

**INSECTICIDAL ACTIVITY AND MECHANISM OF ACTION OF  
PHENYLPROPANOIDS ISOLATED FROM *Piper sarmentosum*  
AGAINST STORAGE INSECT PESTS AND MOSQUITO VECTORS**

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## ABSTRACT

The phytochemical and biological studies were carried out on medicinal and edible plant known as *Piper sarmentosum* L. (Piperaceae). Bioassay guided study of the active hexane and methanol extracts from aerial parts and roots of *P. sarmentosum* L. lead to isolation of four phenylpropanoids namely, asaricin **1**, isoasarone **2**, *trans*-asarone **3** and asaraldehyde **4**. Their insecticidal activity and mechanism of action was investigated against storage pests (*Sitophilus oryzae*, *Rhizopertha dominica* and *Plodia interpunctella*) and mosquito vectors (*Aedes albopictus*, *Aedes aegypti* and *Culex quinquefasciatus*). Potent insecticidal activity against both species was produced by **1** and **2**. Compounds **1** and **2** were highly toxic to *S. oryzae* with LC<sub>50</sub> value of 4.7 and 5.6 respectively. *R. dominica* was slightly more resistance, *P. interpunctella* had highest resistant to **1** and **2** with LC<sub>50</sub> value of  $\leq 17.37$   $\mu\text{g/ml}$  and LC<sub>95</sub>  $\leq 37.7$   $\mu\text{g/ml}$ . asaricin **1** and isoasarone **2** exhibited high repellent activity against *S. oryzae*, *R. dominica* and *P. interpunctella* at 10  $\mu\text{g/ml}$ . during the residual toxicity test it was observed that **1** and **2** had consistent activity within 30 days and their activity declined after that but compound **3** activity was consistent with 60 days of experiment. Likewise, **1** and **2** had potent larvicidal activity against *Ae. albopictus*, *Ae. aegypti* and *Cx. quinquefasciatus*. **1** and **2** similarly were highly potent against *Ae. aegypti*, *Ae. albopictus* and *Cx. quinquefasciatus* larvae with LC<sub>50</sub>  $\leq 8.9$   $\mu\text{g/ml}$  and LC<sub>95</sub>  $\leq 15.1$   $\mu\text{g/ml}$ . The ovicidal activity of **1**, **2** and **3** were evaluated through egg hatching. **1** and **2** showed potent ovicidal activity. Ovicidal activity for both compounds was up to 90% at 25 $\mu\text{g/ml}$ . However repellent and adulticide activities of **1** and **2** against tested female mosquito was moderate. In all insecticidal tests compound **3** had moderate insecticidal activity and compound **4** did not have any insecticidal property. Biochemical investigation revealed that **1**, **2** and **3** inhibit the acetylcholinesterase (AChE). Although **1** and **2**

inhibition was stronger than **3** but correlation study showed that the toxicity of all three compounds was significantly related with their AChE inhibition activity. Further investigation for insect esterase activity showed that all insects had high GST activity and the level of GST was significantly higher in *P. interpunctitela* which had the highest resistance against **1**, **2** and **3**. The levels of non-specific esterases and oxidases activities were not significant between the tested insets. Further the docking studies were done to investigate the binding mode of interaction of **1**, **2** and **3** with AChE and GST enzyme using Autodock/Vina. Between three active sites peripheral anionic site (PAS), catalytic and anionic in AChE, **1**, **2** and **3** interact with more residues within 3 Å using a lower energy at anionic site. Compound **1** was interacted with TYR 370 pocket while **2** and **3** were more stable in THR 154, GLY 155, SER 156, TYR 162 at very low and stable energy level. Docking study on GST interactions with **1**, **2** and **3** suggested that PRO11, GLU64 and TYR105 are more essential residues in GST for **1**, **2** and **3** binding besides known active residues SER65 and ARG66. This can explain the high toxicity of **1** and **2**. Biochemical and docking clearly showed the GST activity role in binding and detoxifying the **1**, **2** and **3**. Our result suggested that **1** and **2** were highly toxic to tested insects by inhibiting AChE enzyme and computation work supported and clarified the binding mode of interaction. Although compound **3** activity was not as significant as **1** and **2** but there is possibility of its usage in mixture as formulation in future study since it is more stable in the environment. On the other hand understanding the role of GST enzyme helped to develop knowledge on insects response to **1**, **2** and **3**. Further studies will warrant possible applications of **1**, **2** and **3** as potential natural insecticide for the control of storage pests and mosquito vectors populations.

## ABSTRAK

Kajian fitokimia dan biologi telah dijalankan ke atas *Piper sarmentosum* L. (Piperaceae). Kajian bioasai berpandu ke atas ekstrak aktif heksana dan metanol daripada bahagian pucuk dan akar *P. sarmentosum* L. membawa kepada pengasingan empat fenilpropanoidal iaitu asaricin **1**, isoasarone **2**, *trans*-asarone **3** dan Arsin **4**. Sebatian **1** dan **2** menunjukkan aktiviti yang kuat terhadap tiga perosak serangga penyimpanan; *S. oryzae* dan *R. dominica* dengan nilai  $LC_{50} \leq 10.6 \mu\text{g/ml}$  dan  $LC_{95} \leq 10.6 \mu\text{g/ml}$ . *P. interpunctella* adalah lebih rintang dengan nilai  $LC_{50} \leq 17.37 \mu\text{g/ml}$  dan  $LC_{95} \leq 37.7 \mu\text{g/ml}$ . Kedua-dua sebatian ini menunjukkan aktiviti penghalau yang tinggi terhadap *S. oryzae*, *R. dominica* dan *P. interpunctella* pada  $10 \mu\text{g/ml}$ . Begitu juga dengan sebatian **1** dan **2** mempunyai aktiviti larvasidal yang kuat terhadap *Aedes albopictus*, *Aedes aegypti* dan *Culex quinquefasciatus*. **1** dan **2** menunjukkan aktiviti yang sama kuat terhadap larva *Aedes aegypti*, *Aedes albopictus* dan *Culex quinquefasciatus* dengan  $LC_{50} \leq 8.9 \mu\text{g/ml}$  dan  $LC_{95} \leq 15.1 \mu\text{g/ml}$ . Dalam kedua-dua kajian aktiviti serangga sebatian **3** terhadap perosak di atas adalah sederhana manakala sebatian **4** adalah tidak toksik. Walau bagaimanapun, sebatian ini menunjukkan aktiviti penghalau dan *adulticidal* yang rendah apabila diuji terhadap nyamuk vektor di atas. Kajian biokimia pada acetilkolinesterase (AChE) menunjukkan bahawa **1** dan **2** mempunyai aktiviti perencatan yang tinggi manakala **3** mempunyai aktiviti yang sederhana. Analisis statistik mengesahkan hubungan bermakna antara perencatan AChE dan ketoksikan **1**, **2** dan **3** yang mana ketoksikan mereka menyebabkan gangguan sistem saraf ke atas serangga yang di uji. Sebaliknya, aktiviti enzim glutathion S-transferases (GST), yang merupakan mekanisme pertahanan utama serangga terhadap ketoksikan adalah diukur. Aktiviti GST adalah jauh lebih tinggi dalam serangga dengan tahap

rintangan yang lebih tinggi. Oksidases dan esterases yang tidak spesifik yang turut terlibat dalam ketahanan serangga juga dikaji tetapi aktiviti-aktiviti mereka adalah tidak ketara. Kajian *docking* telah diteruskan untuk menyiasat interaksi mod ikatan pada **1**, **2** dan **3** terhadap AChE dan enzim GST menggunakan Autodock Vina. Sebatian **1**, **2** dan **3** mempunyai ikatan yang kuat dan stabil pada tapak aktif AChE dan jumlah tenaga interaksi mereka adalah rendah terutama di tapak ikatan anionik (W83). Kajian *docking* terhadap interaksi GST antara **1**, **2** dan **3** mencadangkan bahawa PRO11, GLU64 dan TYR105 adalah sisa penting dalam GST untuk ikatan selain sisa SER65 dan ARG66. Biokimia dan *docking* jelas menunjukkan peranan aktiviti GST dalam ikatan dan detoksifikasi sebatian **1**, **2** dan **3**. Pemahaman lanjut mengenai peranan dan interaksi antara AChE dan enzim GST dan **1**, **2** dan **3** akan membantu untuk meningkatkan kawalan rintangan di dalam vektor. Hakikat bahawa *P. sarmentosum* yang telah digunakan di Asia Tenggara sebagai sumber makanan akan menyokong peranan mesra alam sekitar. Keputusan ujian ketoksikan yang kuat mencadangkan bahawa walaupun GST boleh terikat pada **1** dan **2** tetapi dengan perencatan yang kuat oleh AChE mempunyai potensi yang tinggi untuk mengawal perosak penyimpanan dan nyamuk vektor. Hasil kajian mencadangkan bahawa sebatian **1** dan **2** dengan aktiviti serangga yang kuat mempunyai potensi untuk dibangunkan sebagai insektisid biologi untuk mengawal serangga perosak.

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## LIST OF SYMBOLS AND ABBREVIATIONS

Symbols	Terms
$\alpha$	Alpha
$\beta$	beta
$\lambda_{\max}$	maximum wave length
$\delta$	chemical shift in ppm
$\mu\text{M}$	micromolar
$\mu\text{l}$	microlitre
$\mu\text{g/mL}$	microgram per millilitre
mM	milimolar
mg/ml	milligram per millilitre
g	gram
kg	kilogram
ml	millilitre
m	meter
MHz	mega Hertz
Hz	Hertz
UV	ultraviolet
$\epsilon$	UV extinction coefficient
IR	Infrared
mM	milimolar
ppm	part per million
eV	electron Volt
MeOH	methanol
$\text{CHCl}_3$	chloroform

CH <sub>2</sub> Cl <sub>2</sub>	dichloromethane
CH <sub>3</sub>	methyl group
OCH <sub>3</sub>	methoxyl group
OH	hydroxyl group
KCl	potassium chloride
NaCl	sodium chloride
pH	power of hydrogen
HCl	hydrogen chloride
TLC	thin layer chromatography
PTLC	preparative thin layer chromatography
CC	column chromatography
NMR	nuclear magnetic resonance
FT-NMR	fourier transform nuclear magnetic resonance
cm <sup>-1</sup>	per centimeter
<i>J</i>	coupling constant
<i>d</i>	doublet
<i>s</i>	singlet
<i>dd</i>	doublet of doublets
<i>t</i>	triplet
<i>m</i>	multiplet
1D-NMR	one dimension nuclear magnetic resonance
2D-NMR	two dimension nuclear magnetic resonance
<sup>1</sup> H-NMR	proton nuclear magnetic resonance
<sup>13</sup> C-NMR	carbon 13 nuclear magnetic resonance
COSY	2D homonuclear chemical shift correlation spectroscopy
DEPT	distortionless enhancement by polarization transfer



HMQC	heteronuclear multiple quantum coherence
HMBC	heteronuclear multiple bond coherence
NOE	nuclear overhauser enhancement
GC-MS	gas chromatography-mass spectroscopy
MS	mass spectroscopy
HRMS	high resolution mass spectroscopy
EIMS	electron impact mass spectroscopy
FAB	fast atomic bombardment
ESI	electrospray ionization
m/z	mass to charge ratio
CDCl <sub>3</sub>	deuterated chloroform
MeOD	deuterated methanol
[α] <sub>D</sub>	optical rotation
HPLC	high performance liquid chromatography
RPHPLC	reverse phase high performance liquid chromatography
RPMI	Roswell Park Memorial Institute
CO <sub>2</sub>	carbon dioxide gas
PBS	phosphate buffer saline
EDTA	ethylenediaminetetraacetic acid
FBS	fetal bovine serum
rpm	rotate per minute
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
DMSO	dimethyl sulfoxide
FITC	fluorescein isothiocyanate
ai	active ingredient
°C	degree Celsius (centigrade)

g	gram
kg	kilogram
Cm	Centimeter
ml	milliliter
mg	milligram
L	liter
T	temperature
OD	optical density
R.H.	relative humidity
LC <sub>50</sub>	median lethal concentration
LC <sub>95</sub>	95% lethal concentration
LD <sub>50</sub>	median lethal dose concentration
LD <sub>95</sub>	95% lethal dose concentration
IPM	integrate pest management
ppm	part per million
m	meter
<	Less than
≤	Less than or equal to
>	Greater than
≥	Greater than or equal to

## CHAPTER 1: INTRODUCTION

### 1.1 Background

Insect pests are potential threat to human concerns such as agriculture, livestock production and health. The storage pests such as *Sitophilus oryzae* (El-Maghraby, Nawwar, Bakr, Helmy, & Kamel, 2012), *Rhyzopertha dominica* (Kotze et al., 2014) and *Plodia interpunctella* (Angajala, Ramya, & Subashini, 2014; Kolli, Balakrishnan, Vijayan, & Sundararajan, 2013) reduce the quality and quantity of harvested grains and relative food products. Insects are capable of causing damage to stored grains and grain products in 5–10% in the temperate regions of the world and as high as 20–30% in tropical countries (Nattudurai, Paulraj, & Ignacimuthu, 2012). Mosquito vectors such as *Aedes albopictus*, *Aedes aegypti* and *Culex quinquefasciatus*, which carry and transmit an infectious pathogen, are among the most serious insect pests of medical importance. Mosquitoes are vectors of various disease agents, some of which cause illnesses and deaths in humans and animals every year. Among these diseases are dengue, yellow fever and west nile virus which are endemic and epidemic in many countries (Arroyo, Miller, Catalan, & Monath, 2001).

At present, global challenges are created by synthetic insecticides in controlling the insect pests due to resistance, mammalian toxicity, effect on non-target insects such as honeybees and general environmental hazards (Walker, Golden, & Horst, 2010). The mechanism of toxicity of most insecticides such as organophosphorus and carbamate compounds is based on the inhibition of acetylcholinesterase (Dembélé, Haubruge, & Gaspar, 1999). In insects, acetylcholinesterase (AChE) hydrolyzes the neurotransmitter acetylcholine (ACh) to stop neuronal excitement at the postsynaptic membrane (Kelly, Mutch, Williams, & Blain, 1994; Sánchez, Vera, Montagna, & Magnarelli; Santos et al.,

2014). Such resistance in mosquitoes is usually caused by less inhibition of the target enzyme in the resistant strains or increase in detoxification mechanism (Sokhna, Ndiath, & Rogier, 2013). On the other hand, insects can metabolize and degrade toxic chemicals to survive in hazardous chemical environment. Glutathione S-transferase (GST) is one of the most efficient enzymes in detoxification systems in all animals (Kotze et al., 2014). GST comprise a family of eukaryotic and prokaryotic enzyme best known for their ability to catalyze the conjugation of insecticides (Thom et al., 2001). After observing resistance in insect pests, GST is becoming recognized for its importance in metabolizing the toxic chemical in insects (Almli et al., 2002). Better understanding of the pesticide action on enzymes in insect pests would enable to produce selective and effective insecticides with low mammalian toxicity (Müller, Rocha, Morsch, Neis, & Schetinger, 2002; Forget, Livet, & Leboulenger, 2002; Saeaeue, Morales, Komalamisra, & Vargas, 2011). The use of synthetic insecticides including fumigants to prevent or control insect infestation is being practiced for years. However, resistance to insecticides has appeared in major insect vectors. As of 1992 (Oliver & Brooke, 2014), the list of vector species which showed resistant has increased dramatically (Kaliyaperumal, Askual, & Samuel Fekadu, 2014). These problems led to either the ban or restriction for use of several synthetic insecticides, against insect pests (Kalimuthu et al., 2013). Therefore plant secondary metabolites may provide potential alternatives to currently used synthetic insecticides that are gradually becoming ineffective in controlling insect pests. One of the highly successful and presently used synthetic insecticides is pyrethroids which originally derived from the pyrethrum plant (Beugnet & Chardonnet, 1995). At present, botanical insecticides that can replace pyrethrum in stored-grain pest management or to control mosquitoes are not available (Weaver & Subramanyam, 2000). Several countries including Malaysia do not permit the use of any synthetic insecticide to control stored-grain pests (Abrol, 2014). Besides, the use of

organochlorines against mosquito vectors has also been banned. In addition, mosquitoes show high resistance to other groups of insecticide including organophosphate family (Valkiūnas, Kazlauskienė, Bernotienė, Palinauskas, & Iezhova, 2013). Therefore, there is a need for safer alternatives to conventional synthetic insecticides particularly from natural sources for the protection against insect pests and mosquitoes. Since bioinsecticides of plant origin often exhibit selective toxicity to insects and are more eco-friendly, development of new class of botanical pesticide for insect control is vital (El-Wakeil, 2013). Wide diversity of plant species, especially from the tropical regions of the world could be potential sources of newer bioactive compounds or biopesticides (Rattan, 2010). Malaysia has a wealth of plant species with wide range of bioactive compounds such as alkaloids, phenylpropanoids, terpenes and so on (Gonzalez-Coloma, Reina, Diaz, Fraga, & Santana-Meridas, 2013).

*Piper sarmentosum* is a wild plant, which grows in tropical countries including Malaysia. The aerial part and roots of *P. sarmentosum* are used in traditional medicine as preparation for ancient Malaysian medicine (Dyer, Richards, & Dodson, 2004) and consumed as health drink. Earlier works have shown that roots of *P. sarmentosum* are the sources of novel bioactive compounds and more than a dozen compounds have been isolated and identified (Stohr, Xiao, & Bauer, 1999, Amran et al., 2010). Preliminary studies showed that the aerial part and root extracts of *P. sarmentosum* have insecticidal properties (Damsud, Adisakwattana, & Phuwapraisirisan, 2013). Therefore in this study, a bioassay guided phytochemical investigation was undertaken to discover of new alternatives for synthetic insecticides by study the potent compounds with insecticidal activity in *P. sarmentosum*.

## 1.2 Research Objectives

Based on the facts mentioned above, research objectives of this study are as follows:

1. To extract the aerial part and roots of *Piper sarmentosum* with several solvents with different polarity and evaluate their level of insecticidal activity.
2. To conduct the bioassay guided fractionation and isolation on the most active extracts.
3. To examine the insecticidal property of the isolated compounds against three storage pests (*Sitophilus oryzae*, *Rhyzopertha dominica* and *Plodia interpunctella*) and three mosquito vectors (*Aedes aegypti*, *Aedes albopictus* and *Culex quinquefasciatus*).
4. To determine the mechanism of active compounds through their interaction with metabolic enzymes on tested insects.
5. To use computational docking program for more understanding of interaction of active compounds with active site of acetylcholine and glutathione S-transferase involve with insecticidal activity of insects.

## CHAPTER 2: LITERATURE REVIEW

### 2.1 Chemical Control

According to the reports of Food and Agriculture Organization (FAO), humans have been challenged to control insect and other arthropod pests, plant pathogens, weeds, rodents and other vertebrate pests for thousands of years (Quesada-Moraga, García-Tóvar, Valverde-García, & Santiago-Álvarez, 2004). With the developed society and advancement in science, men have invented some cutting-edge and modern weapons against pests such as chemical pesticides, biological pesticides, remote sensing and measurement, computers and atomic energy. However, it has been only within the last 50 years that dramatic progress has been made in attempt to controlling pests of humans and food (Meyer, & Di Giulio, 2003; Abbott, 1987; Bernier, Furman, Kline, Allan, & Barnard, 2005). Agriculture and relative industry have been revolutionized by the use of chemicals for crop protection, which started in early eighteenth century with the introduction of insecticides based with arsenic and mixture a combination of copper sulfate, lime, and water as fungicide known as bordeaux. At present, it is progressing to the use of new synthetic and sophisticated compounds (Zoubiri & Baaliouamer, 2014). Farmers produce more food with lesser production cost than ever before. The synthetic chemicals have many positive effects on agriculture that conventional agriculture has now started using chemicals. However, synthetic pesticides have it own drawback. These chemicals are produced and used to kill pests and their effects are not limited to target organisms only. Early successes with the first pesticides, for instance dichloro diphenyl trichloroethane (DDT), came swiftly. Yet the race between human and pests was still ongoing. Early results were significant, after the continued victories over blood-feeding arthropods such as mosquitoes and other biting flies and agricultural pests such as scale insects, a decrease in the effectiveness of these new chemical

weapons was catching the attention (Yee, 2008). In fact, their properties as residual toxicant with high toxicity over a wide range of organisms, have led to serious environmental problems. Furthermore, the emergence and spread of increasing resistance in number of insect pests, higher cost of new synthetic pesticides and environmental hazard made it very clear that controlling the vectors and pest could no longer be safe due to the use of individual synthetic pesticides (Kaliyaperumal, Askual, & Samuel Fekadu, 2014; Kannathasan, Senthilkumar, Chandrasekaran, & Venkatesalu, 2007). In recent years high volume of application led the farmers to increase the active ingredients to regain the initial level of pest control. However, levels of pest and vector control decline even with faster paced every year. The resistance in insects pests to current synthetic insecticides make it increasingly more difficult to control their population and it will eventually lead to control failure (Murthy, Georgiev, Park, Dandin, & Paek, 2015; Nair & Burke, 1990; Nattudurai, Paulraj, & Ignacimuthu, 2012).

## **2.2 Pesticide Resistance and Relevant Problems**

The ability to survive in contaminated environments of a population is highly reliant on the ability to adapt to stressors through the process of natural selection. At present, abundant reports of organisms adapting to anthropogenic stressors and chemical exist. Adaptations may be in the form of physiological acclimation (tolerance), changing normal habits or they may be genetically based resistance (Bernier, Furman, Kline, Allan, & Barnard, 2005).

Resistance occurs when pest population, for example insects, is exposed to a several pesticide which mostly has similar active ingredient with relative mechanism of action on insects such as neurotoxicity. Over the period of time when this happens certain individuals that survived are genetically predisposed and will show resistant to the



pesticide. Adaptation occurs in insects as a result of previous contacts can arise from mechanisms or behaviours that decrease the amount of toxicant reaching the target site or decreased responsiveness to the toxicant at the target (Kleinjans, 2003). Consistence applications with higher volume of insecticide will kill larger numbers of susceptible individuals, but those with higher tolerance will survive (Lee, Kim, Kwon, Cha, & Kim, 2014). The results of these survivors will carry the genetic makeup of their parents. These progeny of resistance pests will inherit the ability to survive the exposure to the pesticide will incline rapidly with each survived resistance group of the pests (Ma, He, & Zhu, 2004). Most of pests have high rate of reproduction which can take place within few weeks which may result in production of many generations in a single season or year (Hemingway, 2000). Development of resistance against synthetic chemicals was first observed when *Scirtothrips citri* and *Leptinotarsa decemlineata* were detected to be less sensitive to DDT in 1949 (Rufingier, Pasteur, Lagnel, Martin, & Navajas, 1999). Since use of synthetic pesticides raised, a parallel increase in insecticide resistance and cross-resistance occurred (Burton et al., 2011). On the other hand, cross-resistance typically develops in pesticides that have similar mechanisms of action, such as DDT and pyrethroids, which act on sodium channels in axonal membranes (Sokhna, Ndiath, & Rogier, 2013; Smith et al., 2011). It has been observed that insects pest population which exposed frequently to a pesticide, especially in a wide spectrum has faster resistance develops. Resistance develops in a similar manner in other pests, such as fungus, plant virus and bacteria, weeds and rodents. In most cases resistant population generated after misused of a pesticide with very good controlling result (Cùany et al., 1990; Mogi & Tuno, 2014).

At present those pests that were believed to be under control by using synthetic pesticides are on the rebound. For example, mosquitoes that are able to transmitting

malaria are now resistant to virtually all pesticides used against them (Hemingway & Georghiou, 1983). Populations of the *Helicoverpa zea* which attacks many agricultural crops worldwide such as corn, cotton, tomatoes, tobacco, and peanuts are resistant to multiple pesticides (Yang et al., 2015). In order to overcome this problem, the farmers were forced to use mycotoxins (fumonisins from *Fusarium*, aflatoxins and *A. flavus*) which can cause diseases and are extremely toxic to animals and human, and major problems for corn production (Schjøth, Visconti, & Sundheim, 2009). Recent researches reveal that there are more than 520 species of insects and mites resistant to pesticides (Burton et al., 2011; Kelly, Mutch, Williams, & Blain, 1994). Multiple resistance is increasing rapidly and there are more than 1000 reports on insect resistance (Koekemoer et al., 2010; M. Yang et al., 2008). However, synthetic pesticides are the major part of a pest management program. Pesticide resistance makes the current pesticides weaker and less potent to bring pest numbers below damaging levels for relative human health and agriculture programs (Kleijnans, 2003).

### **2.3 Biochemical Control and Their Importance**

Biopesticide are naturally occurring, genetically modified or natural drives agents which are distinguished from synthetic pesticides by their specific modes of action, low use volume, and specific target (Nawrot, Harmatha, Kostova, & Ognyanov, 1989; Massoud, Labib, & Rady, 2001). A chemical must have the following criteria in order to classify as biochemical to be subject this class of compounds: (1) The chemical mode of action should be as growth regulation, mating disruption, attraction rather than direct toxicity on target pest; (2) Biochemical must be produce naturally (El-Maghraby, Nawwar, Bakr, Helmy, & Kamel, 2012). Pyrethroid is synthesized by human but structurally identical to a naturally occurring chemical (Aïzoun et al., 2013; Tas,kin & Kence, 2004). For a synthetic chemical to be categorized as biochemical, the molecular

structure of the major component of the synthetic chemical must be similar as the molecular structure of the naturally occurring substance (Gunning & Moores, 2001). If there be minor difference between the isomer ratios, which can be found in the naturally occurring compound compared to the synthetic compound, normally won't rule out a chemical and they classified as biochemical pest control agent unless one isomer has significant different toxicity properties than another isomer (Kwon, Clark, & Lee, 2014). Some compounds possess many characteristics of a biological pest control agent, but still does not match the two important established for agents defining as biochemical pest control (Tewari et al., 2014). The Agriculture Organization (FAO) has regulatory authority to evaluate such chemicals on several basis to recognized whether it should be classified as a biochemical pest control agent or a conventional pesticide (Gunning & Moores, 2001). The evaluation would first require exact molecular structure of the naturally occurring secondly the mode of action and finally comparing the effects specific target to non-target organisms (Oliver & Brooke, 2014; Munhenga, Masendu, Brooke, Hunt, & Koekemoer, 2008).

