDIX B

## Bradford Method

This research use bovine serum albumin (BSA) as standard

Apeendix B1 Stock solution:

- 100 mg Coomassie Brilliant Blue R250 in 50 ml 95% ethanol then add 100 ml 85% $H_3PO_4$ and make final volume to 1L by distilled water.

**Appendix Table Table B1** Bradford assay protocol

| Solution reference | Volume |
|---|---|
| Sample | 0.5 ml |
| Bradford solution | 5 ml |
| *Incubate at room temp. for 5 minutes….* | |
| *Determine by spectrophotometer at 595 nm* | |

# CIRRICULUM VITAE

| | |
|---|---|
| NAME | Miss Vasakorn Bullangpoti |
| BIRTH DATE | May 18, 1981 |
| BIRTH PLACE | Bangkok, Thailand |
| EDUCATION | Year Institution Degree<br>2001 Kasetsart University (KU.) B.Sc. (Biology)<br>2004 Kasetsart University (KU.) M.Sc. (Biology) |
| ACADEMIC EXPERIENCE | 1. Presented at 59th International Symposium on Crop Protection May 22, 2007 at Ghent, Belgium.<br>2. Presented at 58th International Symposium on Crop Protection May 23, 2006 at Ghent, Belgium.<br>3. Presented at 11th IUPAC International Congress of Pesticide Chemistry at Kobe, Japan, August 6-11, 2006<br>4. Presented at the 7th Nation Plant Protection Conference. 2-4 November 2005. Lotus Pangsuankaew, Chiengmai, Thailand.<br>5. Presented at the 2nd Asian International Conference on Ecotoxicology and Environmental Safety, September 26-29, 2004, BP Samila Beach Hotel, Songkla Thailand<br>6. Participated in ACCU International Exchange Program under the UNESCO / Japan Funds- In-trust for the promotion of the international cooperation and mutual understanding: for construction of postgraduate network of life scientist in the Asia Pacific region (Japan, China, Thailand and Australia) (2006) |
| SCHOLARSHIP/ AWARD | 1. Praise student Award and praise thesis award (master degree) from Graduate School, KU. (2004)<br>2.Cooperative Research Network (CRN) Scholarship from Commission on Higher Education, Ministry of Education, Thailand (2004)<br>3. Research Funding from Graduated School, KU. (2005):for PhD. Thesis |