Biochemical pest control agents fall into five general biologically functional classes namely, (1) semiochemicals, (2) hormones, (3) natural plant regulators and insect growth regulators, (4) enzymes and (5) microbial pest control agents (Munhenga, Masendu, Brooke, Hunt, & Koekemoer, 2008). In the mid-seventies, WHO and other relative agriculture and health organizations introduced studies into current biological control agents and identification of new ones (El-Maghraby, Nawwar, Bakr, Helmy, & Kamel, 2012). Biological control is vastly known as a suitable technique for controlling the pests due to its least environmental impact and its prevention of consequences of resistance of the pest.

## 2.4 Botanical Pesticide

Botanical pesticides are known to be secondary metabolites that produce in the plants as defense mechanism against herbivore predators and other outside hazardous (Gonzalez-Coloma et al., 2013). Most of the plant secondary metabolites belongs to alkanes, alkenes, alkynes and simple aromatics, lactones, essential oils and fatty acids, terpenes, alkaloids, steroids, isoflavonoids, pterocarpans and lignans (Naranjo & Henneberry, 2002; El-Wakeil, 2013; Moshi & Matoju, 2017). The use of plant material or crude plant extracts and essential oil as botanical insecticides for the protection of agriculture plants and related stored products from insect pests is as old as agriculture itself (El-Wakeil, 2013; Pang et al., 2016). Plants produce a large variety of secondary metabolites which has wide range of activity including pesticide activity (Samuel & Senthilkumar, 2014; Balasubramanian & Tyagi 2017). Before development of synthetic insecticides in the 1940s and their success, botanical insecticides were major weapons used by farmers against crop pests (Weaver & Subramanyam, 2000). Phytochemicals and mostly extracted either from the whole body of little herbs or from different parts like fruits, leaves, stems, barks, roots and flowers of plants. In all cases, the most toxic extracts were concentrated upon and tested against the insect pests (Rattan, 2010; El-Abbassi et al., 2017). More than 2000 plant species have been known for their potential in pest control programs (Sharma, Mohan, & Srivastava, 2006). Several plant families and species insecticidal activity and their chemical constitution were listed in Table 2.1.

**Table 2.1** Several important plants family and species which has been studied for insecticide activity against insects pests.

<b>Family and species</b>	<b>Part used<sup>a</sup></b>	<b>Chemical constitution</b>	<b>References</b>
<b>Annonaceae</b>			
<i>Annona muricata</i> (soursop)	Cold extraction from bark	Gigantetrocin A, Annonomontacin, Bullatalicin, Squamocin, Annonacin	(Leatemala & Isman, 2004) (Florence et al., 2014)
<i>Annona squamosa</i> (sweetsop)	Cold extraction from leaf	Annotemoyin-1, Neoannonin	(Leatemala & Isman, 2004) (Senthilkumar, Gurusubramanian, & Murugan, 2014)
<i>Annona cherimola</i> (chirimuya)	Cold extraction from bark	Itrabin, Asimicin, Almuñequin	(Álvarez Colom, Neske, Popich, & Bardón, 2007) (Gandhi & Gopalkrishna, 1957)
<i>Annona montana</i> (mountain soursop)	Cold extraction from bark	Annonacin, Cis-annonacin-10-One Densicomacin-1, Annonacin-a	(Leatemala & Isman, 2004) (Di Toto Blessing, Álvarez Colom, Popich, Neske, & Bardón, 2010)
<b>Apiaceae</b>			
<i>Pastinaca sativa</i> (parsnip)	Cold extraction from flower	Furanocoumarin, Xanthotoxin 8-methoxy psoralen,	(Berenbaum, 1981) (Zangerl & Berenbaum, 2004)
<i>Pimpinella isaurica</i> (lesser burnet)	Essential oil from leaf	4-2-propenyl-phenyl angelate (PPA), 4-(1-propenyl)-phenyl tiglate (PPT), 4-methoxy-2-(1-propenyl)-phenylangelate (MPPA)	(Zhou, Xie, & Yan, 2011a) (Stroh et al., 1998)
<b>Asteraceae</b>			
<i>Artemisia scoparia</i> (virgate wormwood)	Essential oils from leaf		(Kim, 1997) (Singh, Kaur, Mittal, Batish, & Kohli, 2009)
<i>Roldana barba-johannis</i> (asteraceae)	Cold extracts aerial parts		(Villaseñor, Delgadillo, & Ortiz, 2006)
<i>Vernonia baldwinii</i> (ironweed)	Cold extraction from flower	Sesquiterpene lactone	(Zhou, Xie, & Yan, 2011b)
<i>Artemisia scoparia</i> (capillary wormwood)	Essential oils from leaf		(Seaman, 1982) (Keeley & Jansen, 1994)
<b>Berberidaceae</b>			
<i>Pelargonium peltatum</i> (ivy-leaf)	Cold extraction from flower	Podophyllotoxin	(Harris, 1995) (Bohlmann & Jakupovic, 1990)

**Table 2.1, continued'**

<b>Cactaceae</b>			
<i>Myrtillocactus Geometrizans</i> (bilberry)	roots, aerial parts		(Fischer, Olivier, & Fischer, 1979) (Bohm & Stuessy, 2001)
<b>Caesalpiniaceae</b>			
<i>Detarium microcarpum</i> (sweet detar)	Essential oils from leaf		(Woods, 1992) (Boege & Dirzo, 2004)
<i>Buddleja madagascariensis</i> (smokebush)	Cold extraction from bark	Quercetin	(Jacques & Miège, 1978) (Woods, 1992) (Boege & Dirzo, 2004)
<b>Chrysanthemum</b>			
<i>Chrysanthemum cinerariaefolium</i> (chrysanth)	Cold extraction from flower	Cinerin I, Cinerin II, Jasmolin I, Jasmolin II, Pyrethrin I and Pyrethrin II,	(Hitmi, Barthomeuf, & Sallanon, 1999) (Brewer, 1974)
<b>Ericaceae</b>			
<i>Gaultheria procumbens</i> (checkerberry)	Cold extraction from leaves	Phenolic (benzoate ester) Methyl salicylate	(Ezcurra, Gómez, & Becerra, 1987) (Stout, 2007)
<b>Fabaceae</b>			
<i>Cassia nigricans</i> Vahl (greene)	Essential oils		(da Costa, de Siqueira Neves, de Oliveira Silva, & Fagundes, 2011) (Fagundes, 2014)
<i>Glycyrrhiza glabra</i> (sweet root)	Cold extraction from bark		(Pereira, Moura, & Da-Silva, 2014) (Gorb, Voigt, Eigenbrode, & Gorb, 2008)
<i>Derris elliptica</i> (tuba)	Essential oils from leaf	Rotenone	(Zhou, Xie, & Yan, 2011b)(Toxopeus, 1952)
<b>Meliaceae</b>			
<i>Acalypha indica</i> (indian nettle)		Azadirachtin	(Kubo & Klocke, 1982) (Isman et al., 1996)
<i>Aglaia cordata</i> (medang bebulu)	Cold extraction from bark	Rocaglamide	(Talukder & Howse, 1993) (Rembold & Puhlmann, 1993)
<i>Agalia oilo</i> (kiah)	Cold extraction from leaves		(Akhtar, Yeoung, & Isman, 2008) (Mikolajczak & Reed, 1987)

**Table 2.1, continued'**

<i>Agalia odorata</i> (chinese Rice Flower)	leaves extracts		(Serit et al., 1992) (Champagne, Isman, Downum, & Towers, 1993)
<i>Agalia maiiae</i> (karnataka)	Essential oils from leaf		(Floyd et al., 2003) (Perez et al., 2010)
<i>Agalia iloilo</i> (tagalog)	Cold extraction from bark		(Kubo & Klocke, 1982) (Akhtar, Yeoung, & Isman, 2008)
<i>Agalia ohgophy</i> (priyangu)	Cold extraction from fruit		(Talukder & Howse, 1993) (Mikolajczak & Reed, 1987)
<i>Azadirachta excels</i> (Jacobs )	Cold extraction from bark	Azadirachtins O-Q (1-3), azadirachtin B (4), azadirachtin L-5, azadirachtin M -6-11alpha-azadirachtin H -7, 11β-azadirachtin H -8 and azadirachtol-9	(Champagne, Isman, Downum, & Towers, 1993)(Floyd et al., 2003)
<i>Azadirachta indica</i> (neem)	Essential oils from leaf Seeds		(Isman et al., 1996) (Akhtar, Yeoung, & Isman, 2008)
<i>Carapa guianensis</i> (andiroba)	Essential oils from leaf		(Serit et al., 1992) (Perez et al., 2010)
<i>Cabrlea canjerana</i> (canharana)	Cold extraction from leaves	Methyl angolensate, 3-β -deacetylfissinolide, 7-deacetoxy-7-oxogedunin, and beta-photogedunin	(Ebada, Lajkiewicz, Porco, Li-Weber, & Proksch, 2011) (Hall, 2011)
<i>Cedrela odorata</i> (chinaberry )	Cold extraction from fruit		(Rembold & Puhlmann, 1993)(Akhtar, Yeoung, & Isman, 2008)
<i>Cedrela salvadorensis</i> (salvadorinetrela)	Cold extraction from leaves		(Rembold & Puhlmann, 1993)(Akhtar, Yeoung, & Isman, 2008)
<i>Cipadessa fruticosa</i> (red bell bush)	Essential oils from leaves		(Rembold & Puhlmann, 1993)(Akhtar, Yeoung, & Isman, 2008)
<i>Cedrela dugesii</i> ( spanish cedar)	Cold extracts from bark	NA	(Hall, 2011)
<i>Dysoxylum beddomei</i> (mahogany )	Cold extraction from bark	triterpenes 3beta,24,25-trihydroxycycloartane and beddomeilactone	(Ebada, Lajkiewicz, Porco, Li-Weber, & Proksch, 2011)
<i>Dysoxylum Malabaricum</i> (white cedar )	Cold extraction from leaves		(Hall, 2011) (Willers et al., 2014)

**Table 2.1, continued'**

<i>Entandrophragma candollei</i> (harms)	Cold extracts from leaves		(Perez et al., 2010) (McKenna, Hammad, & Farran, 2013)
<i>Lansium domesticum</i> (lanzones)	Cold extracts from seeds		(Prophiro et al., 2012) (Obara, Höft, & Höft, 2004)
<i>Melia azedarach</i> (chinaberry)	Cold extracts from leaves, seeds		Lemes, Grattapaglia, Grogan, Proctor, & Gribel, 2007)
<i>Melia dubia</i> (kannada)	Cold extraction from leaves		(Ebada, Lajkiewicz, Porco, Li-Weber, & Proksch, 2011) (Hall, 2011)
<i>Melia volkensii</i> (mature)	Cold extraction from fruit		(Serit et al., 1992) (Perez et al., 2010)
<i>Sandoricum koetjape</i> (red santol)	Cold extraction from bark		(Rembold & Puhlmann, 1993) (Akhtar, Yeoung, & Isman, 2008)
<i>Teucrium tomentosum</i> (germanders)	Cold extraction from leaves		(Perez et al., 2010) (McKenna, Hammad, & Farran, 2013)
<i>Yucca periculosa</i> (izote)	Cold extraction from bark	3,3 0 ,5,5 0 - tetrahydroxy-4-methoxystilbene	(Ebada, Lajkiewicz, Porco, Li-Weber, & Proksch, 2011) (Hall, 2011)
<b>Lamiaceae</b>			
<i>Ajuga remota</i> (bungle)	Cold extraction from bark	Ajugarin I	(Olesen, Forfang, & Báez, 1998) (Ford & Johnson, 2008)
<i>Ocimum basilicum</i> (basil)	Essential oils		(Zhang, Robert, Wang, & Guo, 2007) (Vos, Edwards, & van Staden, 1994)
<i>Ocimum gratissimum</i> (african basil)	Essential oils from leaves		(Nilsson, Jonsson, Rason, & Randrianjohany, 1985)
<i>Scutellaria galericulata</i> (hooded skullcap)	Cold and hot extract from bark		(Olesen, Forfang, & Báez, 1998) (Ford & Johnson, 2008)
<i>Teucrium fruticans</i> (tree germander)	Cold extraction from seeds	NA	(Mauchline et al., 2008)
<i>Teucrium glabra</i> (germander)	Cold extraction from bark	NA	(Nilsson, Jonsson, Rason, & Randrianjohany, 1985) (Zhang, Robert, Wang, & Guo, 2007)
<i>Teucrium hirta</i> (koko kahiki)	Cold extraction from fruit	NA	(Potgieter, Edwards, Miller, & Van Staden, 1999) (Conti, Canale, Cioni, Flamini, & Rifici, 2011)



**Table 2.1, continued'**

<i>Thymus vulgaris</i> (thyme)	Cold extraction from bark	Thymol Monoterpene	(Conti, Canale, Cioni, Flamini, & Rifici, 2011) (Schiller, Zedler, & Black, 2000)
<b>Liliaceae</b>			
<i>Allium porrum</i> (wild onion)	Cold extracts from roots	Aginosid	(Ellouze et al., 2012)
<b>Loganiaceae</b>			
<i>Strychnos nux-vomica</i> (Telugu)		Strychnine	(Ellouze et al., 2012)
<b>Pinaceae</b>			
<i>Pinus species</i> (hoop pine)	Cold extraction from bark	Abietic acid	(Lombardero, Alonso-Rodríguez, & Roca-Posada, 2012)
<b>Piperaceae</b>			
<i>Piper guianacastensis</i> (forest peppe)	Cold extraction from bark	Methyl 4-hydroxy-3-(3-methyl-2-butenyl) benzoate	(Scott, Jensen, Philogène, & Arnason, 2008) (Kitayama et al., 2013)
<i>Piper longum</i> (indian pepper)	Cold extracts from roots	asarinine	(Ramos, Vanin, & Kato, 2009) (Maduell, Callejas, Cabrera, Armengol, & Orduz, 2002)
<i>Piper retrofractum</i> (balinese long pepper)	Cold extraction from fruit, seed and stems	Pipericide, pellitorine, guineensine, retrofractaminde	(Scott, Jensen, Philogène, & Arnason, 2008) (Kitayama et al., 2013)
<i>Piper nigrum</i> (black peper)	Cold extraction from fruit	Piperin	(Bernard et al., 1995) (Ramos, Souza, Kato, & Batista, 2012)
<i>Piper longum</i> (long pepper)	Cold extraction from fruit	Piperin	(Bernard et al., 1995) (Ramos, Souza, Kato, & Batista, 2012)
<i>Piper retrofractum</i> (pepper)	Cold extraction from bark	Pipericide, pellitorine, guineensine and retrofractaminde	(Ramos, Souza, Kato, & Batista, 2012) (Ramos, Vanin, & Kato, 2009)
<i>Piper sarmentosum</i> (kadok)	Cold extraction from fruit	NA	(Zainal Ariffin et al., 2009) (Ugusman, Zakaria, Hui, & Megat Mohd Nordin, 2011)
<b>Polygonaceae</b>			
<i>Persicaria hydropiper</i> (laksa Plant)	Cold extracts from roots	Sesquiterpene and Polygodial	(Prota, Mumm, Bouwmeester, & Jongsma, 2014)
<b>Rutaceae</b>			
<i>Citrus limon</i> (lemon)	Essential oil from leaves and seeds	Limonene	(Cordeiro, Sugahara, Stein, & Leite Junior, 2014)
<i>Evodia rutaecarpa</i> (evodia )	essential oil	NA	(Liu & Ho, 1999)
<b>Salicaceae</b>			

**Table 2.1, continued'**

<i>Ryania speciosa</i> (hogla)	Cold extraction from stems	Ryania	(Lümmen, 2013)(Akhtar, Yeoung, & Isman, 2008)
<b>Scrophulariaceae</b>			
<i>Digitalis purpurea</i> (foxglove )	Cold extracts from roots	Cardenolide, Digitoxin	(Verboven, Brys, & Hermy, 2012) (Roca-Pérez, Boluda, Gavidia, & Pérez- Bermúdez, 2004)
<b>Solanaceae</b>			
<i>Lycopersicon esculentum</i> (tomato)	Cold extraction from seeds	Tomatine	(Sohrab, Bhattacharya, Rana, Kamal, & Pande) (Degenhardt, Refi-Hind, Stratmann, & Lincoln, 2010)
<i>Nicotiana tabacum</i> (tobacco)	Cold extracts from leaves	Nicotine	(Delporte, Van Holle, & Van Damme, 2013) (Shang et al., 2015)
<i>Withania somnifera</i> (winter Cherry)	Cold extraction from roots	Withanolide E	(Gaur & Kumar, 2010)
<b>Simaroubaceae</b>			
<i>Quassia amara</i> (bitter wood)	Cold extracts from roots	quassin	(Martin & Madassery, 2005) (Roubik, Holbrook, & Parra, 1985)
<b>Verbenaceae</b>			
<i>Lantana camara</i> (wild-sage)	Cold extraction from aerial part	NA	(Roubik, Holbrook, & Parra, 1985) (Rajashekar, Vijay Kumar, Ravindra, & Bakthavatsalam, 2013)

Despite the beneficial characteristics of the botanical extracts, vast availability of plants species and large amount of research which have been conducted to prove their biological efficacy, there are only a small number of products that have been commercialized lately (Gonzalez-Coloma et al., 2013). As can be seen in Table 2.1 there many plants with identify active compounds but there are some limitations inherent to botanical pesticide success. The major problem is many researches have been stopped after identifying the insecticidal activity of extracts or essential oil. Rattan (2010) has reported that research rarely goes to the point of identifying the main active components of the plant extracts. Comprehension of the main active component could solve other problems such as

storage, stability, quality and quantitative reproducibility of secondary metabolites (Ramos, Souza, Kato, & Batista, 2012).

#### **2.4.1 Identification and characterisation of plant compounds**

Identification of the plants natural compounds with their biological activity and determination of their yield is one of the most important steps of discovering of botanical pesticides (Satariah, 1999). The procedure of identification of active compounds is involved with extraction, fractionation, isolation and finally identification of the compounds (Suvitha, Periandy, & Gayathri, 2015). There are many methods for the extraction and isolation of plant bioactive compounds. Using of solvents with different polarity is the most common way of extraction which mostly followed by column chromatography using silica gel. After fractionation of extracts each fraction could sentence for chromatography analysis such as Thin-layer chromatography (TLC) and High-performance liquid chromatography (HPLC) which can be combined with Ultraviolet–visible spectroscopy (UV), diode array (PDA), Mass spectrometry (MS) and Tandem mass spectrometry (MS/MS) detection (Perales et al., 1985).

There are many spectroscopic analyses to identify isolated compounds chemical structure. Nuclear magnetic resonance spectroscopy (NMR), Infrared spectroscopy (IR), Ultraviolet–visible spectroscopy (UV) and mass spectrometry (MS) are among most popular methods of molecular structure analysis (Charlet et al., 2002). One of the advantages of these methods is the very little amount of pure compound needed for the analysis, therefore application of spectroscopic methods are more and more common in the analysis of bioactive substances present in plants extracts (Chimichi et al., 1999; Mazur et al., 1996).

## 2.5 Well Known Commercialized Botanical Insecticide

The following paragraph shall discuss briefly some well known botanical insecticide.

### 2.5.1 Azadirachtin

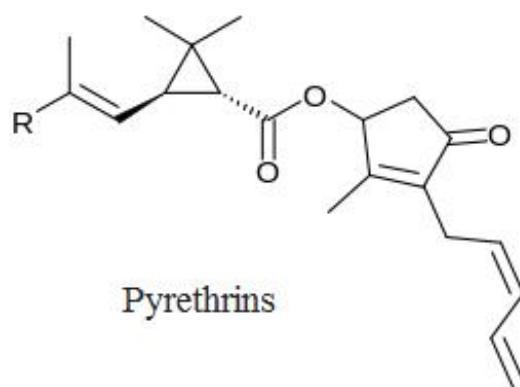
Azadirachtin belongs to limonoid family which is isolated from the Neem tree. It has been reported to have strong toxicity, antifeedant and growth inhibition activity against several insects pest (Kubo & Klocke, 1982) (Figure 2.1).



**Figure 2.1:** Azadirachtine.

### 2.5.2 Pyrethrins

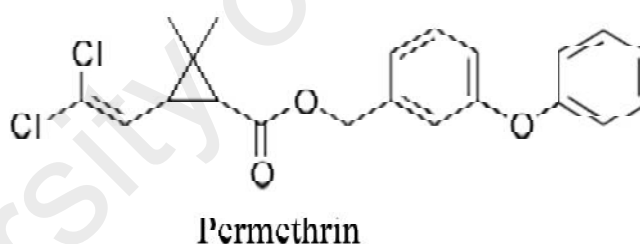
This compound belongs to terpenoid family and normally derived from *Chrysanthemum cinerariifolium* that have potent insecticidal activity which attack the nervous systems of all insects (Malcolm, 1988; Zhu et al., 2014) (Figure 2.2).



**Figure 2.2:** Pyrethrins.

### 2.5.3 Permethrin

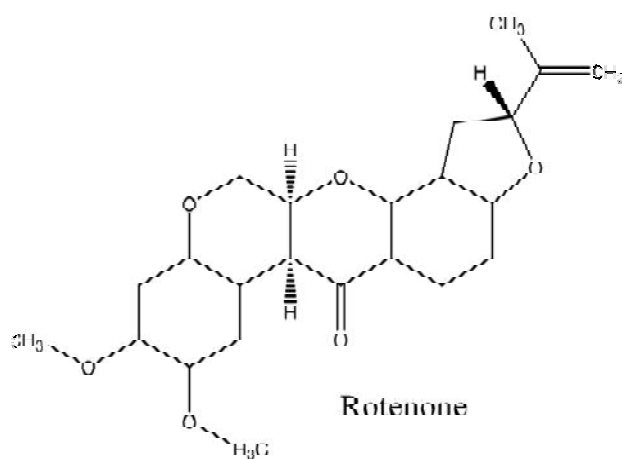
Permethrin is part of the synthetic chemical from pyrethroids family which has been used as insecticide. Its mechanism of action is by prolonging sodium channel activity of insects nervous system (Burton, 2011) (Figure 2.3).



**Figure 2.3:** Permethrin.

### 2.5.4 Rotenone

Rotenone is a botanical insecticide isolated from tuba plants (*Derris elliptica*). It is the main type of flavonoid aglycones in the root of tuba (Nawrot, Harmatha, Kostova, & Ognyanov, 1989) (Figure 2.4). The mechanism of its action is through digesting and contact with the insects. The negative side of rotenone is to be toxic to the fish and humans. The positive side which is depredate fast in contact with sunlight.



**Figure 2.4:** Rotenone.

### 2.5.5 Nicotine

*Nicotiana tabacum* produces the toxic alkaloid nicotine which have been used as insecticide (Delporte, Van Holle, & Van Damme, 2013; Shang et al., 2015) (Figure 2.5).

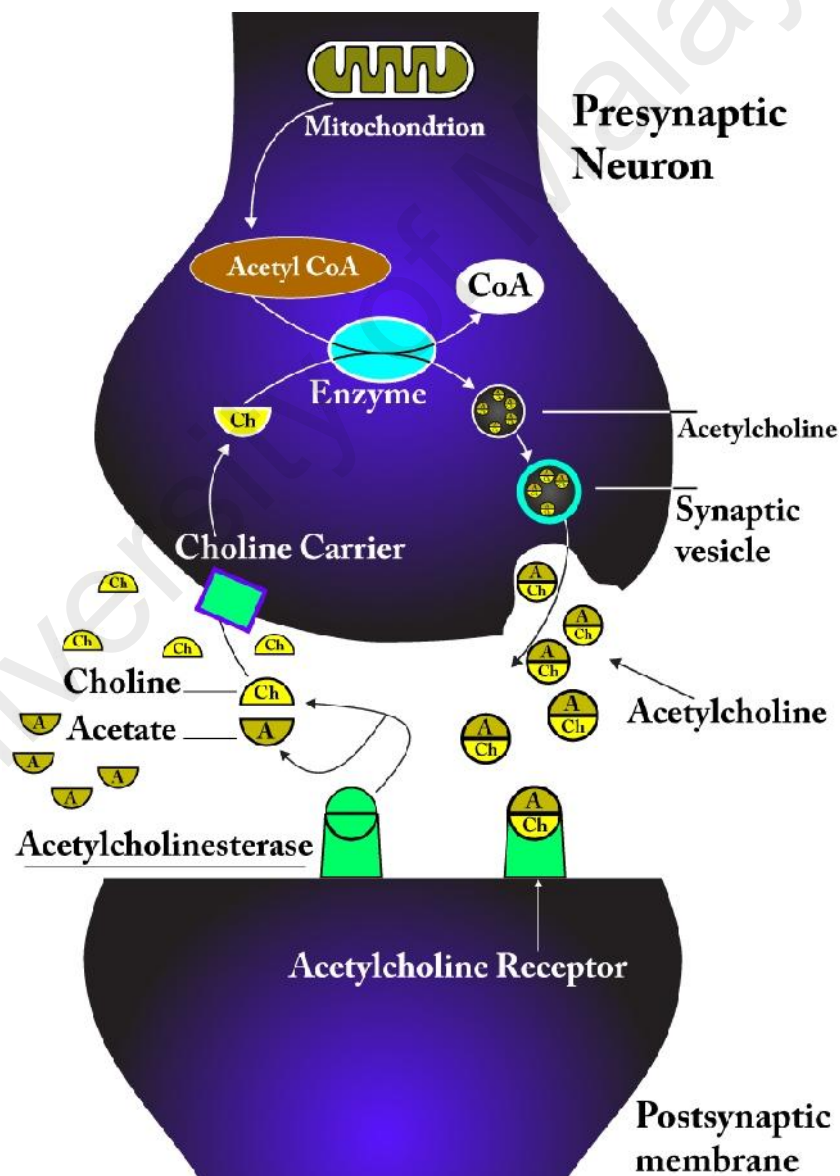
**Figure 2.5:** Nicotine.

There are many other botanical compounds which have not been commercialized. It includes alkaloids and phenylpropanoids compounds from several families including Meliaceae, Annonaceae and Piperaceae and many others (Aronson, 2014; Ramos, Souza, Kato, & Batista, 2012; Ramos, Vanin, & Kato, 2009)

### 2.6 Insecticidal Mode of Action

Most insecticides including organochlorine and organophosphates work on the nervous system; however, they keep the nerve cells from communicating with each

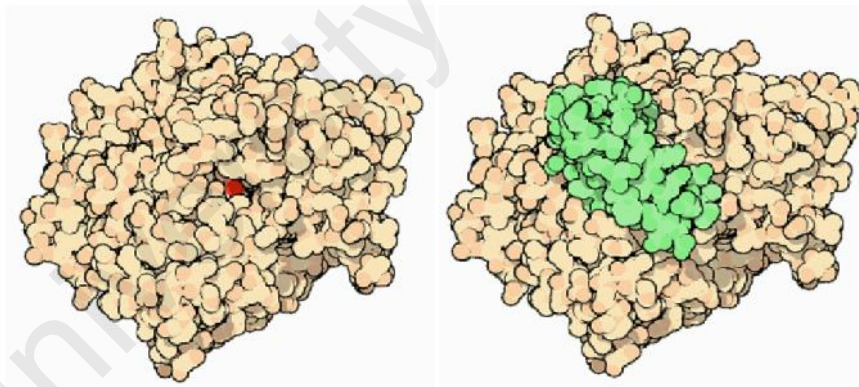
other (Alfthan, Kenttämää, & Zukale, 1989; Scott, Jensen, Philogène, & Arnason, 2008; Korayem, Hasabo, & Ameen, 1993). Usually, nerve cells in the organs of living organism send electrical impulses down to the end of the cell where they are transferred to the synapse to another nerve cell. Nerves communicate with one another and with muscle cells by using neurotransmitters. These are molecules in charge of transferring the messages that are released from the nerve cell and rapidly diffuse to neighboring cells, stimulating a response once reaching the target (Naik, Vaidya, & Namjoshi, 2013; Bachrouch, Ferjani, Haouel, & Jemâa, 2015; Korayem, Hasabo, & Ameen, 1993).



**Figure 2.6:** Acetylcholinesterase mechanism in nerve synapses.

## 2.7 The mechanism of Action of Acetylcholinesterase

A chemical which is known as acetylcholine (ACh) moves from one cell to the other and binds with the new cell, sending the electrical pulse down the new cell. Organophosphate and organochlorine insecticides are similar to nerve gas agents that are closely related which prevent the ACh from coming loose from the new cell, so pulses are interrupted and can't receive any more impulses (Figure 2.6) (Hemingway & Georghiou, 1983; French-Constant & Bonning, 1989). This attacks Acetylcholinesterase (AChE) and because AChE has an essential function targeting it can cause serious damage to nervous system. Most of the insecticides attack the nervous system causing ACh to accumulate in the nerve synapse that paralyzes the insects (Figure 2.7) (Fournier & Mutero, 1994). Over the years of controlling and suppressing insect pests' population, most of the synthetic pesticides were targeting insects AChE enzyme for disabling or killing the pest (Mathieu-Daudé, 2014).



**Figure 2.7:** The active site tunnel of acetylcholinesterase , from PDB entry 1b41 and 1eve, showing the active site serine in red. (4.2.214)

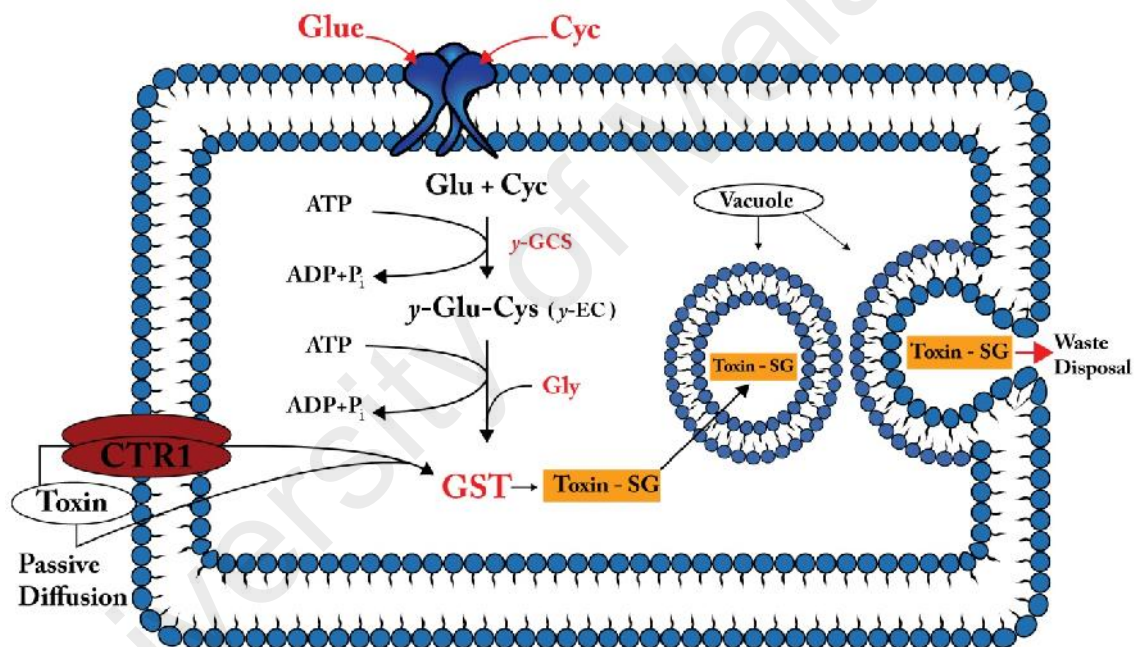
Attacks on the nervous system disturb insects function and cause death (Chen, Han, Qiao, & Qu, 2007; Xu et al., 2008). Synthetic insecticides such as malathion are in this class and were famous for treating Mediterranean fruit fly *Ceratitits capitata* infestations of the several mosquito's vectors. Similarly, carbamates have relative



properties to organophosphates; however, it won't last long in the environment due to degradation (Ffrench-Constant & Bonning, 1989; Mathieu-Daudé, 2014). Pyrethroids has synthesized which mimic the action of chemicals in the Chrysanthemum flower and considered to be among the safest insecticides because it breaks down when exposed to light. They are mostly used against lice and other household pests (Beugnet & Chardonnet, 1995). Neonicotinoids are the synthetic versions of nicotine. Neonicotinoids have specific effect on insects nervous system causing them become jumpy, with leg tremors, stimulate wing motion, unbalance movement, paralysis and eventually death (Basit, Saeed, Saleem, & Sayyed, 2013). Pyrethroids, Neonicotinoids and similar botanical pesticides such as rotenone are not highly toxic to mammals including humans because they work on a neural pathway that is more abundant in insects (Mohamed et al., 2017; Pham et al., 2017). Nevertheless, some of these chemicals have been banned because they might be contributing to toxicity against honey bees which plays vital role in crop pollination (Gregorc & Poklukar, 2003).

All organism possess defense mechanism against toxic compounds. Glutathione S-transferase (GST) is the first line of defense mechanism against acute and chronic toxicities of in insects (Almli et al., 2002; Hegazy, Tars, Hellman, & Mannervik, 2008). GST involves in cellular protection by direct or enzymatically reducing in any outside toxic elements such as free radicals, reactive species, other toxicants and heavy metals. GSTs are a similar to second phase of detoxification enzymes which detoxify both reactive species and toxic compounds. Primarily detoxification starts from catalysing GSH-dependent conjugation and redox reactions with the toxicant (Zhang, Modén, Tars, & Mannervik, 2012). When the toxin or heavy metal ion (HMI) enter the cell it will catalyzed the reaction involving binding glucuronic acid to toxicant. The binding of glucuronic acid will attach to toxins will make them more water-soluble and less active.

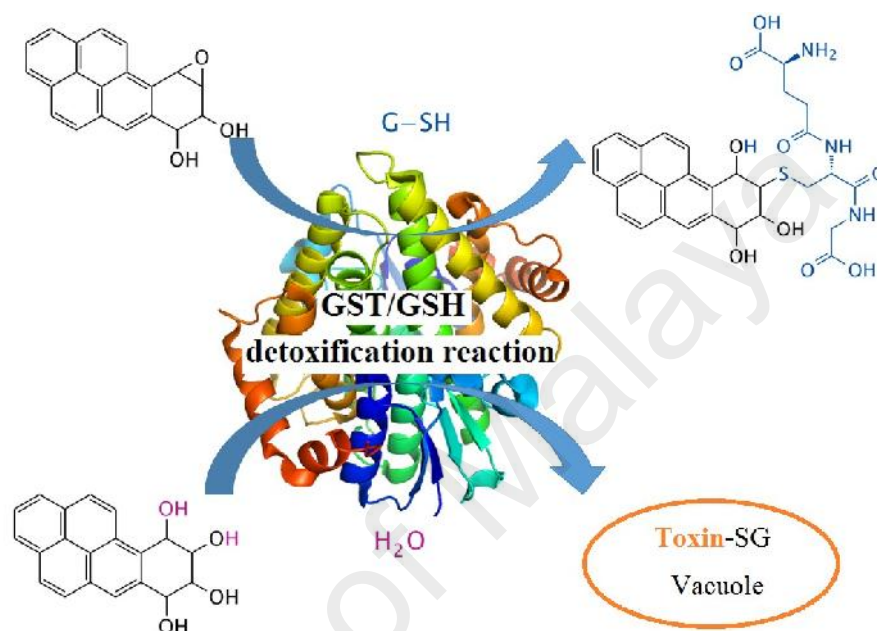
The energy for this reaction comes from ATP and GST catalyzes the transfer of glutathione to phase one products. The Glutathione enzyme is a complex tripeptide of amino acids such as L-glutamine and glycine. GST has a sulfhydryl (SH) group on the cysteinyl portion, that is highly capable of electron donation (Pugazhendhi et al., 2017). As electrons are lost, the molecule gets oxidized and loses its potential for cell toxicity, and two such molecules bind by glutathione disulfide or oxidized glutathione (GSSG) (Mittova et al., 2003; Thuillier et al., 2013; Shemarova, Maizel, & Khovanskikh, 2000) (Figure 2.8).



**Figure 2.8:** Glutathione S-transferase detoxification process.

Pesticides' mechanism of action and resistance in the insects can be directly related to the GST's function. This means resistant insects produce more active and potent GSTs to detoxify the toxic elements (Hegazy, Tars, Hellman, & Mannervik, 2008). Ironically, pesticide or compounds which cannot be bonded and detoxified by GSTs will be more desirable in controlling and fighting resistant insects (Wei, Clark, & Syvanen, 2001) (Figure 2.9). Such high resistance in mosquitoes which are vectors of

fatal diseases such as malaria, dengue fever and west Nile virus or other agriculture insects pests with high GST enzyme activities that help them to detoxify them self from common insecticides (Kalha et al., 2014; Jadeja et al., 2011).



**Figure 2.9:** Glutation S-transferas detoxification process in cell. Modify from from PDB entry bjl22.

Family of oxidase enzymes and non-specific esterases in insects and animals involved in catalytic reaction in which each of the two atoms of oxygen is used for a different function in the reaction (Nongonierma, Mooney, Shields, & FitzGerald, 2013). Depending on insects and specific insecticide they can be involved in the resistance and pesticide mode of action. Non-specific esterases (NSEs) are widely distributed in many types of cells (Kolliopoulou & Swevers 2010; Koradaa, Naskara, & Edison, 2010). NSEs are demonstrated and assayed by the cleavage of a short-chain acyl group from a chromogenic leaving group. Actual function of oxidase and NSEs are poorly understood (Houk & Hardy, 1981; Chattopadhyay, Sengupta, Verma, Sen, & Saratchandra, 2001). The fact that there are many types of enzymes involve in detoxification such as specific

or non-specific esterases, monooxygenases and transferases has long been long proved (Hirai et al., 2002; Touhara, Bonning, Hammock, & Prestwich, 1995).

## 2.8 Malaysian Flora

Natural resources from tropical rain forest could be a resourceful bank of biological active compounds. Many research programs in the tropics established focussing on these readily accessible resources. Malaysia is among the countries with highest plant diversity, many species has been reported with medicinal properties (Nair & Burke, 1990; Zhu et al., 2014). While some species are more known than others, still there are many which are unknown for relative activities. The importance of vast diversity of medicinal plants is not only in medicinal value but for the noble activities as biopesticide (Villaverde, Sevilla-Morán, Sandín-España, López-Goti, & Alonso-Prados, 2014). For instance, extraction and toxicity test from several tropical timber operations led to the discovery of potent insecticides in the stem wood of the Malaysian tree, *Azadirachta excelsa* (Nurdiana et al., 2013). *P. amalago var. medium*, *P. glabratum*, *P. mikanianum*, and *P. mollicomum* are belonging to the genus Piper (Yuncker, 1975), and bioassay-guided Piperaceae studies have been indicated their insecticidal potential (Scott et al., 2005, 2008). Moreover, there are formulations of Piperaceae derivatives already in registration process in Brazil (Scott et al., 2004). Piperamides are one of the major classes of compounds found in the Piper genus, and these compounds have potent insecticidal activity by acting as neurotoxic compound (Dyer & Palmer, 2004). Piper genus have important characteristic for the management of insects resistant populations (Scott et al., 2004).

One of the very interesting plant which has been highly neglected in this area is *Piper sarmentosum*. This plant has been shown to have several medicinal activity and

its extracts with insecticidal activity but however it has never been investigated further (Ee et al., 2009).

## 2.9 *Piper sarmentosum*

*Piper sarmentosum*, known as kaduk in Malaysia belongs to Piperaceae family is widely distributed in the region (Figure 2.10). Piperaceae consists of more than 2000 species (Bezerra et al., 2008). *P. sarmentosum* is a perennial climbing shrub in tropical countries. It is a terrestrial herb growing up to 60 cm height, green trunk and jointed at the nodes. The leaves are heart-shaped, thin and dark green in colour with length and width of 7-15 cm and 5-10 cm, respectively. The leaves of *P. sarmentosum* were used as vegetable or food wrapping (Damsud, Adisakwattana, & Phuwapraisirisan, 2013).



**Figure 2.10:** *Piper sarmentosum* plants grow in plantation near by University Malaya.

*P. sarmentosum* contains many secondary metabolites such as phenylpropanoids (Hafizah et al., 2010) tannins and alkaloids compounds (Gutierrez, Gonzalez, & Hoyo-Vadillo, 2013); calcium, iron, vitamin B1, B2, C, E,  $\beta$ -carotene (Rukachaisirikul et al.,

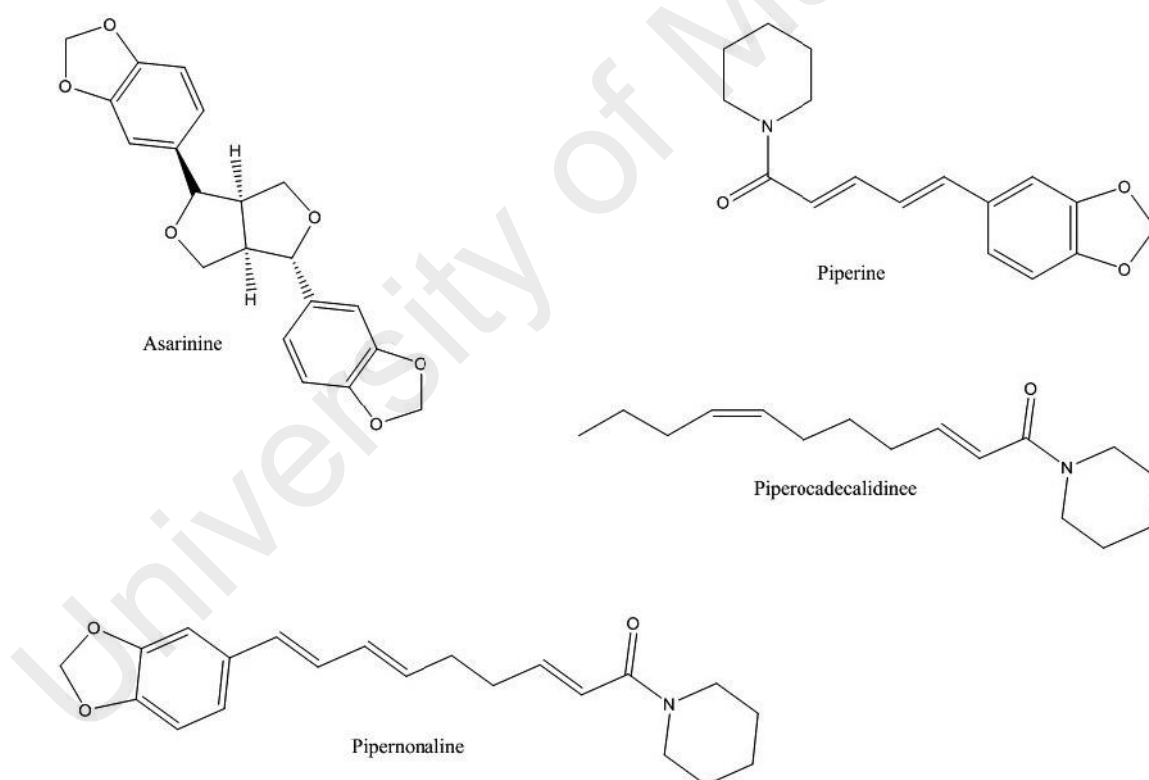
2004) and  $\beta$ -sitosterol (Scott, Jensen, Philogène, & Arnason, 2008). The plant content of oxalic acid is high which make complex with calcium (Zakaria, Patahuddin, Mohamad, Israf, & Sulaiman, 2010). Traditionally locals in Malaysia apply Kaduk leaves to the forehead of children to lessen headache. The root of *P. sarmentosum* has been used for toothache and antifungal dermatitis on the feet. The roots chewed with betel nut and the juice swallowed were beneficial for cough and asthma as well as to treat toothache. In other remedy warm leaves coated with coconut oil are applied to the muscle and joint to ease the pain (Lee et al., 2001; Zainal Ariffin et al., 2009; Sawangjaroen, Sawangjaroen, & Poonpanang, 2004).

## **2.10 Pharmacological and Insecticide Activity**

Ridtitid et al, (1998) studied the active component of eleven Thai medicinal plants to screen for their cathartics and antispasmodics activities. The pharmacological study revealed that methanol extracts of *P. sarmentosum* was able to lower the intestinal tension and also inhibit the acetylcholine related to intestinal tension in isolated rats (Ridtitid et al, 1998; Lee, 2000, Peungvicha et al., 1998). In another study methanol extracts was highly potent against *Entamoeba histolytica* infecting the intestines of mice (Sawangjaroen, Sawangjaroen, & Poonpanang, 2004). *P. longum* and its component, Piperine have accounted as a non-toxic immunomodulatory with antitumor property (Lee, 2000). The water extract of whole plant of *P. sarmentosum* has been noted for a hypoglycemic effect in rats (Peungvicha et al., 1998). The essential oil of *P. sarmentosum* is known to demonstrate larvicidal activity against mosquitoes (Silvia et al., 2008)

## 2.11 Chemical Constituents

Several researches have been conducted to study the chemical constituents of petroleum ether, methanol and hexane extracts of *Piper sarmentosum*. These studies identified several phenylpropanoids and alken such as piperine and asarinine from *Piper nigrum* and *P. longum* (Figure 2.11). Both compounds isolated from of *P. longum* showed strong fungicidal activities against gray leaf spot caused by *P. grisea*, and were characterized by the spectroscopic analyses (Ramos, Souza, Kato, & Batista, 2012; Ramos, Vanin, & Kato, 2009) (Figure 2.11).



**Figure 2.11:** Structures of isolated compounds from *Piper sarmentosum*.

## 2.12 *Sitophilus oryzae*

The rice weevil, *Sitophilus oryzae* L. (Coleoptera: Curculionidae) (Figure 2.12) are among the most destructive insect pests of stored grains worldwide (Arthur, 1996, 2002). Rice weevils are perfectly able to infect grain kernel which is possible for most storage pests. Because of this ability, they are classified as of primary colonizers (Fang et al.; L. Fang, Subramanyam, & Arthur, 2002). *S. oryzae* larvae feed from inside the stored products and not much affected by current controlling agents which applied to the external kernel part (Ziaee, Moharramipour, & Francikowski, 2014). Rice weevils originated in India. It was then spread to Europe and other parts of the world through grain trade. Rice weevils are perhaps most widely distributed of known insect, being found in all parts of the world where grains are consumed (Arthur, 2002).

### 2.12.1 *Sitophilus oryzae* Characteristics

The adult rice weevils have wing and about 3 to 4 mm long. They usually have four red to yellow marks on thorax that are round or irregular in shape. Wings are fully developed and used for flight. Male rice weevils has shorter, bigger and more distinct snout than females.

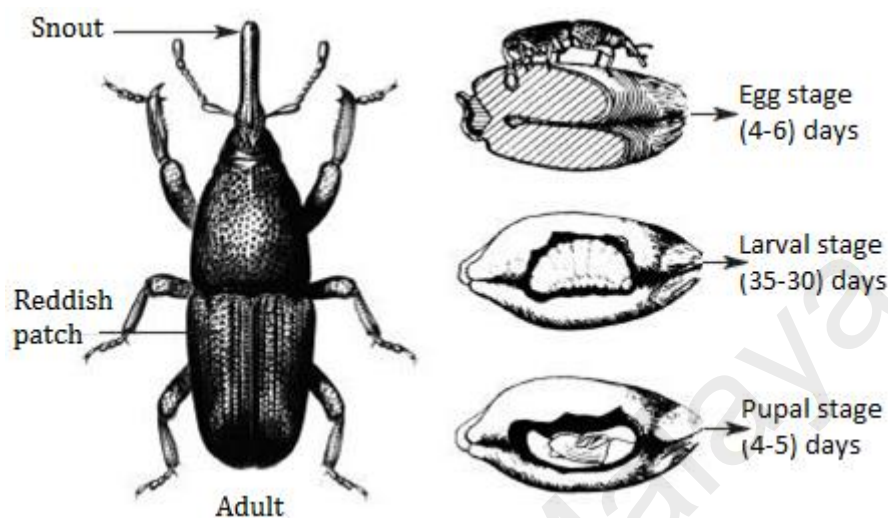


**Figure 2.12:** Adult of rice weevils.  
Source: Turney (1962).

All larvae are legless and thickened in the middle with a humpback look. Larvae are fairly smooth, 7 to 8 tiny finger like sensory projections on the lower mouthpart



(Figure 2.13). All adults are brown in colour meanwhile larvae have creamy body with white and black head (Ziaee, Moharramipour, & Francikowski, 2014).



**Figure 2.13:** *Sitophilus oryzae* life cycle. [http://www.entomology.ucr.edu/ebeling/ebeling7.html#insects infesting whole grain](http://www.entomology.ucr.edu/ebeling/ebeling7.html#insects%20infesting%20whole%20grain) (28.8.2013)

The rice weevil is the most serious stored grain pests worldwide that attacks wheat, corn, oats, barley, beans, nut, wild bird seed, rye, buckwheat, macaroni, starch, cereal product and all 16 types of stored grains. They cause more damage in warm climates such as Malaysia (Coombs et al. 1977; El-Nahal, 1989). They can also infest grain in the field. The larvae hollow out kernels of grain and usually attack whole kernels. The larvae of *S. oryzae* complete their development inside a seed kernel or relative products such as macaroni and flour. An adult female rice weevil making a hole by eating into the storage products and then deposits one egg in the cavity, covering it with substance from its ovipositor (Holloway, 1986). The egg turn in to larvae inside the seed, feeding from inside and hollowing it out then turn into pupates within the channel (Delobel & Grenier, 1993). Adult females lay more than 300 eggs in a lifetime. The egg develops to larva through 3 to 4 stages (instars) in about 18 days then pupates for about 6 days and then develops into an adult in the grain kernel. They stay for 3 to 4 days until body hardened and mature (Hou et al., 2006). The life of the adult rice

weevils is varied depending on many different factors, in average is about 3 to 6 months (Mouhouche & Fleurat-Lessard, 2004).

### 2.13 *Rhyzopertha dominica*

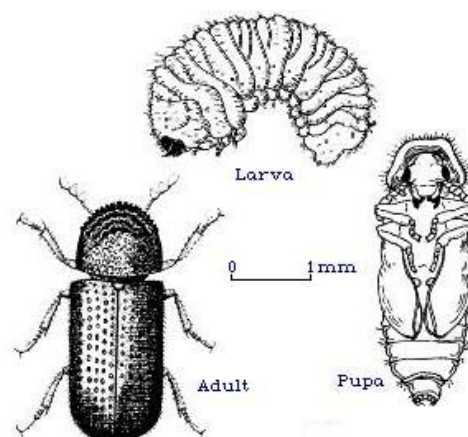
Lesser grain borer, *Rhyzopertha dominica* (Talukder & Howse, 1993b) belongs to the Bostrychidae family of beetles which are wood boring insects (Figure 2.14). *R. dominica* adults have very strong mandibles that are strong tools to caving into the seeds (Forget et al., 2003).



**Figure 2.14:** *Rhyzopertha dominica* adults feeding on grain. <http://www.azoresbioportal. angra.uac.pt /listagens. php?lang=en&sstr =8&id= A00259&dis =pico> (28.8.2013)

In tropical countries, lesser grain borer is more common and destructive due to temperate climates. However, it spread to most areas in transported grain. It is a problem of grain only rather than cereal products and is considered one of the harmful pests of stored grain in Australia (Forget, Livet, & Leboulenger, 2002).

### 2.13.1 *Rhyzopertha dominica* Characteristics



**Figure 2.15:** Lesser grain borer life cycle. USD United States Department of Agriculture. <http://www.ars.usda.gov/Research/docs.htm?docid=16093> (28.8.2013)

The lesser grain borer life cycle is divided to egg, larvae, pupae and adult stages. *R. dominica* larvae develop inside stored food products such as grains then adults immerged and damage grain by causing cavity into the kernels and leaving them covered in flour-like powder (Steel, Elmouttie, & Hamilton, 2012). Eggs are laid individually outside grains which may have been damaged by adult feeding (Figure 2.15). Larvae grow into the grain to complete their development. In the perfect condition, *R. dominica* females lay more than 400 eggs during their lifetime. Healthy eggs required up to 25 days to develop to adult (Daglish, Holloway, & Nayak, 2013). The larvae have light cream colour, with strong mouthparts for biting and six legs. The young larvae are highly active in stored grain but become less active and gradually more C-shaped as they grow (Semeao, Campbell, Hutchinson, Whitworth, & Sloderbeck, 2013). The larvae size increases by reaching the four instar stages and will become inactive inside the grain. The stored whole grain has preferable environment for larva to develop and usually takes 28 days at 29°C and more than 30 days at 25°C (Forget, Livet, & Leboulenger, 2002; Forget et al., 2003). Both larvae and adults dispose high amount of waste pellets. The fourth instar larvae will turn to pupae within the feeding

channels inside the grains and approximately within 7 days and then they turn into adults. When the pupal developed to adult, they will chew through the layers of grain and emerge (Athanassiou, 2008). The adult female will be capable of oviposition after 2 weeks of emergence and it can last up to 4 months. *R. dominica* adults are strong flyers when conditions are warm and are often carried by air currents from infested storages (Wakil, Riasat, & Lord, 2013).

#### **2.14 *Plodia interpunctella***

*Plodia interpunctella* (Hübner), the Indian meal moth is one of the common moths infesting stored grains and grain products (Figure 2.16). It can develop and infest many different commodities such as grains, beans, meals, dried fruits and relative grain products such as flour (Lewis, Lizé, & Wedell, 2013), and can be an economic insect pest in storage and commercial food processing factories (Ndomo-Moualeu, Ulrichs, Radek, & Adler, 2014; Razazzian, Hassani, Imani, & Shojai, 2015).



**Figure 2.16:** The Indian meal moth. Harvard University; Bugwood.org (28.8.2013).

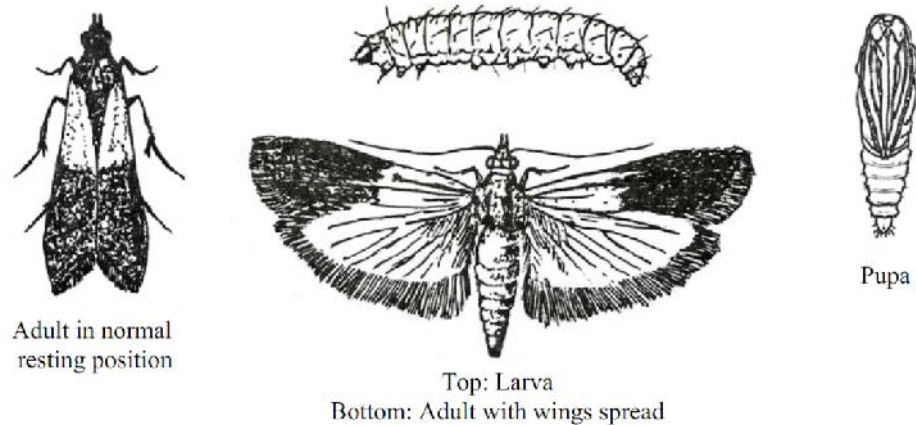
### 2.14.1 *Plodia interpunctella* Characteristics

The Indian meal moth is a strong flyer with wingspan of 18 to 20mm (Figure 2.17). The colour of the outer wings is partially reddish brown to bronze, the part of the wings closer to the body is greyish. The mature larvae (caterpillars) length is about 13 mm long. They are a dirty white colour which sometimes exhibits light pink (Figure 2.17). The pupa is in a soft silken cocoon with brown colour produce by the larvae (Razazzian et al., 2015; Tang et al., 2017).



**Figure 2.17:** *Plodia interpunctella* 4<sup>th</sup> instar larvae. Harvard University; Bugwood.org (28.8.2013).

Infestations and damage to food materials and finished products lead up to 30% lost and rejection of products by consumers. A female *P. interpunctella* could lay more than 200 eggs during her lifetime. Eggs are laid individually on the food materials and within a few days, the tiny whitish caterpillars emerge. If the stored food product packaging be loose or damaged it allowed the moths to reproduce, and larvae and adults develop to pupate and spread the infestation (Mewis & Ulrichs, 2001). The products that well packaged are unlikely for infestation since the larvae or adult will die from lack of air or buildup of moisture that would have allowed the development of fungus to destroy the larvae and adults (Lee et al., 2017; Predojević, et al., 2017).



**Figure 2.18:** *Plodia interpunctella* life cycle. Harvard University; Bugwood.org (28.8.2013).

These larvae feed for a few weeks and when they are mature, they start to ceiling meet, and spin the silken cocoon for secure the pupae stage in which they developed and transferred to adult. Then from pupate the adult moth emerges, mating occurs and the life cycle repeats itself. In warm weather, the cycle may take only 6 to 8 weeks (Mewis & Ulrichs, 2001) (Figure 2.18).

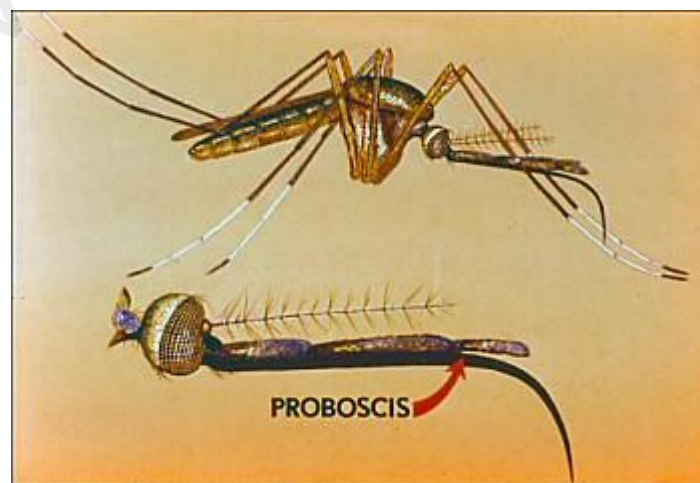
## 2.15 Mosquito Vectors, Pests of Medical Importance

The impact of insects as vectors and other arthropods on human health are enormous. Most of these vectors feed on blood (hematophagous) and one of the most common insects in this group is mosquito (Valkiūnas, Kazlauskienė, Bernotienė, Palinauskas, & Iezhova, 2013).

### 2.15.1 Classification of Mosquitoes

Mosquitoes are among the well known group of insects from family Culicidae of the order Diptera (Tadkowski, Jones, & Firman, 1977). They are notorious to man as pests and vectors of some of the most deadly human diseases. Mosquitoes are small insects with two winged and nearly three quarter of all mosquito species was found in

the humid tropics and subtropics (Van Essen, Kemme, Ritchie, & Kay, 1994). More than 3000 species of mosquitoes from 34 genera has been identified till today (Edwards, Severson, & Hagedorn, 1998; Valkiūnas, Kazlauskienė, Bernotienė, Palinauskas, & Iezhova, 2013). Three subfamilies have been recognized among the Culicidae: the Toxorhynchitinae, Anophelinae and Culicinae (Aouinty, Oufara, Mellouki, & Mahari, 2006). Culicinae are the major vectors of arboviruses and filariasis, and important vectors of human disease. The female mosquito possesses a proboscis which is curved backwards, making it capable of piercing skin and transmitting disease (Bagavan, Rahuman, Kamaraj, & Geetha, 2008) (Figure 2.19). The most important genera in subfamily in the matter of Culicinae are *Culex*, *Aedes*, *Mansonia*, *Haemagogus* and *Sabethes* from medical point of view (Kamaraj, Rahuman, & Bagavan, 2008; Prajapati, 1997). *Aedes* is a well known vector of dengue infection which is better known as yellow fever. Some *Aedes* species are also vectors of other parasitic disease such as filariasis and viral disease (WHO, 1981). *Aedes aegypti*, *Aedes albopictus* and *Culex quinquefasciatus* are among the most important vectors which is described briefly below (Vassilakos, Athanassiou, Chloridis, & Dripps, 2014).



**Figure 2.19:** Female mosquito proboscis schematic looks.  
<https://www.utep.edu/LEB/mosquito/index.htm> (28.8.2013)

### 2.15.2 *Aedes aegypti* (Linnaeus)

*Aedes aegypti* is a tropical mosquito and it is believed to be originated from Central Africa where it was found in great quantity (Anees, 2014) (Figure 2.20). *Ae. aegypti* is a domestic breeder which could be distributed to all parts of the world by finding breeding places on sailing ships on those days (Bagavan, Rahuman, Kamaraj, & Geetha, 2008; Bryant & Raikhel, 2011; Kamaraj, Rahuman, & Bagavan, 2008). *Ae. aegypti* is categorized as one of the most efficient vectors for viruses because it is highly antropophilic, vigorously developed well and preferring to live indoors in close contact with humans (WHO, 1992; WHO, 1996). It is commonly found in urban areas especially in the most densely populated districts (Tadkowski, Jones, & Firman, 1977). *Ae. aegypti* breeds in domestic water containers with clean water (WHO, 1992; WHO, 1996).



**Figure 2.20:** *Aedes aegypti* adults. <http://www.uruguayeduca.edu.uy/Portal.Base/Web/verContenido.aspx?ID=201725> (28.8.2013)

### 2.15.3 *Aedes albopictus* (Skuse)

*Aedes albopictus* (Figure 2.21) known as secondary vector of dengue has been found in all types of country, urban, suburban, rural and it is originated in the tropical forest of Southeast Asia (Boyer, Foray, & Dehecq, 2014; Petrić, Bellini, Scholte,



Rakotoarivony, & Schaffner, 2014) where many closely related species are known to exist (Knudsen, 1995).



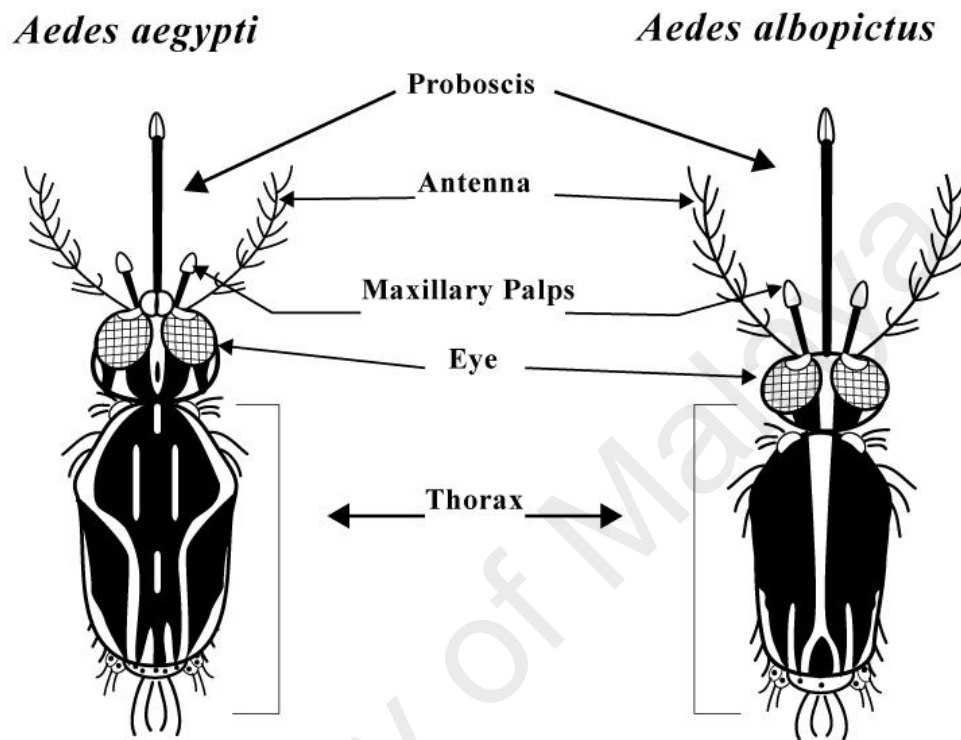
**Figure 2.21:** *Aedes albopictus* adults. [http://civr.ucr.edu/asian\\_tiger\\_mosquito.html](http://civr.ucr.edu/asian_tiger_mosquito.html) (28.8.2013)

*Ae. albopictus* is a highly adaptable species because it seems to be able to easily colonize after transported to a new region which making it hard to control them (Schleifer, 2009). Photoperiod has direct effect on egg deposition of *Ae. albopictus* plus their eggs capability of growing in colder temperature allows them to colonize in northern latitudes. In addition, egg cold hardiness of temperate strains *Ae. albopictus* enables them to survive the very cold winter temperatures in the northern latitudes (Yee, 2008).

#### **2.15.4 Identification of Adult *Aedes aegypti* and *Aedes albopictus***

The easy recognition of Adults *Aedes aegypti* and *Aedes albopictus* is by the silver white patterns of scales on the dorsal side of the thorax (Figure 2.22). The anatomy of *Aedes aegypti* contains of two straight lines of pattern encircled by curved lines on the side (Schleifer, 2009; Lounibos et al., 2001; Mogi & Tuno, 2014). On the other hand *Aedes albopictus*: single broad line of white scales situated in the middle of

the thorax (Petrić, Bellini, Scholte, Rakotoarivony, & Schaffner, 2014; Dumont & Tchuenche, 2012; Conti, Canale, Bertoli, Gozzini, & Pistelli, 2010) (Figure 2.22).



**Figure 2.22:** Schematic comparison between *Aedes aegypti* and *Aedes albopictus* (modified from WHO, 1995).

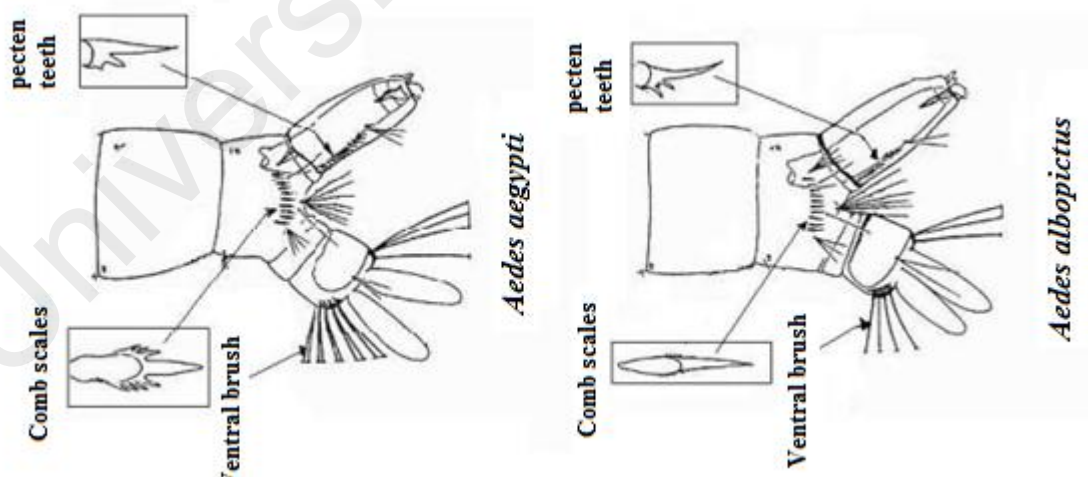
#### 2.15.5 *Aedes aegypti* and *Aedes albopictus* Larval Biology

Mosquito larvae have four larval stages that require five to 10 days for completion (Schleifer, 2009). The variation of duration depends on temperature or larval diets (Bagavan, Rahuman, Kamaraj, Rahuman, & Bagavan, 2008). Mosquito larvae cannot be found in running waters because the larvae are unable to resist wave action (WHO, 1992). Mosquito larvae are found in all types of aquatic habitats and water. The larvae commonly are found in waters containing micro flora, fauna and debris of plant as well as animal origin (Knudsen, 1995). Mosquito larvae move about mainly in two ways, by shaking the body and using their mouth brushes as driving force (WHO, 1970).

Mosquito larvae normally plunge in to the lowest point of water in case of sudden disturbance of the surface or if a shadow crosses them (Lounibos et al., 2001; Schleifer, 2009). *Ae. aegypti*, *Ae. albopictus* and other mosquito species have four larval instars, each instar will finished with shedding the old skin (Rahuman, Gopalakrishnan, Venkatesan, & Geetha, 2008).

### 2.15.6 Identification at the Larval Stage

*Aedes aegypti* and *Aedes albopictus* larval stages are similar to each other. These two *Aedes* species can be differentiated by the shape of the comb scales on the eighth parts of the abdomen and the shape of the pecten teeth on the siphon (Figure 2.23). In *Ae. aegypti* larvae, the comb teeth have highly grown sideways denticles but the pecten teeth have less expound denticles (Samuel, Lalrotluanga, Muthukumar, Gurusubramanian, & Senthilkumar, 2014). Whereas, in *Ae. albopictus* larvae, the comb teeth have no lateral denticles but the pecten teeth have three perfectly expound pointed denticles (Angajala, Ramya, & Subashini, 2014).



**Figure 2.23:** Schematic comparison of *Aedes aegypti* and *Aedes albopictus* larvae ( modified from WHO, 1995)

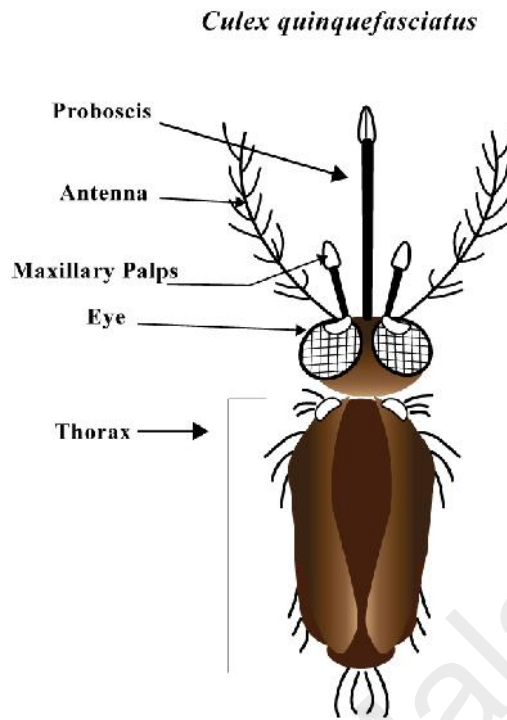
### 2.15.7 *Culex quinquefasciatus*

*Culex quinquefasciatus* has adapted to metropolitan conditions with a larval stage capable of developing in extremely contaminate water collections. *Cx. quinquefasciatus* is a medium-sized brown mosquito and one the most common species to the whole world which is highly attracted to humans as a source of food (Cheikh et al., 2008) (Figure 2.24) (WHO, 1992). Their biting can be responsible for great discomfort and allergic responses (Zhu et al., 2014). It can transmit pathogens such as *Wuchereria bancrofti* (agents of lymphatic filariasis) and it is known for being the vector of neurotropic viruses (Elango, Rahuman, Kamaraj, Bagavan, & Zahir, 2012).



**Figure 2.24:** *Culex quinquefasciatus* adults [https:// www.vectorbase.org/organisms/culex-quinquefasciatus](https://www.vectorbase.org/organisms/culex-quinquefasciatus)

The St. Louis and Japanese encephalitis viruses, eastern and western equine encephalomyelitis viruses, Rift Valley virus and West Nile virus are efficiently transmitted by *Cx. quinquefasciatus* (Pasteur, Georghiou, & Iseki, 1984; McCarroll et al., 2000). Therefore, it is important to control the population of this mosquito in order to curb the transmission of its associated pathogens.



**Figure 2.25:** *Culex quinquefasciatus* physical characteristic.

### 2.15.8 *Culex quinquefasciatus* Larvae

The *Cx. quinquefasciatus* larval has a short head and plump becoming darker toward the base. Larvae mouth covered with yellowish brushes which used for sifting organic materials. The larvae middle part is composed of eight segments, the siphon and the saddle (Figure 2.25). Each segment has an individual feathery setae pattern (Pan et al., 2009; Zhao et al., 2014). The siphon is on the upper side of the abdomen, and in *Cx. quinquefasciatus* the siphon is approximately four times longer than its extensive with multiple setae tufts (Kannathasan et al., 2007; Raghavendra et al., 2011; Wan-Norafikah et al., 2013). Controlling of disease contracted by vectors are still solely depends on the anti-vector measures for example by elimination of mosquito larvae by using synthetic larvicide. However effectiveness of current insecticide including permethrin are decline most field strain larvae of the same species showed to permethrin (Yu et al., 2015).



**Figure 2.26:** Larva of *Culex quinquefasciatus* [http://entnemdept.ufl.edu/creatures/aquatic/southern\\_house\\_mosquito.htm](http://entnemdept.ufl.edu/creatures/aquatic/southern_house_mosquito.htm)

*Culex quinquefasciatus* pupae stage is similar to *Aedes aegypti* and *Aedes albopictus*. The pupae's cephalothorax (fused head and thorax) has various colour with comma shaped (Figure 2.26) (Elimam, Elmalik, & Ali, 2009).

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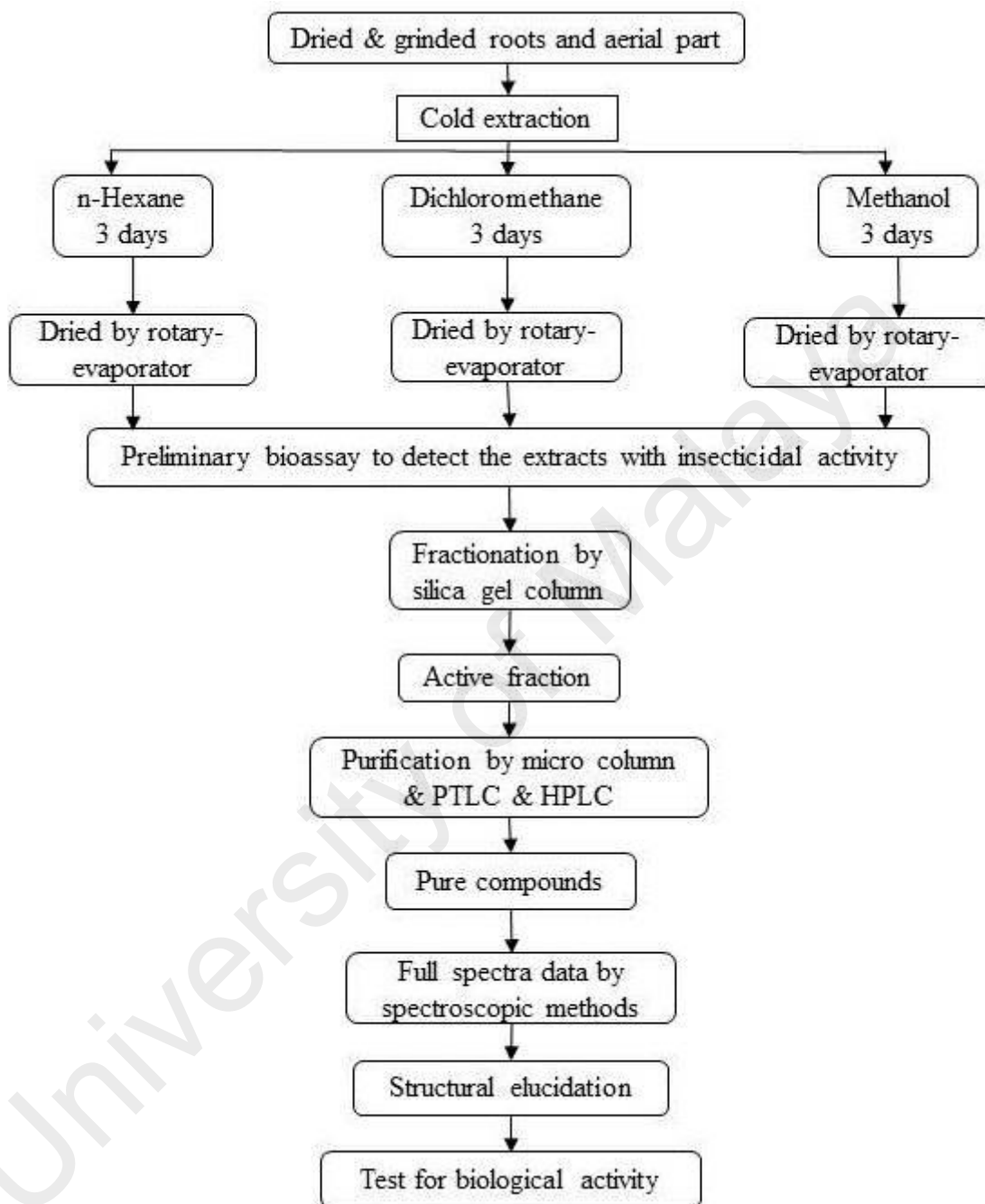
## CHAPTER 3: BIOASSAY GUIDED FRACTIONATION OF THE CRUDE EXTRACT OF *PIPER SARMENTOSUM*

### 3.1 INTRODUCTION

Bioassay guided study on the insecticidal activity of the extracts and active components of *Piper sarmentosum* was carried out. In this chapter, *P. sarmentosum* was studied for its chemical constituents responsible for its insecticidal activity, isolation and elucidating their molecular structure. The aerial part and roots of the plant were investigated. The isolation techniques were carried out using the conventional methods, i.e. column chromatography (CC), preparative thin layer chromatography (PTLC) and high performance liquid chromatography (HPLC).

After selecting the most potent extracts (Using preliminary toxicity test on *Sitophilus oryzae*, *Rhyzopertha dominica* and *Plodia interpunctella* , (Chapter 4), a preliminary separation procedure using column chromatography was done to fractionate the crude extract and to verify the active fractions before the final isolation of active compounds (Abd El-latif, 2015; Addo et al., 2010). A plant extract contains many compounds that may be seen in visible light or under ultraviolet conditions but the aim of this study was to target the active compounds with insecticidal activity (Abd El-latif, 2015). For this matter the isolated compounds from active fraction were tested for their insecticidal activity (Chapter 4).

The compounds separated from the active fraction was structurally elucidated through several spectroscopic methods; UV, IR, MS (GCMS, LCMS and HRMS), 1D (<sup>1</sup>H-NMR, <sup>13</sup>C-NMR, and DEPT) and 2D-NMR (COSY, HMQC, HMBC and NOESY). The known compounds were identified by comparing their spectral data with those described in the literature (Suvitha, Periandy, & Gayathri, 2015).



**Figure 3.1:** General procedures for extraction and isolation of active compounds.



### 3.2 LITERATURE REVIEW

Plants from *Piperaceae* family are belong to the order of flowering plants (*Piperales*) (Nwaubani, Opit, Otitodun, & Adesida, 2014). *Piperaceae* family comprises up to 2000 species are well known for their wide range of active compounds and their medicinal properties. Traditionally, this plant was used as expectorant, carminative, throat refresher, flatulent asthma reliever and appetite enhancer (Nwaubani, Opit, Otitodun, & Adesida, 2014; Ziaee, Moharramipour, & Francikowski, 2014). In Malaysia, *P. sarmentosum* is known as kaduk and it has many usage in folk medicine. Previous research on bioactive component from this plant have resulted in the isolation of a number of alkaloids, phenyls and phenylpropanoids (Suvitha, Periandy, & Gayathri, 2015).

Many studies have been conducted to investigate other biological activities including the anti-*Plasmodium falciparum*anti, anti-inflammatory antibacterial effects of *Piper anducum* (Gutierrez, Gonzalez, & Hoyo-Vadillo, 2013; Scott, Jensen, Philogène, & Arnason, 2008). Some solvent extracts with low polarity from *Piper sarmentosum* have also shown considerable antibacterial and antifungal activity (2008 Zainal Ariffin et al., 2009). Based on the literature review, the extracts of *P. sarmentosum* have been known to demonstrate insecticidal activity against several insect pests (Zakaria, Patahuddin, Mohamad, Israf, & Sulaiman, 2010). Isolation and identification of active component from plant is a very important step in introducing the new biopesticide (Massoud, Labib, & Rady, 2001). However, to date, there is no report on its specific isolated compounds from *P. sarmentosum* with insecticidal activity against storage pests and mosquitoes.

### 3.3 MATERIALS AND METHODS

The chemicals and instruments used in this study explained as follow.

#### 3.3.1 Solvents

For chromatographic separation; TLC, CC, PTLC analysis and UV, IR, MS, LCMS spectroscopy and HPLC, analytical grade solvents (Merck, United States) were used. For NMR, detectable nuclei  $CDCl_3$  was used. Hexane, dichloromethane (DCM) and methanol (MeOH) industrial grade (Chemo lab, Malaysia) were used for bulk extractions where these solvents were distilled twice before use.

#### 3.3.2 Instruments

**I) NMR:** spectra were obtained using JEOL LA 400 FT NMR, JEOL ECA400 FT NMR and BRUKER LA 400 FT NMR Spectrometer System using deuterated chloroform as solvent. Chemical shifts were reported in ppm and coupling constants were given in Hertz (Hz).

**II) Mass Spectroscopy:** Mass spectra were obtained on Agilent Technologies 6530 Accurate-Mass Q-TOF LC/MS, with ZORBAX Eclipse XDB-C18 Rapid Resolution HT 4.6 mm i.d. x 50 mm x 1.8  $\mu$ m column.

**III) Ultraviolet Spectroscopy:** UV spectra were recorded on a Shimadzu UV-Visible Recording Spectrophotometer using HPLC grade ethanol as solvent with mirror UV cell.

**IV) Infrared Spectroscopy:** The infrared (IR) spectra were obtained through Perkin Elmer FT-IR Spectrometer Spectrum RX1 using chloroform as solvent.

**V) Optical Activity:** It was determined on JASCO (Japan) P1000 automatic digital polarimeter.

### **3.4 Chromatography**

The methods used for separation and isolation of the compounds from *P. sarmentosum* are described as follows.

#### **3.4.1 Thin Layer Chromatography (TLC) and Preparative Thin Layer Chromatography (PTLC)**

Thin Layer Chromatography (TLC) was carried out using aluminum supported silica gel 60 F<sub>254</sub> plates. TLC was used to see the spots of the isolated compound. UV Light Model UVGL-58 Mineralight Lamp 230V, 50/60 Hz was used to examine spots or bands on the TLC after spraying with the required reagents. Preparative Thin Layer Chromatography (PTLC) was used in the separation of compounds. For this aspect silica gel 60 F<sub>254</sub> glass plates of size 20 cm x 20 cm (Merck 1.05715.0001) were used. Separation of compounds were observed under UV Light as described for TLC.

#### **3.4.2 Column Chromatography (CC)**

To set up the solvent system, all industrial grade solvents were distilled and for packing the column, silica gel 60, 230-400 mesh ASTM (Merck 9385, United States) was used. A slurry of silica gel 60 (approximately 30:1 silica gel to sample ratio) in hexane and DCM (1:1) solvent system was poured into a glass column of appropriate size with gentle tapping to remove trapped air bubbles. The hexane crude extract was initially dissolved in minimum amount of solvent and loaded on top the packed column. The extract was eluted with an appropriate solvent system at a certain flow rate. In case of methanol extracts were mixed with silica gel was loaded to the column using dry

packing method. Fractions were collected in conical flasks and evaporated for the next step. Fractions with similar relative RF spots on TLC were combined.

### 3.4.3 High Performance Liquid Chromatography (HPLC)

To separate compounds **1** (Asaricin) and **2** (Isoasarone), Waters HPLC System (United States) equipped with isocratic Module, System Fluidics Organizer and UV detector set at the range from 200-400 nm was used. Chromatographic analysis and separations were performed on ZORBAX Eclipse Plus C18 (9.6 mm i.d. x 250 mm x 3.5 µm) HPLC columns. HPLC grade methanol and deionized water were used as mobile phase solvents with HPLC grade formic acid as buffer. All solvents and samples used in this procedure were filtered through 0.45 µm nylon membrane filter (Waters, United States) prior to use. MassLynx software was used in data collection and analysis.

### 3.5 Detector Reagent — Vanillin-Sulphuric Acid Vapour

To detect the spots on TLC, 1.0 g vanillin were solved in in 10 mL of concentrated H<sub>2</sub>SO<sub>4</sub>. Then, the mixture was added upon cooling to 90 mL of ethanol before spraying onto the TLC plate. The TLC plate was then placed on heater and heated at 50 °C until full development of colours had been observed. The active compounds Asaricin **1**, Isoasarone **2** and *Trans*-asarone **3** occurrence was purple and compound **4** was grey which all were later determined as phenylpropanoid.

### 3.6 Plant Collection and Preparation for Extraction

*Piper sarmentosum* was collected in the vicinity of University of Malaya in year 2011. A voucher specimen (KU 0110) was deposited in the University of Malaya's herbarium. The aerial part and roots of *P. sarmentosum* were separately dried and grinded.

### 3.7 RESULTS

The results of extraction, isolation and elucidation of phenylpropanoids were done in several steps as follow.

#### 3.7.1 Roots and Aerial part extraction

Dried, powdered roots (1kg) of *P. sarmentosum* were extracted successively with hexane (3L, 2x), DCM (3L, 2x) followed by MeOH (3L, 2x) at room temperature, giving 9.79 g, 7.08 g and 16.42 g of extracts, respectively. Extraction from the aerial parts was done in the same way and results were hexane: 10.19 g, DCM: 6.2 g and MeOH: 18.03 g.

Since this experiment was bioassay guided study after preparation of hexane, methanol and dichloromethane extracts from aerial part and roots of *P. sarmentosum*, all extracts were subjected for toxicity test against the storage pests. The procedure was explained in detail in Chapter 4. Since the amount of possible active component inside the extracts were unknown the extracts were applied at high concentration of 1 mg/ml; 1000ppm (El-Maghraby, Nawwar, Bakr, Helmy, & Kamel, 2012). Preliminary toxicity test against storage pest; *S. oryzae*, *R. dominica* and *P. interpunctella* revealed that the hexane and methanol extracts of aerial part and roots showed strong insecticidal activity (1mg/ml caused 100% mortality to subjected pests). Therefore, hexane and methanol extracts were subjected for fractionation. The common chromatography methods were used for fractionation and final separation at this level (Figure 3.1).

In total, six types of extracts were active: roots hexane extract (RHE), roots methanol extract (RME), aerial parts hexane extract (AHE) and aerial part methanol

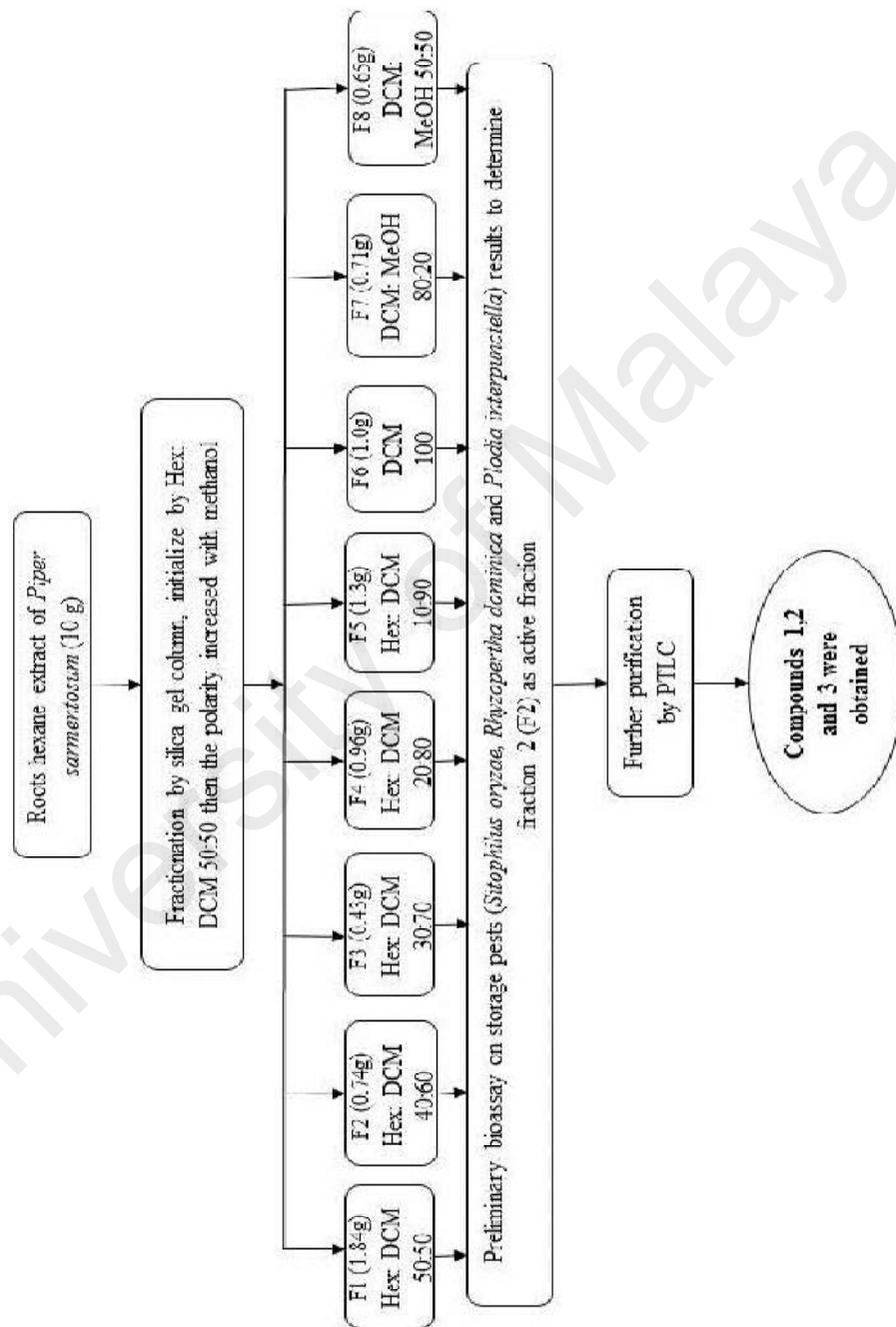
extracts (AME). All were subjected to column chromatography for fractionation and further determination of active fraction.

### 3.7.2 Isolation of roots hexane extracts (RHE)

The 10 grams of RHE soluble materials were subjected to a silica gel column (hexane/ DCM/ MeOH, 50:50:0 → 0:100:0 → 0:0:100) and 8 fractions were obtained.

The second fraction eluted (hexane 40: DCM 60 to hexane 30: DCM 70) was potent against the *S. oryzae*, *R. dominica* and *P. interpunctella* (Chapter 4). The second fraction was further purified on PTLC using DCM /hexane (40:60) as mobile phase which led to the isolation of Asaricin (67 mg) **1**, Isoasarone (40 mg) **2** and *Trans*-asarone **3** (180 mg) (Figure 3.2).

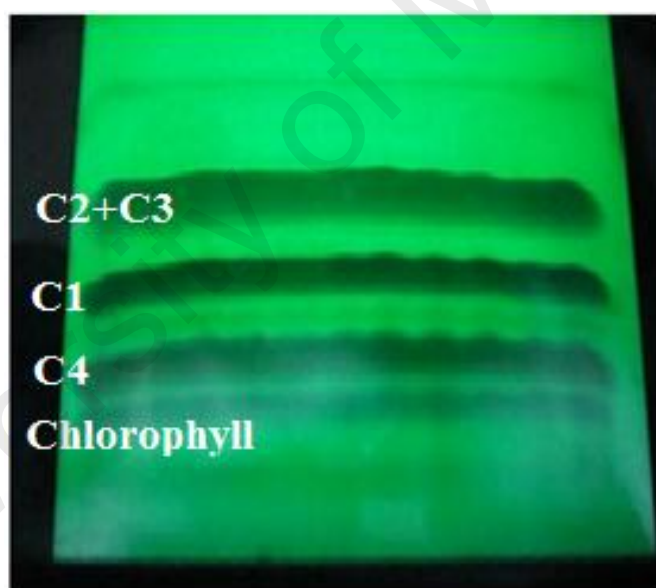
University of Malaya



**Figure 3.2:** General procedures for bioassay guided fractionation and isolation of roots hexane crude extract (RHE).

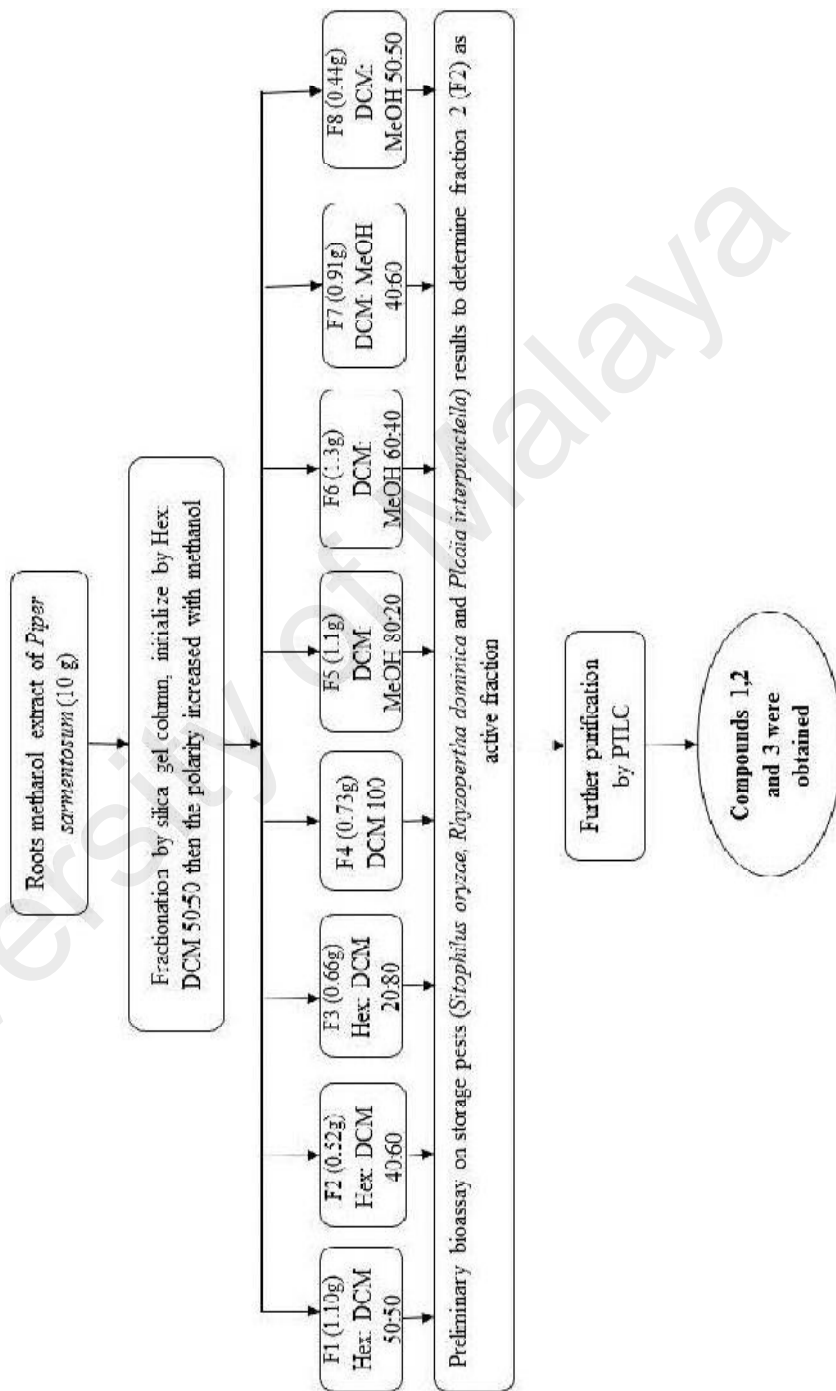
### 3.7.3 Isolation of roots methanol extracts (RME)

Ten grams of RME soluble materials was subjected to column chromatography as described in section 3.5 (III). Dry packing method was used for RME in which material dissolved in and then mixed with silica gel and then dried up using the rotary evaporator. The mixture was loaded to the column (hexane/ DCM/ MeOH, 50:50:0 → 0:100:0 → 0:0:100) 8 fractions obtained (Figure 3.4). The second fraction eluted by hexane/DCM (40:60 to 20:80) was further purified on a PTLC with hexane/DCM (60:40). From this fraction, compounds **1** (22 mg), **2** (67 mg) and **3** (280 mg) isolated (Figure 3.3).



**Figure 3.3:** Isolation of the compounds 1) Asaricin, 2) Isoasarone, 3) *Trans*-asarone and 4) asaraldehyde from roots methanol extracts on PTLC.





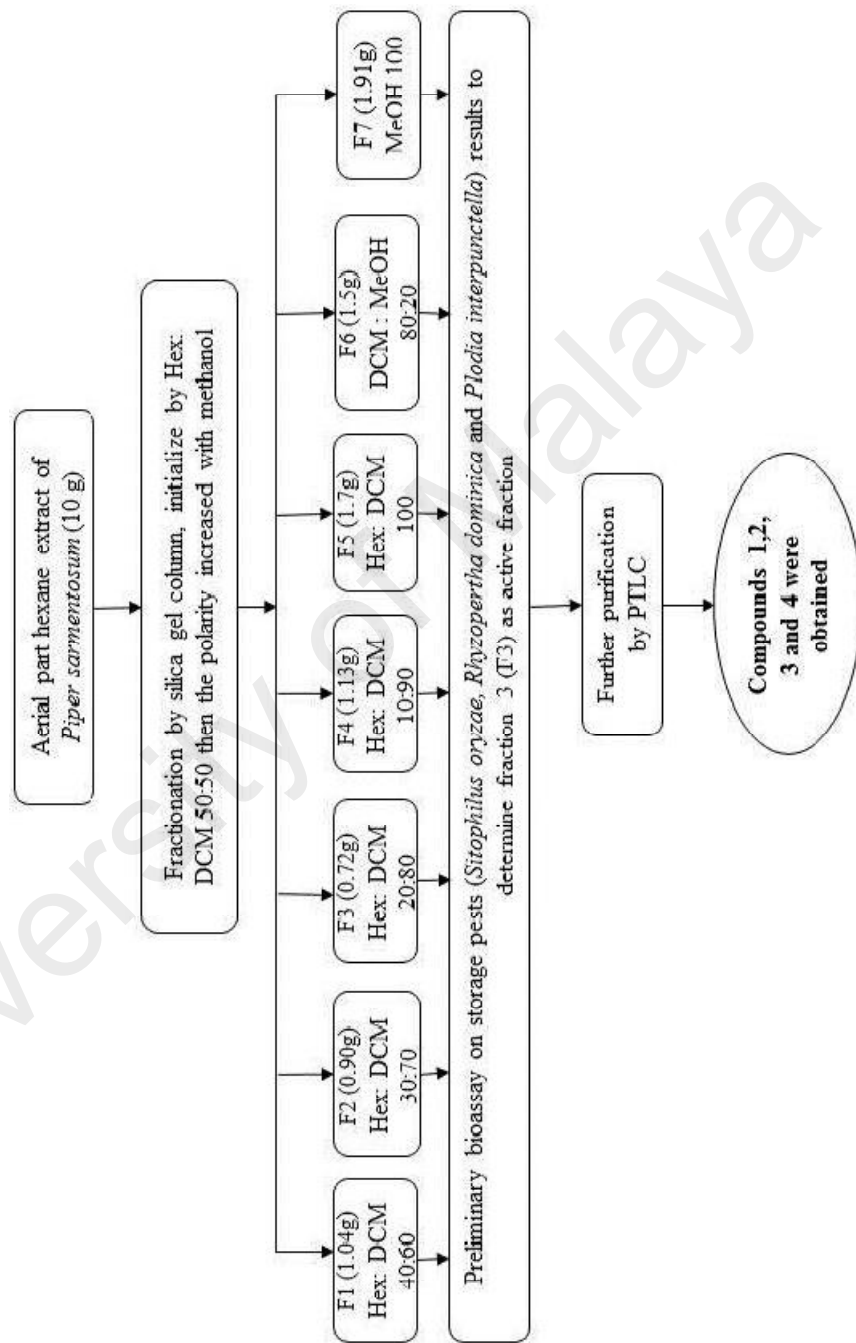
**Figure 3.4:** General procedures for bioassay guided fractionation and isolation of roots methanol crude extracts (RME).

### 3.7.4 Isolation of aerial part hexane extracts (AHE)

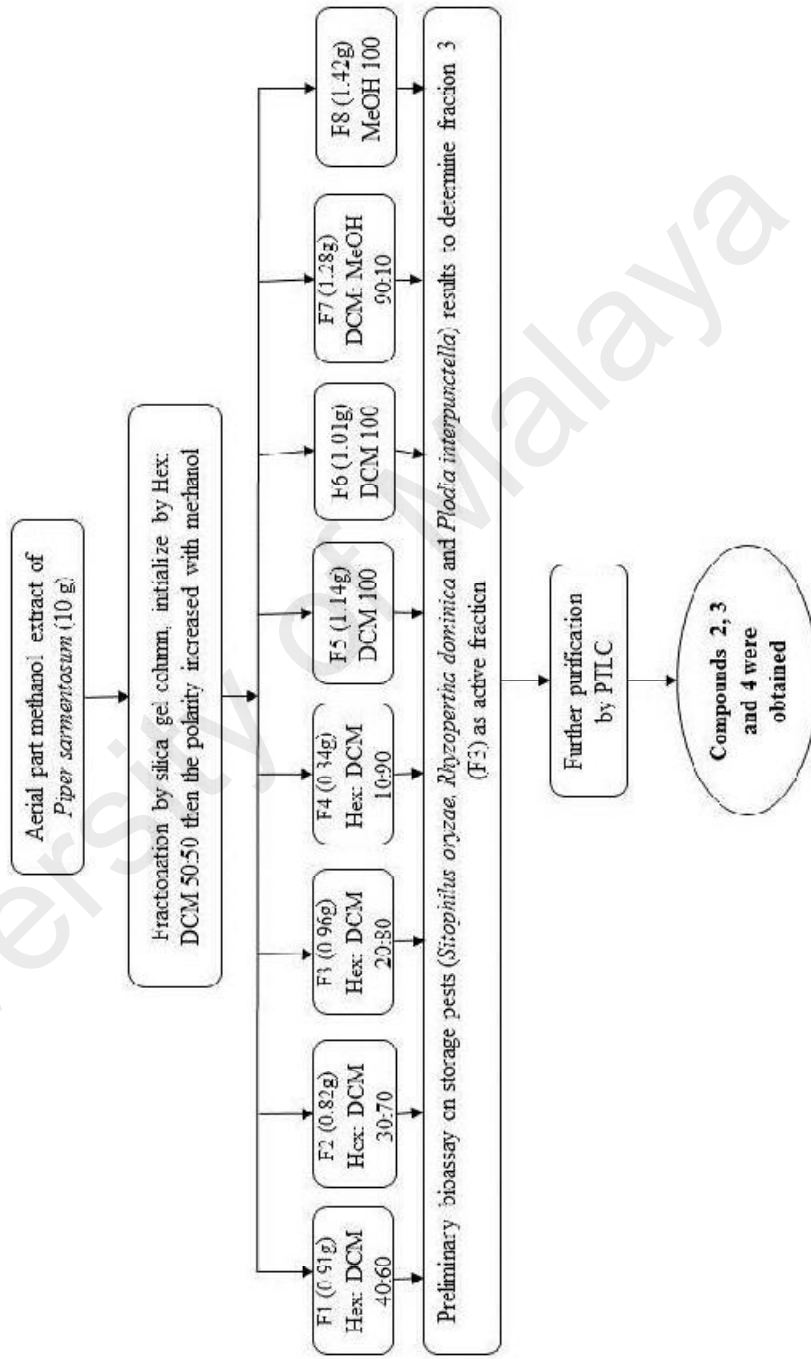
Ten grams of AHE was subjected to a silica gel column using hexane/ DCM/ MeOH, (40:60:0 → 0:100:0 → 0:0:100) as mobile phase. The flow of separation in the aerial part hexane extracts was similar to RHE (as described in section 3.6.2). However, amount of chlorophyll and pigments in aerial part extracts was more. A total of seven fraction were obtained and submitted for preliminary test (Chapter 4). In the fractionation of aerial part hexane extracts, only the third fraction [hexane/DCM (20:80)] was active. This fraction was further purified on PTLC using hexane/DCM (40:60) to give asaricin (34 mg) **1**, isoasarone (55 mg) **2**, *Trans*-asarone (243 mg) **3** and asaraldehyde (15 mg) **4** (Figure 3.5).

### 3.7.5 Isolation of aerial part methanol extracts (AME)

Dry packing technique was used for packing the column. Eight fractions were obtained from the fractionation of methanol extracts. After preliminary tests and confirm the insecticidal activity of the third fraction it was further purified with PTLC using hexane/ DCM (40:60) to separate compounds **2**) isoasarone (49 mg), **3**) *Trans*-asarone and **4**) asaraldehyde (31 mg) (Figure 3.6).



**Figure 3.5:** General procedures for bioassay guided fractionation and isolation of aerial parts hexane crude extract (AHE)

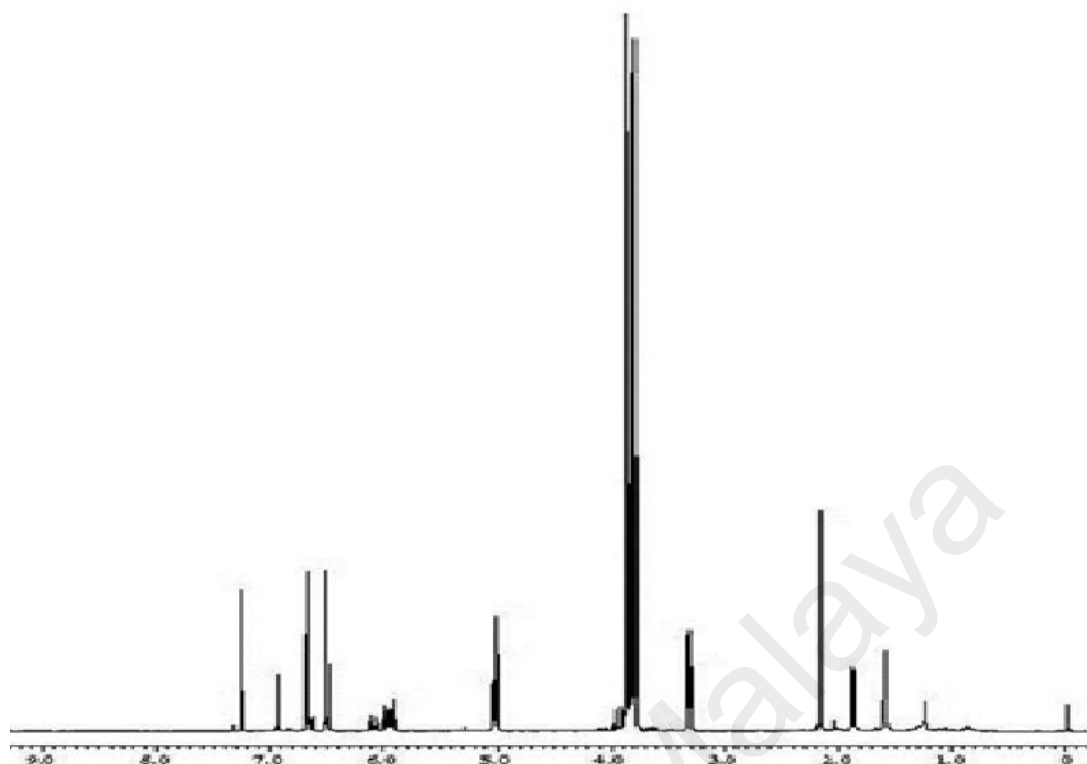


**Figure 3.6:** General procedures for bioassay guided fractionation and isolation of aerial parts methanol crude extract (AME)

### 3.8 Final isolation and purification of compounds 1, 2, 3 and 4

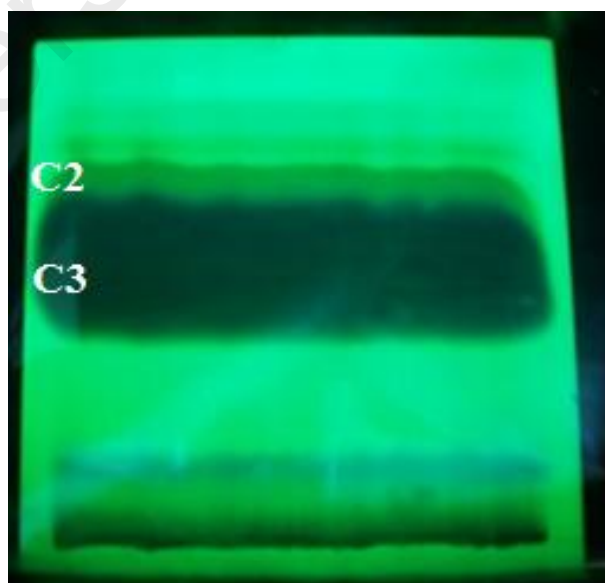
A total of four compounds, designated as compound 1) asaricin, 2) isoasarone, 3) *Trans*-asarone and 4) asaraldehyde were isolated from the active fractions. At this point, compounds 2 and 3 were combined and only after analysis of NMR proton, it was revealed that the spectrum was showing the mixture. Two techniques were used to separate the compounds 2 and 3. The first technique was using HPLC isocratic system which was developed with the initial conditions as methanol/ ionised water (70:30) on a C-18 reverse phase column. The UV max plot and the total ion chromatogram of the pure compound, compound 2 had retention time of 41.92 minute and compound 3 had retention time of 42 which had a UV maximum absorption at 214 nm and 286 nm.

The HPLC methods was very efficient but problem with running time and amount the compounds that could isolated by each run. Since the running each bioassay there was several mg of each compounds was needed therefor HPLC was not the best choice for separation. To overcome this matter using PTLC for separation was the only last option. As it can view in Figure 3.3 compounds 2 and 3 was appeared as uniform line which gave the idea that its one major. For separation of these two compounds hexane was used as the based non polar mobile phase and acetone was added at very low dosage to slowly increase the polarity to see the possibility of separation of these two compounds.



**Figure 3.7:**  $^1\text{H}$ -NMR result of mixture of compounds **2** and **3**.

The second technique was developed using PTLC and hexane and acetone (1.99:0.01) as mobile phase. As it can be viewed in the Figure 3.18, compounds **2** and **3** were separated.



**Figure 3.8:** Separation of compound **2** and **3** using PTLC under UV light.

### 3.8.1 Phenylpropanoids from the aerial part of *Piper sarmentosum*

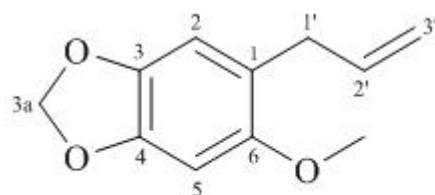
The active extracts of *P. sarmentosum* aerial part (Hexane and Methanol) were subjected to column chromatography followed by HPLC to yield 4. All the isolated compounds from the active fractions were belonged to phenylpropanoids family. The isolated phenylpropanoids molecular structure has aromatic phenyl group with six-carbon, aromatic phenyl group and three carbon propene tail of cinnamic acid. This unique structure makes aromatic phenyl hydrophilic and tail hydrophobic.

The four isolated phenylpropanoids were known Phenylpropanoids; asaricin (**1**), isoasarone (**2**), *trans*-asarone (**3**) and asaraldehyde (**4**). The yield percentage of the isolated Phenylpropanoids is shown in Table 3.1.

**Table 3.1:** Chemical constituents isolated compounds from active fractions of *Piper sarmentosum* (aerial parts and roots).

Compounds	RME Yield%	RHE Yield%	AME Yield%	AHE Yield%
Asaricin	0.22	0.67	NA	0.34
Isoasarone	0.67	0.40	0.49	0.55
<i>Trans</i> -asarone	2.8	1.8	3.21	2.43
Asaraldehyde	NA	NA	0.31	0.15

### 3.8.2 Asaricin (Compound 1)



Asaricin 1

Figure 3.9: Compound 1.

Asaricin 1 was isolated as an optically inactive yellow oil. The LCMS-IT-TOF mass spectrum revealed a pseudomolecular ion peak  $[M+H]^+$  at  $m/z$  192.21118. The molecular mass was supported by the  $^{13}\text{C}$  NMR spectrum (Table 3.2) which confirmed the presence of only 11 carbon signals. The IR spectrum exhibited characteristic absorption peaks of aromatic ( $2925\text{ cm}^{-1}$ ) and methylenedioxy ( $1040$  and  $933\text{ cm}^{-1}$ ) stretching vibration (Banerji, Sarkar, Datta & Sengupta, 2002).

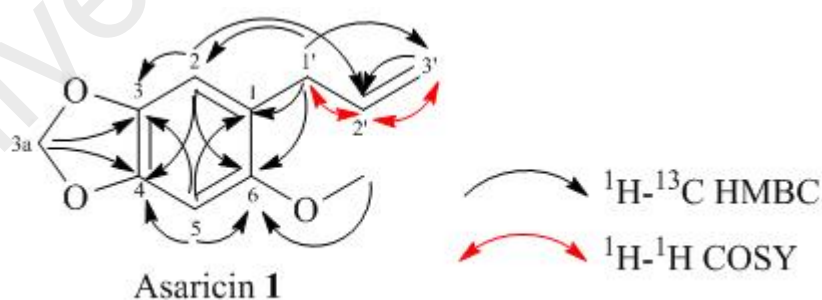


Figure 3.10: HMBC and COSY correlation of asaricin 1.

In the  $^1\text{H}$ -NMR spectrum (Table 3.2), the presence of two aromatic protons at  $\delta_{\text{H}}$  6.64 (1H, *s*, H-2) and  $\delta_{\text{H}}$  6.51 (1H, *s*, H-5) which appeared as singlets, suggested that the protons are at meta and para position. Two sets of multiplets were observed at  $\delta_{\text{H}}$  5.87-



5.97 (1H, *m*) and  $\delta_{\text{H}}$  4.99-5.06 (2H, *m*) corresponding to vinyl protons, H-2' and H<sub>2</sub>-3' respectively. The presence of an up field doublet at  $\delta_{\text{H}}$  3.28 (2H, *d*,  $J = 6.8$  Hz, H<sub>2</sub>-1') which correlated with the carbon signal at  $\delta_{\text{C}}$  34.0 (C-1') in the HSQC spectrum coupled with the vinyl proton of H-2' indicated the existence of an allyl group. In addition, a downfield singlet was observed at  $\delta_{\text{H}}$  5.88 (2H, *s*, H<sub>2</sub>-3a) thus suggesting the presence of a methylenedioxy group. A singlet representing the methoxyl protons attached to C-6 appeared at  $\delta_{\text{H}}$  3.75 (1H, *s*).

The <sup>13</sup>C-NMR spectrum of asaricin **1** showed a total of eleven carbon signals; one methyl, three methines, three methylenes and four quaternary carbons. The methoxyl carbon resonated at  $\delta_{\text{C}}$  56.6 and the HMBC spectrum showed correlation of the methoxyl protons (OMe) with C-6 ( $\delta_{\text{C}}$  152.2), therefore indicated the connectivity of methoxyl group with the aromatic carbon, C-6 (Figure 3.10). Besides, the HMBC correlation of H<sub>2</sub>-3a with C-3 ( $\delta_{\text{C}}$  146.4) and C-4 ( $\delta_{\text{C}}$  101.1) inferred that the methylene group is attached to the quaternary carbons C-3 and C-4 of the benzene ring. The methylene protons of the allyl group was connected to the benzene ring at C-1 which can be deduced from the HMBC correlation between H<sub>2</sub>-1' with C-1 (Figure 3.10). The spectroscopic data obtained (Table 3.2) were consistent with those found in the literature (Satariah, 1999), thus confirming the identity of asaricin **1** (Figure 3.9).

**Tables 3.2:**  $^1\text{H}$ -NMR (400 MHz) and  $^{13}\text{C}$ -NMR (100 MHz) spectral data of asaricin **1** in  $\text{CDCl}_3$ .

Position	$^1\text{H}$		$^{13}\text{C}$	
	$\delta_{\text{H}}$ (multiplicity, $J$ in Hz)		$\delta_{\text{C}}$	
	Experimental ( $\text{CDCl}_3$ )	Reference [19] ( $\text{CDCl}_3$ )	Experimental ( $\text{CDCl}_3$ )	Reference [19] ( $\text{CDCl}_3$ )
1	-	-	120.8	120.7
2	6.64 ( <i>s</i> )	6.64 ( <i>s</i> )	109.7	109.6
3	-	-	146.4	146.2
3a	5.88 ( <i>s</i> )	5.88 ( <i>s</i> )	101.0	100.9
4	-	-	141.0	140.9
5	6.51 ( <i>s</i> )	6.52 ( <i>s</i> )	95.0	94.8
6	-	-	152.2	152.0
1'	3.28 ( <i>d</i> , 6.8)	3.28 ( <i>d</i> , 6.6)	34.0	33.9
2'	5.87 - 5.97 ( <i>m</i> )	5.89 - 5.97 ( <i>m</i> )	137.3	137.2
3'	4.99 - 5.06 ( <i>m</i> )	4.99 - 5.06 ( <i>m</i> )	115.3	115.2
6-OMe	3.75 ( <i>s</i> )	3.75 ( <i>s</i> )	56.6	56.5

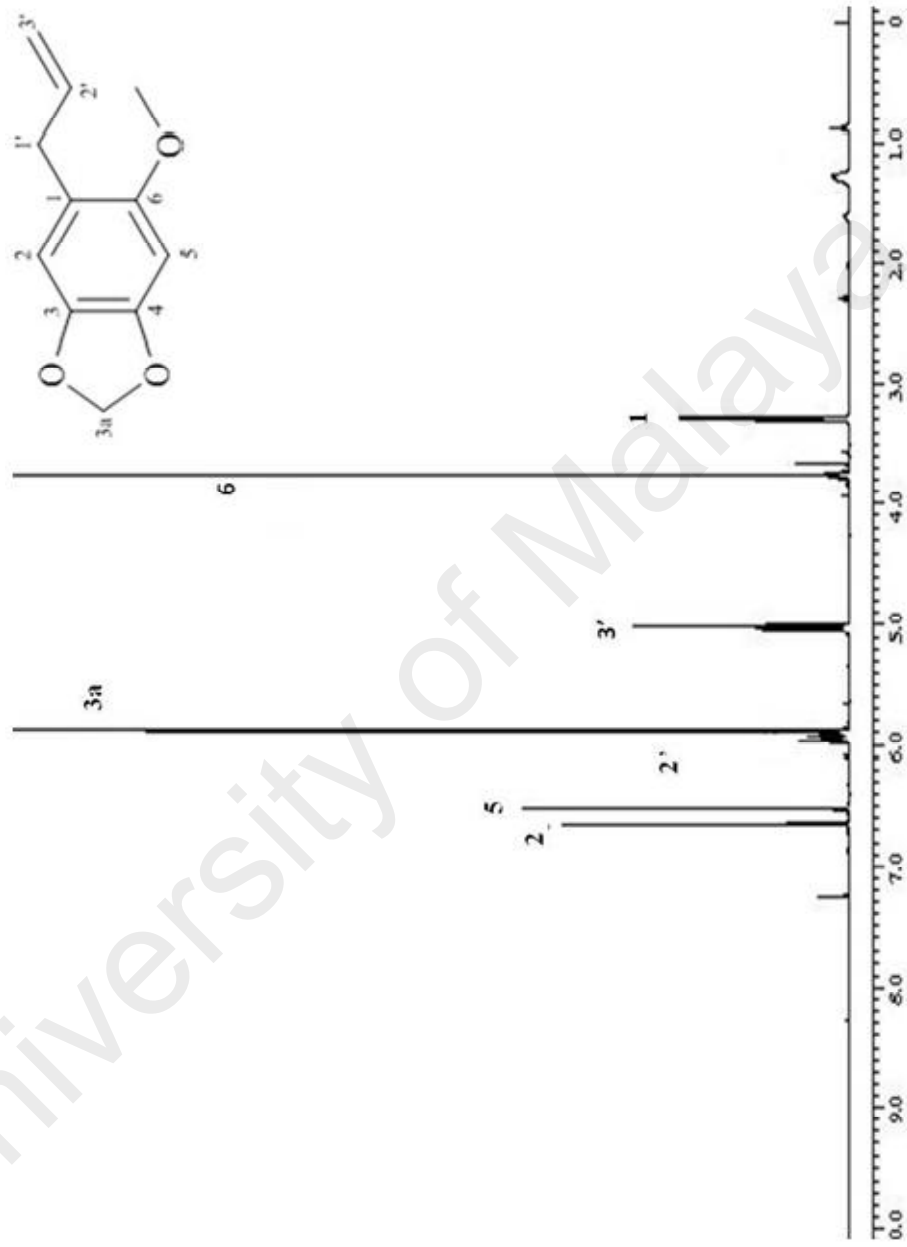


Figure 3.11: Compound 1 <sup>1</sup>H-NMR.

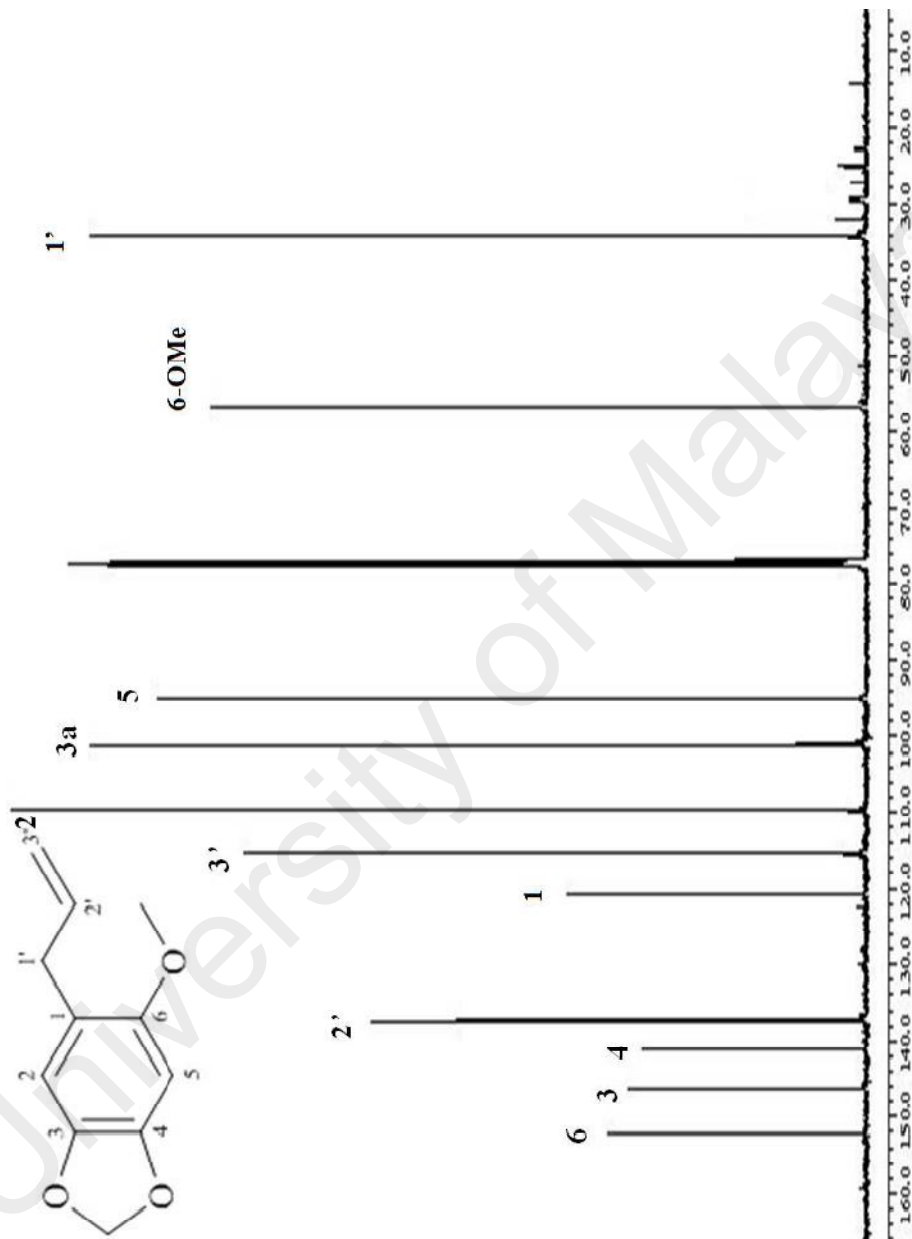
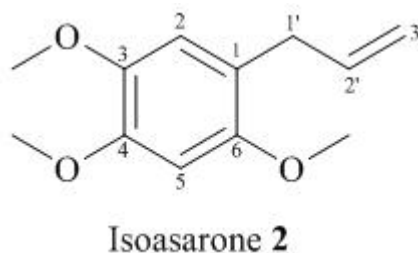


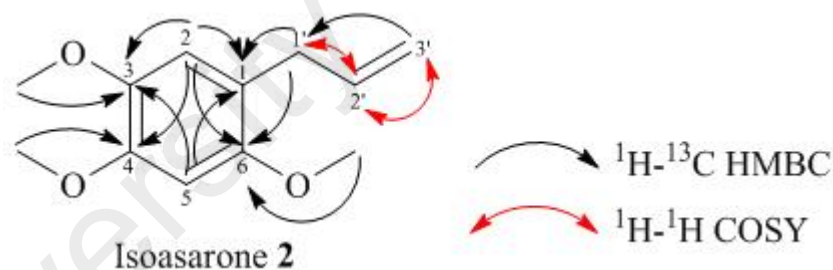
Figure 3.12: Compound 1  $^{13}\text{C-NMR}$ .

### 3.8.3 Isoasarone (Compound 2)



**Figure 3.13:** Compound 2.

Isoasarone **2** was also isolated as an optically inactive yellow oil. Its molecular formula was confirmed as C<sub>12</sub>H<sub>16</sub>O<sub>3</sub> from the LC-MS-IT-TOF which revealed a pseudomolecular ion peak [M+Na]<sup>+</sup> at *m/z* 231.0258. Absorption bands at 1205 and 1038 cm<sup>-1</sup> were observed in IR spectrum which were due to asymmetric and symmetric C-O-C stretching vibration (Atterwill and Neal, 1978).



**Figure 3.14:** HMBC and COSY correlation of isoasarone 2.

In the <sup>1</sup>H-NMR spectrum, the two aromatic protons, a methoxyl protons and an allyl group of isoasarone **2** exhibited the similar pattern with that of asaricin **1** except the absence of methylenedioxy group in **2**. Instead two more singlets were observed at δ<sub>H</sub> 3.84 and 3.89 corresponding to methoxyl protons which attached to C-3 and C-4 respectively as compared to **1**.

The <sup>13</sup>C-NMR spectrum of **2** showed a total of twelve carbon signals; three methyl, three methines, two methylenes and four quaternary carbons. The three

methoxyl protons that correlated with C-3 ( $\delta_C$  56.8), C-4 ( $\delta_C$  56.4) and C-6 ( $\delta_C$  56.8) respectively were confirmed in HMBC spectrum (Figure 3.14).

The analysis of spectroscopic data obtained (Table 3.3) and compared with the reported literature values (Santos and Chaves, 1998), which confirmed the structure of isoasarone **2** (Figure 3.13).

**Table 3.3:**  $^1\text{H}$ -NMR (400 MHz) and  $^{13}\text{C}$ -NMR (100 MHz) spectral data of isoasarone **2** in  $\text{CDCl}_3$ .

Position	$^1\text{H}$		$^{13}\text{C}$	
	$\delta_{\text{H}}$ (multiplicity, $J$ in Hz)		$\delta_{\text{C}}$	
	Experimental ( $\text{CDCl}_3$ )	Reference [20] ( $\text{CDCl}_3$ )	Experimental ( $\text{CDCl}_3$ )	Reference [20] ( $\text{CDCl}_3$ )
1	-	-	120.2	120.3
2	6.70 ( <i>s</i> )	6.70 ( <i>s</i> )	114.1	114.3
3	-	-	143.2	143.3
4	-	-	148.1	148.2
5	6.54 ( <i>s</i> )	6.54 ( <i>s</i> )	98.2	98.4
6	-	-	151.5	151.6
1'	3.30 ( <i>d</i> , 6.4)	3.30 ( <i>dm</i> , 6.4)	33.8	33.8
2'	5.92 - 6.02 ( <i>m</i> )	5.97 ( <i>m</i> )	137.5	137.5
3'	5.02 - 5.07 ( <i>m</i> )	5.04 ( <i>m</i> )	115.4	115.4
3-OMe	3.84 ( <i>s</i> )	3.83 ( <i>s</i> )	56.8	56.8
4-OMe	3.89 ( <i>s</i> )	3.88 ( <i>s</i> )	56.4	56.5
6-OMe	3.81 ( <i>s</i> )	3.81 ( <i>s</i> )	56.8	56.8

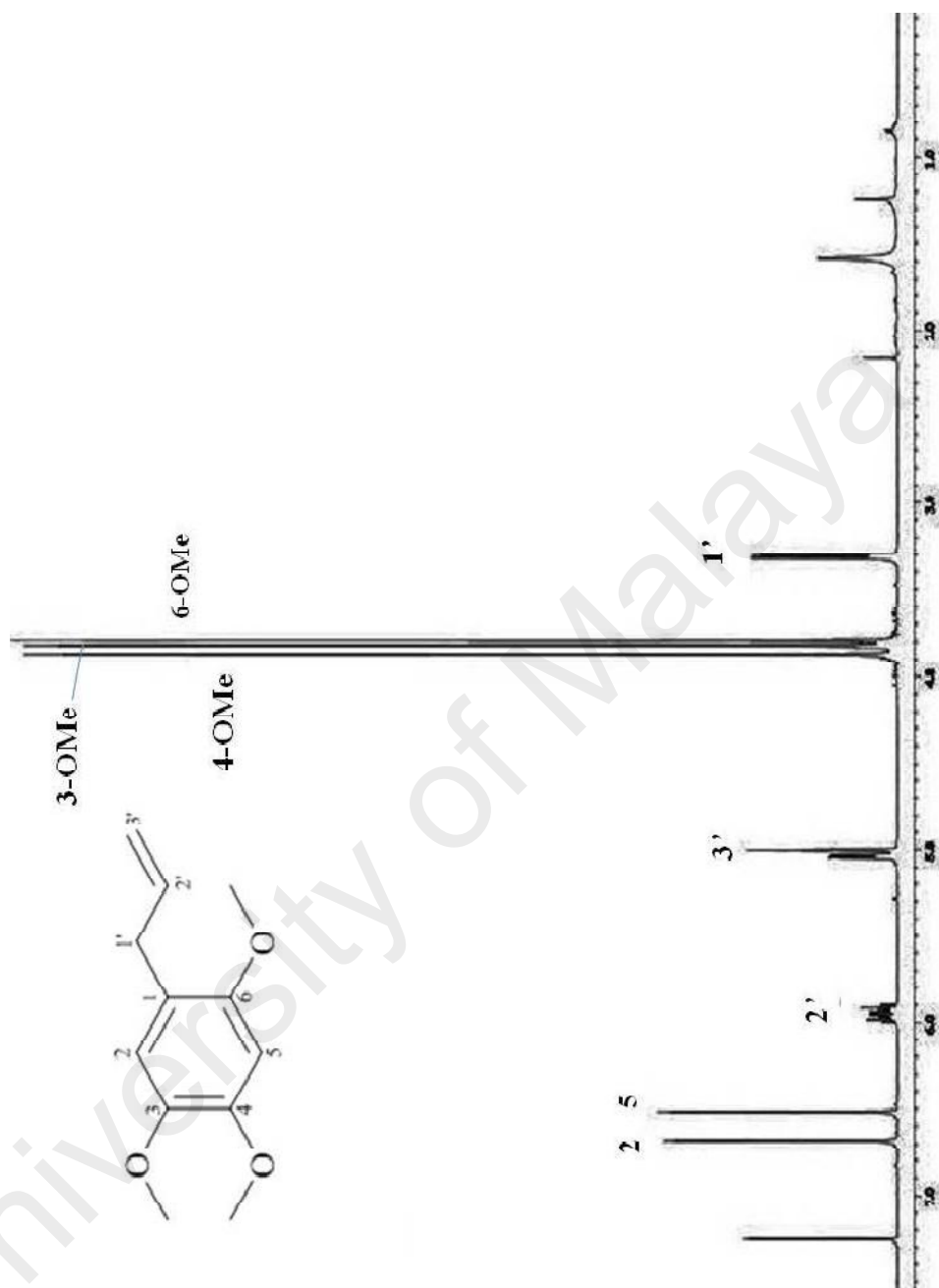


Figure 3.15: Compound 2  $^1\text{H-NMR}$ .

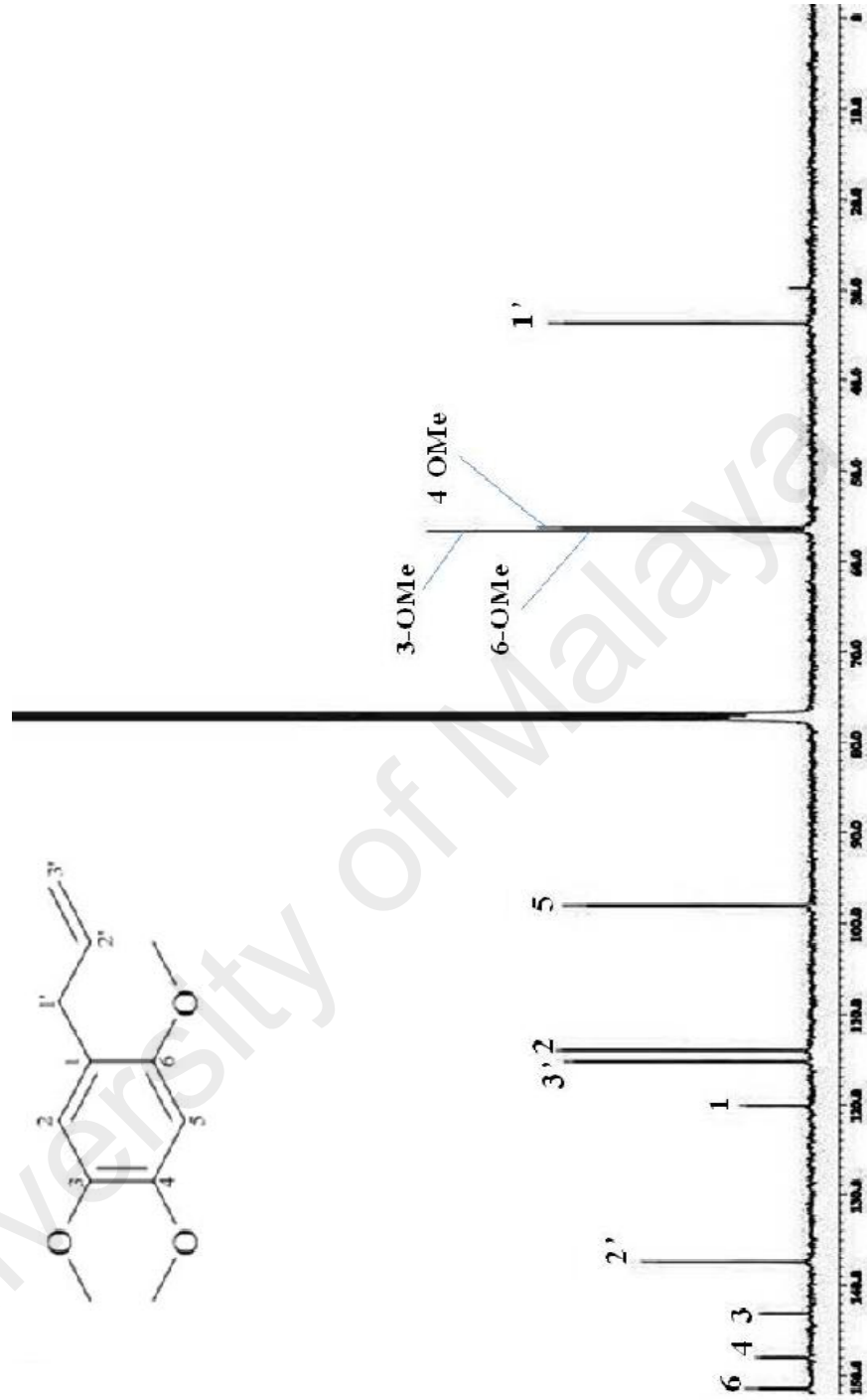
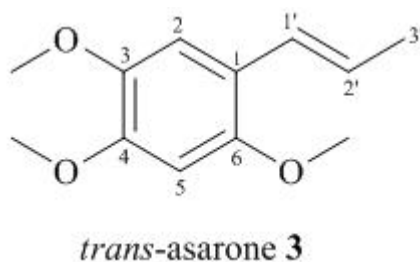


Figure 3.16: Compound 2  $^{13}\text{C}$ -NMR.

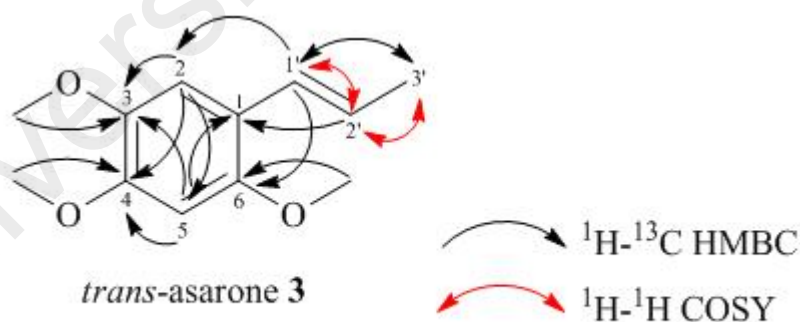


### 3.8.4 *Trans*-asarone (Compound 3)



**Figure 3.17:** Compound **3**.

*Trans*-asarone **3** was also isolated as an optically inactive yellow oil. The LCMS-IT-TOF mass spectrum displayed a pseudomolecular ion peak  $[M+H]^+$  at  $m/z$  208.2625 which was in agreeable to the molecular formula of  $C_{12}H_{16}O_3$ . The IR spectrum of *trans*-asarone **3** showed absorption peaks at 1202, 1033 and  $970\text{ cm}^{-1}$  which indicated asymmetric and symmetric C-O-C and *trans*-double bond stretching, respectively (Atterwill & Neal, 1978; Patra & Mitra, 1981).



**Figure 3.18:** HMBC and COSY correlation of *trans*-asarone **3**.

The  $^1\text{H}$  and  $^{13}\text{C}$ -NMR spectra of *trans*-asarone **3** showed very similar profile with that of isoasarone **2**. However, in  $^1\text{H}$ -NMR spectrum, a slight difference were observed in the signals of H-1', H-2' and H<sub>3</sub>-3'. In **2**, one may observe the presence of

three olefinic protons as compared to only two olefinic protons in **3**. In addition, the  $^{13}\text{C}$ -NMR spectrum of **2** showed the presence of one  $\text{sp}^2$  methine and one  $\text{sp}^2$  methylene whereas in **3**, two  $\text{sp}^2$  methine were observed ( $\delta_{\text{H}}$  6.11, 1H, *dq*,  $J = 16.0, 6.9$  Hz, H-2' and  $\delta_{\text{H}}$  1.89, 3H, *dd*,  $J = 6.9, 1.8$  Hz, H<sub>3</sub>-3') (Figure 3.19). These suggest that the terminal olefinic in **2** is replaced with a 1'-propenyl group where the double bond is in the middle of the chain.

The  $^{13}\text{C}$ -NMR and DEPT spectra of **3** showed a total of twelve carbon signals; four methyl, four methines and four quaternary carbons (Figure 3.20). The COSY correlation between H<sub>3</sub>-3' and H-2' indicated the methyl group is attached to the *trans*-double bond of H-2' (Figure 3.19). Complete  $^1\text{H}$  and  $^{13}\text{C}$ -NMR assignments (Table 3.4) were established by thorough analysis of COSY, HMBC and HSQC data (Figure 3.18). From the analysis of the spectroscopic data obtained and comparison with the literature values (Patra & Mitra, 1981), the identity of *trans*-asarone **3** was ensured.

**Table 3.4:**  $^1\text{H}$ -NMR (400 MHz) and  $^{13}\text{C}$ -NMR (100 MHz) spectral data of *trans*-asarone **3** in  $\text{CDCl}_3$ .

Position	$^1\text{H}$		$^{13}\text{C}$	
	$\delta_{\text{H}}$ (multiplicity, $J$ in Hz)		$\delta_{\text{C}}$	
	Experimental ( $\text{CDCl}_3$ )	Reference [21] ( $\text{CDCl}_3$ )	Experimental ( $\text{CDCl}_3$ )	Reference [21] ( $\text{CDCl}_3$ )
1	-	-	119.2	118.3
2	6.95 (s)	6.91 (s)	109.9	109.2
3	-	-	143.5	142.6
4	-	-	148.9	148.0
5	6.50 (s)	6.45 (s)	98.1	97.3
6			150.8	149.9
1'	6.66 ( <i>dq</i> , 16.0, 1.8)	6.64 ( <i>dq</i> , 16.0, 1.5)	125.2	124.4
2'	6.11 ( <i>dq</i> , 16.0, 6.9)	6.02 ( <i>dq</i> , 16.0, 6.2)	124.6	123.4
3'	1.89 ( <i>dd</i> , 6.9, 1.8)	1.87 ( <i>dd</i> , 6.2, 1.5)	19.0	18.7
3-OMe	3.86 (s)	3.81 (s)	56.9	56.1
4-OMe	3.89 (s)	3.84 (s)	56.3	55.1
6-OMe	3.83 (s)	3.77 (s)	56.7	55.7

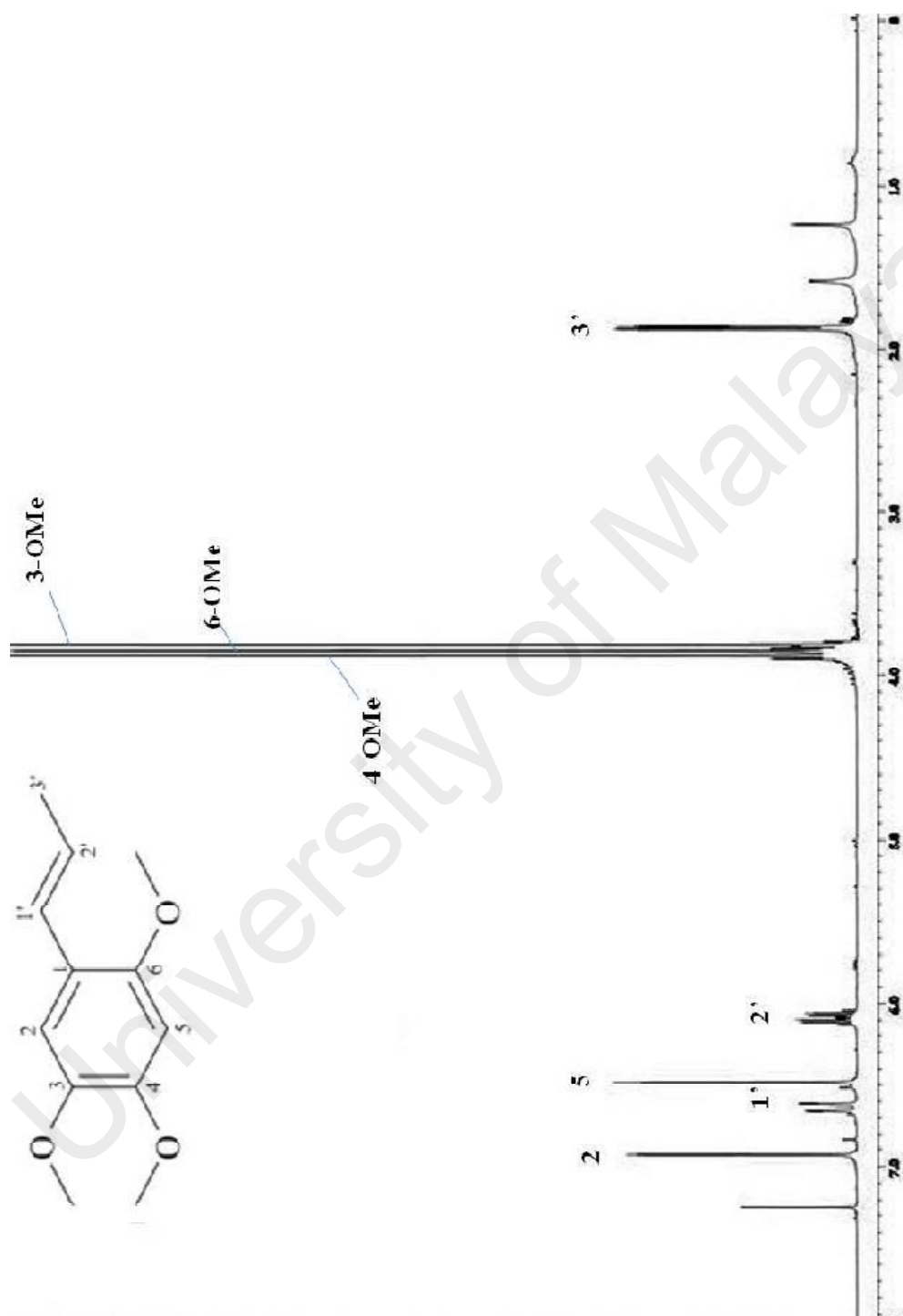


Figure 3.19: Compound 3  $^1\text{H-NMR}$ .

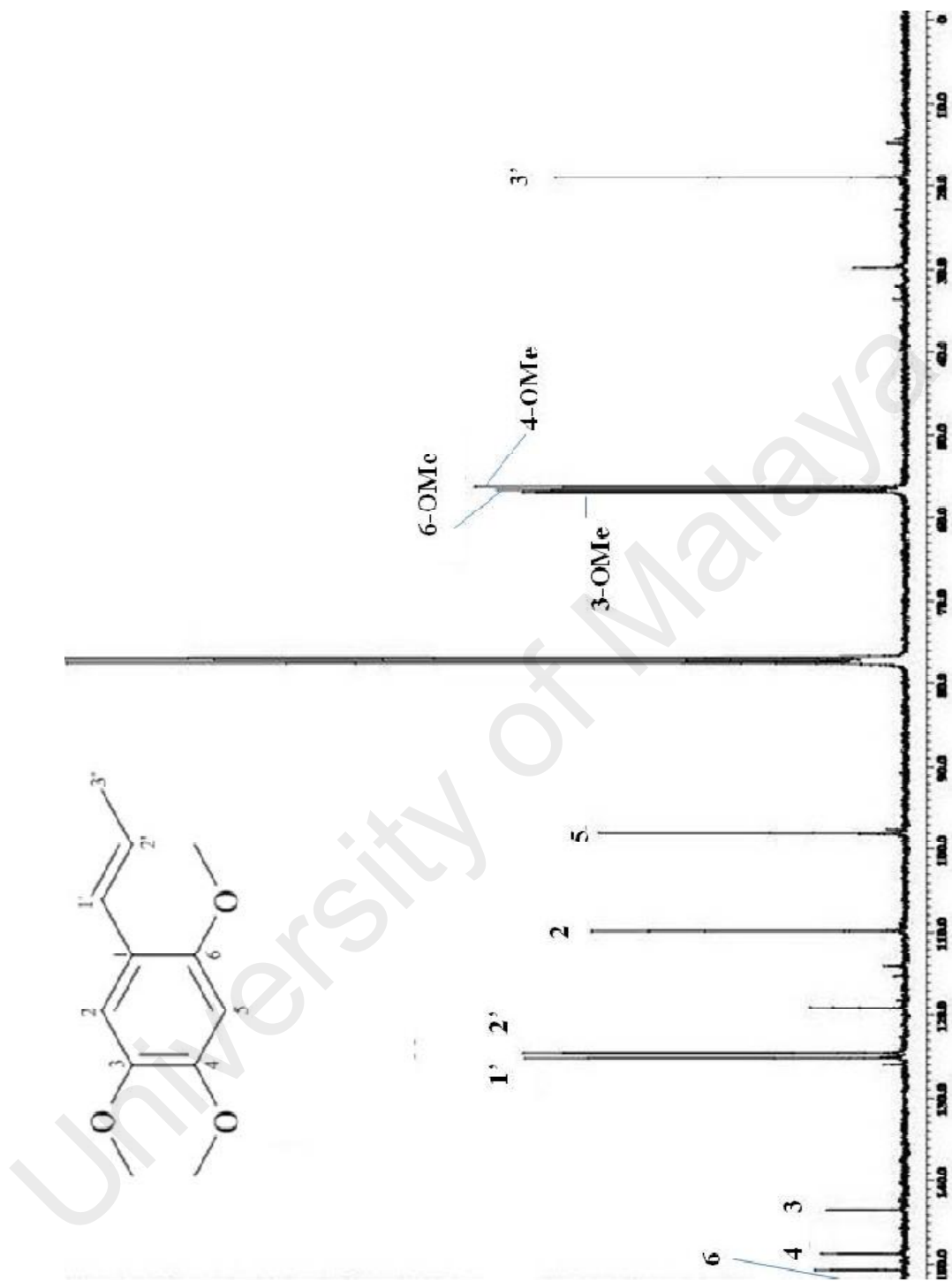
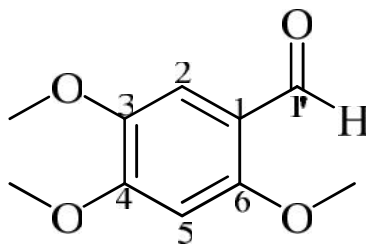


Figure 3.20: Compound 3  $^{13}\text{C-NMR}$ .

### 3.8.5 Asaraldehyde (Compound 4)



Asaraldehyde

**Figure 3.21:** Compound 4.

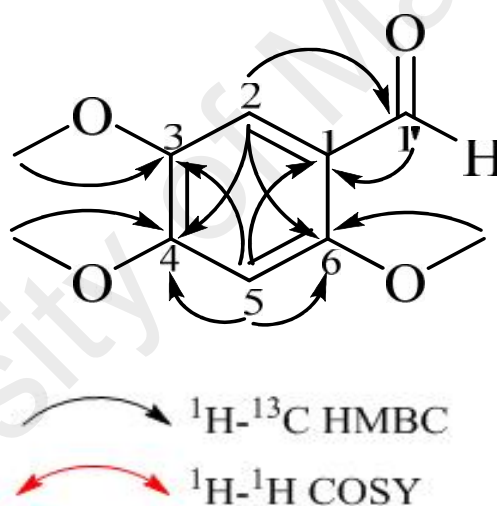
Asaraldehyde **4** was also isolated as an optically inactive yellow oil. Its molecular formula of  $C_{10}H_{12}O_4$  was determined through LC-MS-IT-TOF spectrum which showed a pseudomolecular ion peak  $[M+H]^+$  at  $m/z$  196; calcd. for  $C_{10}H_{12}O_4$  196).

The  $^1H$  and  $^{13}C$ -NMR spectra of asaraldehyde **4** and isoasarone **2** showed a significant resemblance except a singlet proton signal was observed at very downfield region of  $\delta_H$  10.33 (1H, *s*, H-1') (Figure 3.23) indicated the presence of a carbonyl proton instead of two sets of multiplets and one doublet which referred to allyl group in that of isoasarone **2** (Table 3.5). Three methoxyl groups were also observed in  $^1H$ -NMR of **4** which resonated at  $\delta_H$  3.89,  $\delta_H$  3.94 and  $\delta_H$  3.99.

The  $^{13}C$ -NMR and DEPT spectra of **4** showed a total of ten carbon signals; three methyl, three methines and four quaternary carbons. The HMBC correlation (Figure 3.24) of H-1' with C-1 ( $\delta_C$  117.4) indicated the carbonyl proton is attached to quaternary C-1 of benzene ring forming benzaldehyde unit. HMBC spectrum showed

correlation of three methoxyl groups;  $\delta_H$  3.89,  $\delta_H$  3.94 and  $\delta_H$  3.99 with C-3 ( $\delta_C$  143.6), C-4 ( $\delta_C$  158.8) and C-6 ( $\delta_C$  155.8) respectively supported the connectivity of the three methoxyl groups with aromatic carbon C-3, C-4 and C-6 (Figures 3.22 and 3.24) (Figure 3.24).

Comprehensive study of the 1D and 2D-NMR spectra (Table 3.5) and comparison with the literature values led to the conclusion that the investigated compound is indeed asaraldehyde 4.



**Figure 3.22:** HMBC correlation of asaraldehyde 4.

**Table 3.5:**  $^1\text{H}$ -NMR (400 MHz) and  $^{13}\text{C}$ -NMR (100 MHz) Spectral Data of asaraldehyde **4** in  $\text{CDCl}_3$ .

Position	$^1\text{H}$		$^{13}\text{C}$	
	$\delta_{\text{H}}$ (multiplicity, $J$ in Hz)		$\delta_{\text{C}}$	
	Experimental ( $\text{CDCl}_3$ )	Reference [4] ( $\text{CDCl}_3$ )	Experimental ( $\text{CDCl}_3$ )	Reference [4] ( $\text{CDCl}_3$ )
1	-	-	117.4	117.6
2	7.34 ( <i>s</i> )	7.30 ( <i>s</i> )	109.2	109.3
3	-	-	143.6	143.8
4	-	-	158.8	158.9
5	6.50 ( <i>s</i> )	6.48 ( <i>s</i> )	96.1	93.6
6	-	-	155.8	156.0
1'	10.33 ( <i>s</i> )	10.29 ( <i>s</i> )	188.3	188.2
3-OMe	3.89 ( <i>s</i> )	3.88 ( <i>s</i> )	56.4	56.4
4-OMe	3.94 ( <i>s</i> )	3.90 ( <i>s</i> )	56.4	56.4
6-OMe	3.99 ( <i>s</i> )	3.95 ( <i>s</i> )	56.5	56.6



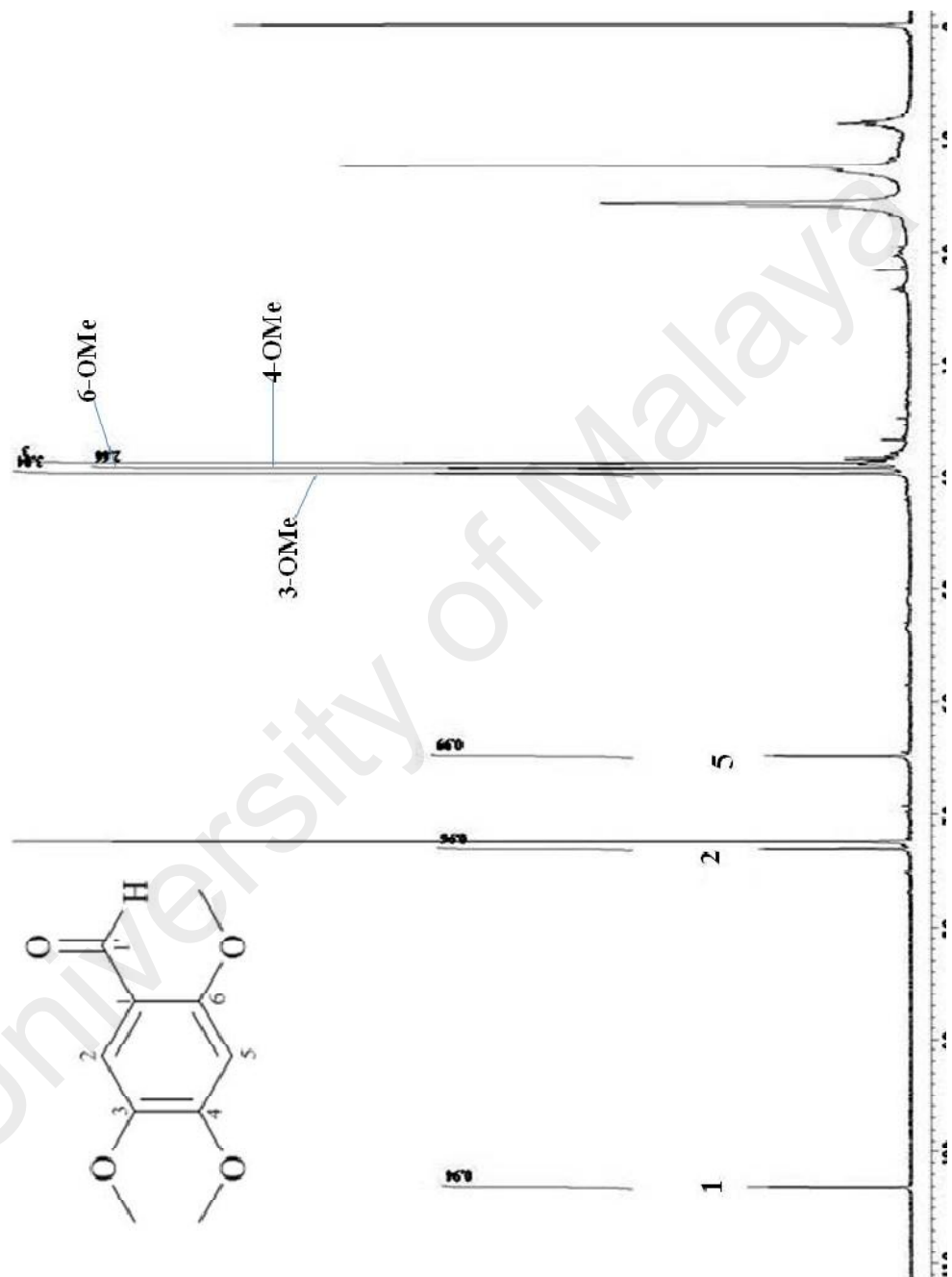


Figure 3.23: Compound 4 <sup>1</sup>H-NMR.

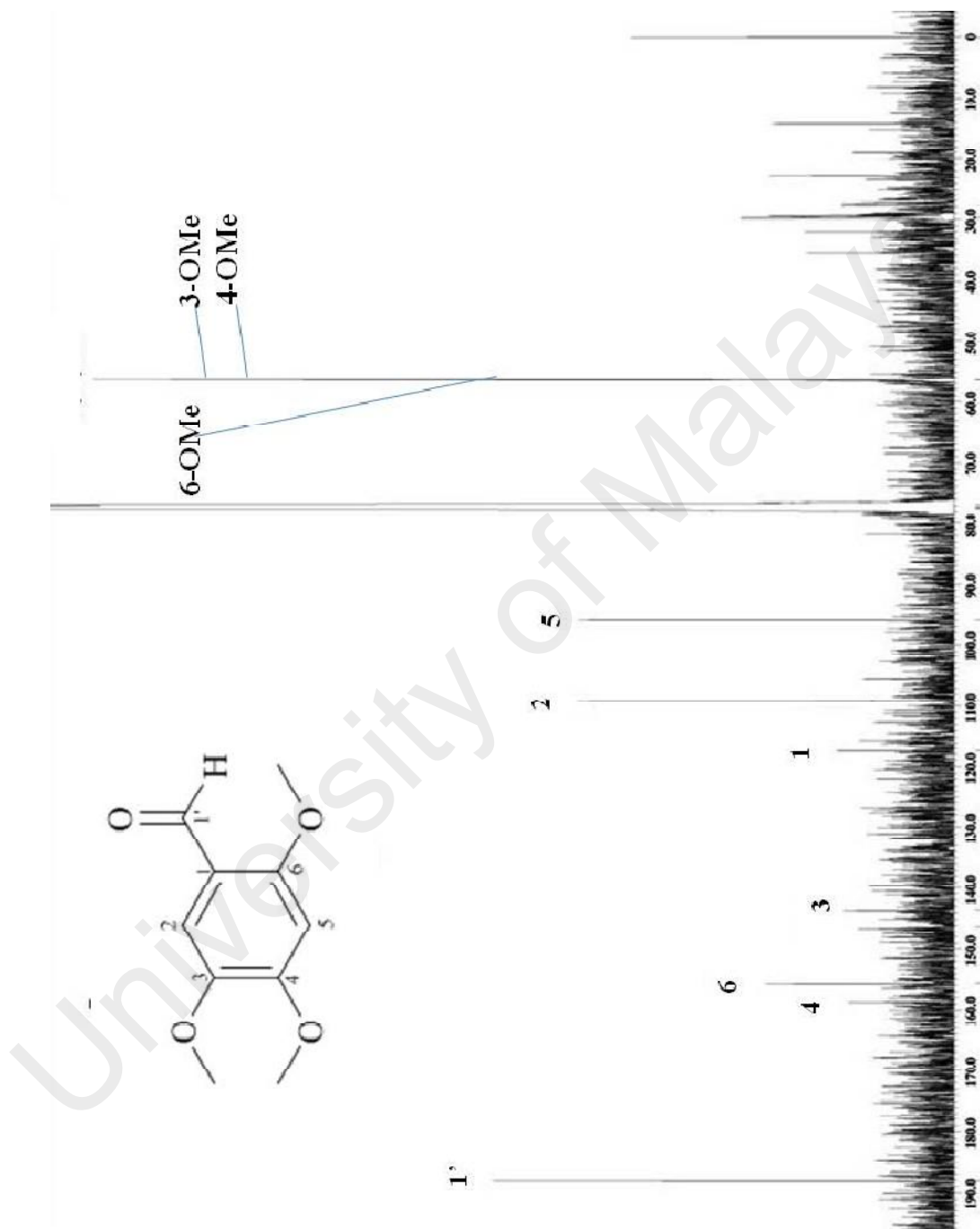


Figure 3.24: Compound 4  $^{13}\text{C-NMR}$ .

### 3.9 DISCUSSION

Bioassay guided fractionation and isolation of the hexane and methanol extracts of *Piper sarmentosum* led to the isolation of four phenylpropanoids, designated as compound 1-4. The structures of the compounds were identified as asaricin (**1**), isoasarone (**2**) and *Trans*-asarone (**3**) upon comparison of their spectroscopic data with those reported in the literature (Bárbara & Maria 2000; Satariah, 1999). Compound 4 did not show any insecticidal activity. Asaricin, isoasarone and 2, *trans*-asarone have also been found earlier in the oils of some species from Piperaceae family, for instance *P. lenticellosum*, *P. aduncum* and *P. guineese* oil in Colombia (Conti, Canale, Bertoli, Gozzini, & Pistelli, 2010). Asaricin has been found most commonly in the oils of plants of the *A(sia)sarum* sp. (syn. *Heterotropa* sp.), Aristolochiaceae, *Illicium* sp., *Cornus officinales* and *Ligusticum pteridophyllum* (Gonzalez-Coloma, Reina, Diaz, Fraga, & Santana-Meridas, 2013).

### 3.10 CONCLUSION

Initial extraction followed by preliminary test (Chapter 4) of the aerial part and roots using different solvents proved that hexane and methanol extracted the compounds with insecticidal activity. The hexane and methanol extracts of the *P. sarmentosum* were purified using a silica gel column and PTLC. HPLC of the active fractions revealed the compounds with insecticidal activity. It can be deduced from the results that the total yield of compounds **1** and **2** from roots extract was more than that of in aerial parts extract. Purification and isolation of compounds with insecticidal activity from the aerial parts and roots of *P. sarmentosum* were successful. The isolated active compounds from both hexane and methanol extracts were similar. The active compounds from the hexane and methanol extracts from aerial part and roots were compounds **1** and **2**. Compound **1** was major compound and compound **2** was minor.

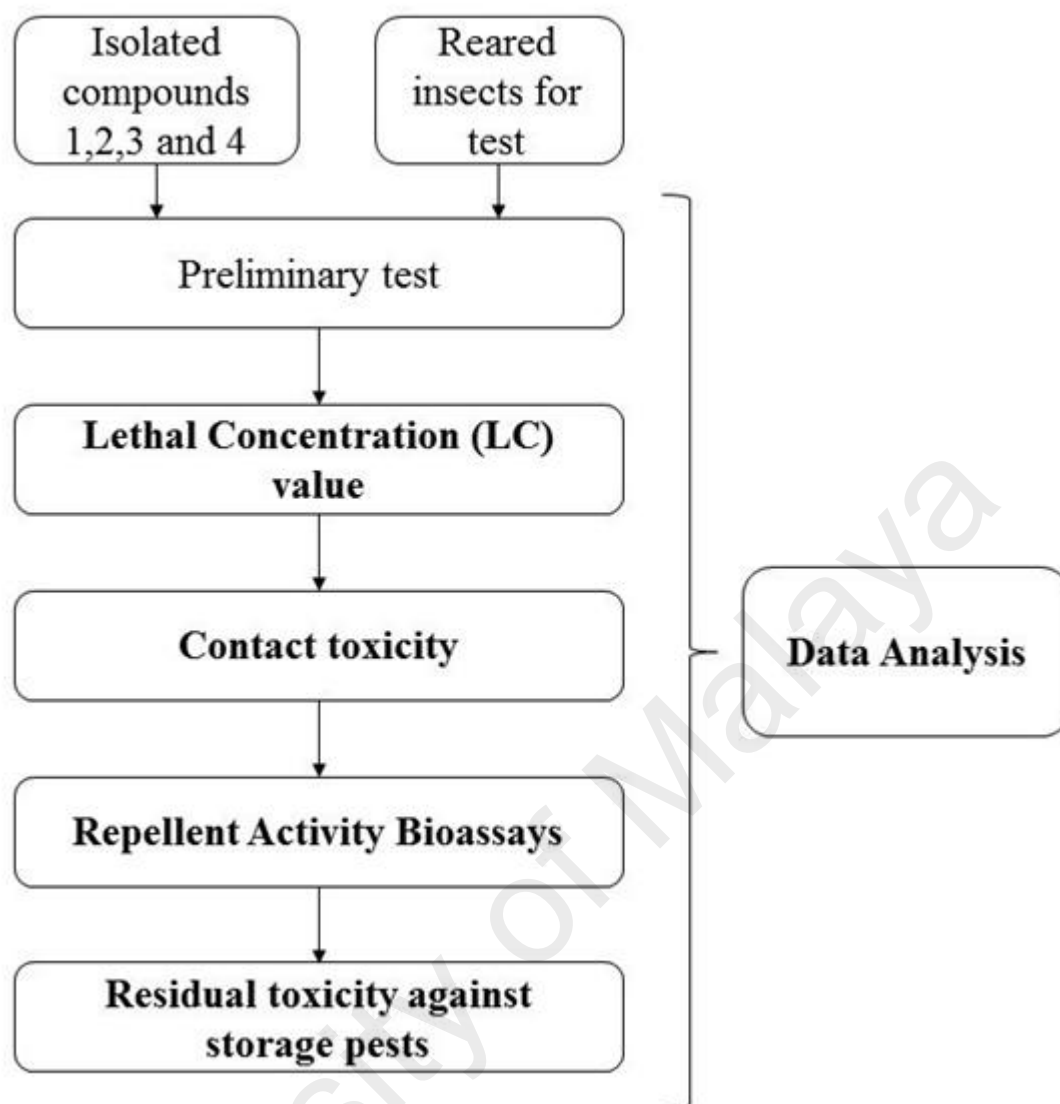
Since *P. sarmentosum* is widely grown in Malaysia, it will be easy and sustainable to use its active component as bioinsecticide.

University of Malaya

**CHAPTER 4: DETERMINATION OF INSECTICIDAL ACTIVITY OF *P. sarmentosum* ISOLATED COMPOUNDS ON THREE MAJOR STORAGE PESTS**

**4.1 INTRODUCTION**

Synthetic insecticides are the most commonly used as control factors in managing stored grain insect pests. Most of the effective insecticides are highly hazardous which may cause injury or death to human beings and other non-targeted organisms. To find the alternative biopesticide for controlling the three major storage pests *Sitophilus oryzae*, *Rhyzopertha dominica* and *Plodia interpunctella* the four isolated phenylpropanoids from the active extracts were tested for their insecticidal activities. Therefore, the objectives of this chapter were; (i) to carry out a bioassay-guided of hexane, dichloromethane and methanol extracts toxicity, (ii) to highlight the active fractions between all fractionation of the active extracts (iii) to evaluate the toxicity of isolated compounds from active fraction. The aim of this study was to provide a clearer understanding of which compounds were responsible for the toxicity of methanol and hexane extracts against *S. oryzae*, *R. dominica* and *P. interpunctella*. For that matter several bioassays were conducted relatively in this chapter to determine how the mode of action of isolated compounds; asaricin **1**, isoasarone **2**, *trans*-asarone **3** and asaraldehyde **4**. Four different bioassay were conducted to evaluate **1**, **2**, **3** and **4** activity; 1) Evaluating the lethal concentration dose (LC), 2) contact toxicity assay, 3) repellent activity bioassays and 4) residual toxicity against storage pests (Figure 4.1).



**Figure 4.1:** General procedures of bioassay and activity tests of compounds 1, 2, 3 and 4 in this chapter.

## 4.2 LITERATURE REVIEW

Stored product insect pests cause gain lost to stored products by reducing the quantity and quality of grain during post-harvest storage. The damage to stored grains and relative grain products by insects could be raised to 30% in tropical countries including Malaysia (Nattudurai et al., 2012; Damsud et al., 2013). *Sitophilus oryzae* (El Maghrbi & Hosni, 2014), *Rhyzopertha dominica* (Kotze et al., 2014) and *Plodia interpunctella* (Hübner) (Insecta: Lepidoptera: Pyralidae) (Ndomo-Moualeu et al., 2014; Mgbeahuruike et al., 2017), in particular, are considered as main pests of stored dry

food. These insects can infest a wide range of stored grains such as rice, wheat and corn. The adults of *S. oryzae* (Figure 4.2) and *R. dominica* are known to attack and consume the intact grains while their larvae feed on the kernel and develop inside of it (Oberlander, Silhacek, Shaaya, & Ishaaya, 1997; Napoleão et al., 2013). *P. interpunctella*, also known as the Indian meal moth larva, during its adult stage does not cause much damage as compared to its larva stage. *P. interpunctella* is an external feeder, larva continuously produces a silk web around the surface and inside of the food and feeds within the web (Bowditch & Madden, 1996, Ziaee, Moharramipour & Francikowski, 2014).



**Figure 4.2:** Raw spaghetti infected with *Sitophilus oryzae*.

Methyl bromide and phosphine are widely used formulated chemical fumigants for disinfestations storage products and will also be restricted in future due to their harmful effects. Methyl bromide has been highly restricted because of its ozone depleting potential, which leads to harmful effects of radiation on the organisms on the Earth. One of the protocols in Montreal has agreed to restrict the use of these fumigants

by 2005 in developed countries and by 2010 in developing countries ( Riahi et al., 2015) Pyrethroid is one of the options for protecting storage grain products in the future. However, stored grain pests will most likely develop resistance to pyrethroids. Unfortunately, there is no suitable alternatives to replace pyrethroids (Vayias, Athanassiou, Milonas, & Mavrotas, 2009). Thus, it was necessary to develop safe alternative for stored grain pest management. It has been suggested that biopesticide from plant origins could have a greater potential in future. Botanical insecticides in general have less toxicity on mammals, environment and have more selective effects on target pests.

Several types of aromatic plants have been investigated for their anti-feedant and insecticidal activity including several plants from *Piperaceae* family (Da Silva, Yunes, De Souza, Monache, & Cechinel-Filho, 2010). *Piper sarmentosum* is widely distributed in Malaysia and often used as food flavouring agents, traditional medicines (Aronson, 2014) and pest control agents (Nair, & Burke, 1990). Investigations on bioactive agents of *Piper* species have led to the isolation of several classes of physiologically active compounds such as alkaloids, amides, pyrones, dihydrochalcones, flavonoids, phenylpropanoids, lignans and neolignans (Parmar et al., 1997). Since in the past studies the investigation were limited to active extracts and essential oil consequently the study were setup to investigate it more in depth (Tankam & Ito, 2013).

## **4.3 MATERIALS AND METHODS**

### **4.3.1 Rearing of insects**

The pure culture of rice weevil, *Sitophilus oryzae* and lesser grain borer, *Rhyzopertha dominica* were obtained from Crop Protection Institute, University Putra Malaysia (Figure 4.3). *Plodia interpunctella* were separated from imported Indian



basmati infested rice obtained in Kuala Lumpur, Malaysia. The insects were reared on the mixture of susceptible maize and rice grains. All grains were sterilized in hot air oven at 60°C for 4 h. This method was modified from Semeao et al., 2013. New grains were used every two weeks. Grains were frozen 7 days prior to eliminate existing insects inside and then changed to normal temperature prior to be fed to insects.



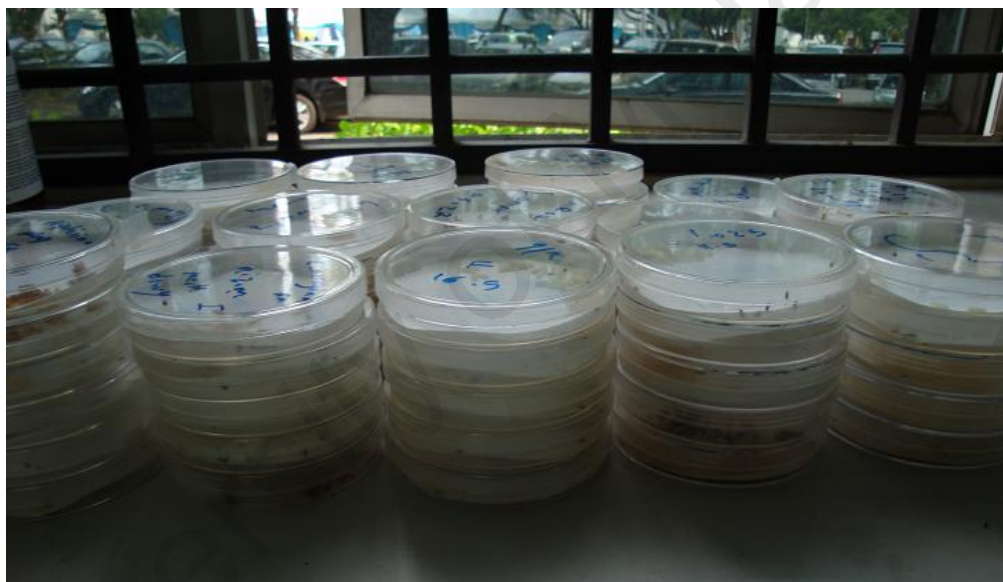
**Figure 4.3:** *Sitophilus oryzae* adults hatched from the infected raw spaghetti.

Approximately 100 adults were released in a (1 liter capacity) plastic containing 250 g of conditioned grains. The adults were removed after two weeks and the grains were kept in laboratory condition at  $27 \pm 2^\circ\text{C}$  and 80% RH for the development of progenies. Adult *P. interpunctella* were fed with 5% honey solution till mating and produce eggs. Two week-old adults of *Sitophilus oryzae* and *Rhyzopertha dominica* and third instar larvae of *P. interpunctella* were used in the experiments.

#### 4.3.2 Insecticidal Effects on Treated Grain

Preliminary bioassay was conducted by dissolving 1 mg of each extract (hexane, dichloromethane and methanol) in 1 ml acetone and introducing them onto 5 g of rice

grain using a pipette. The control received 1 ml of acetone. The treated grains were dried under the fume hood. Twenty unsexed adults of *S. oryzae* and *R. dominica* were separately introduced into the respective petri dishes containing 5 g of treated rice respectively. Ten 3<sup>rd</sup> instar larvae of *P. interpunctella* were carefully collected with a fine brush and placed in each petri dish (10 cm diameter x 1.5 cm) (Figure 4.4). All treatments were monitored to obtain the mean mortality percentage for 96 h. Experiments were replicated four times. The same technique was used to determine bioactivity of each fraction obtained from active extracts against tested insects.



**Figure 4.4:** Bioassay was conducted inside the disposable petri dish.

#### 4.3.3 Lethal Concentration (LC) Value of Compounds 1-4

The compounds isolated from the active fractions (as determined in section 3.6) were evaluated for toxicity towards *S. oryzae*, *R. dominica* and *P. interpunctella*. 5 g of rice grains were treated with different concentrations (w/v) of each compound. Stock solutions (w/v) of asaricin **1**, isoasarone **2**, *trans*-asarone **3** and asaraldehyde **4** were prepared dissolving the desired quantity of each compound in acetone to obtain final concentration. Compounds **1** and **2** concentration for *S. oryzae* and *R. dominica* was 0.5

to 15 µg /ml and compounds **3** and **4** was 50 to 400 µg /ml. on other hand concentration of compounds **1** and **2** for *P. interpunctella* was 5 to 40 µg /ml and compound **3** was 200 to 1000 µg /ml. Controls were treated with acetone. The treated grains were allowed to dry at room temperature (27 °C). The petri dishes were covered and sealed with parafilm. Mortality was recorded after 24, 48 and 72 hours of exposure.

#### **4.3.4 Contact toxicity**

Direct contact application was used to study the contact toxicity of compounds **1-4**. One mg of each compound was dissolved in 1 ml of acetone and used as the stock solution to determine the contact toxicity and the lethal concentration (LC<sub>50</sub>) for further tests (Vassilakos, Athanassiou, Chloridis, & Dripps, 2014). After a serial dilution, dosages between 0.2 mg/ml (200 ppm) to 1mg/ml (1000 ppm) were carefully introduced onto filter papers (Whatman No. 1). Controls received 1 ml of acetone. After drying under a fume hood for 15 min, each filter paper was placed at the bottom of a petri dish. Twenty adults of *S. oryzae* and *R. dominica* and twenty larvae of *P. interpunctella* were introduced into each petri dish respectively. Then, petri dishes were covered and sealed with parafilm.

#### **4.3.5 Repellent Activity Bioassays**

Repellency tests were conducted by optimizing Talukder & Howse (1993, 1994) in two separate experiments. For the first experiment, two disposable petri dishes were connected by making small hole on the side of each and connected by straw sealed with parafilm to prevent the insect from escape. Filter-paper circles of 10 cm in diameter were used for each petri dish. Pure compound was applied on one at a concentration of 10 µg/ml. One ml of solution was uniformly applied with a pipette, in such a way as to have a treated substrate of 0.12 µg/cm of each pure compound. The treated filter paper

was air-dried until the solvent was totally evaporated. The treated and the untreated filter paper were placed on each connected petri dishes. Ten adults of *S. oryzae* were placed on petri dish with threaded filter paper. Weevils present in each side were counted at hourly intervals for 6 h after treatment.

In the second experiment, pure compound was applied on the rice instead of applying on filter paper. In this experiment, one of the petri dishes contained 5 g of treated raw rice grains while the other petri dish contained the untreated rice. The insects in each side were counted in 5, 10, 15 and 20 h after introducing the tested insects to treated petri dish. Data were converted to express percentage repulsion (PR) using the following formula:

$$PR (\%) = (Nc - 50) \times 2$$

Where  $Nc$  is the percentage of weevils present in the control half.

The same concept was carried out for other tested insects. Mean separation of repellent activity of the different pure compounds and the comparisons between them were made using Tukey test ( $P \leq 0.001$ ) using SAS program (Lopez et al. 2014).

#### **4.3.6 Residual toxicity against storage pests**

Residual toxicity against adults of *S. oryzae* and *R. dominica* and larvae of *P. interpunctella* were carried out in separate plastic cups for a period of 60 days after 15 g of rice treated with compounds **1-4**. The length of exposure and the dosage of each compound were chosen as the concentration required causing 95% mortality of the individuals (Islam & Talukder, 2005). The  $LC_{95}$  value from the previous study (Chapter 4: **4.2.3**) was used to evaluate the persistence of toxicity during the 60 days period. After each treatment was completed, the plastic cups were closed with caps, secured and

held at room temperature (27 °C). The control was only treated with acetone. These bioassays were carried out over a period of 60 days whereby on the 7th day, 30 insects were placed in each plastic cup. After 72 h, the insects were removed from each cup. Insects' mortality were recorded and dead insects discarded. All cups were tightly closed until next bioassay. The procedure was repeated on the 15<sup>th</sup>, 20<sup>th</sup>, 30<sup>th</sup>, 40<sup>th</sup>, 50<sup>th</sup> and 60<sup>th</sup> day of assessment. All bioassays were replicated 4 times.

#### 4.3.7 Data Analysis

Bioassay data within range of 5-95% were pooled and subjected to probit analysis by using Polo Plus (Finney, 1971) (LeOra Software, Berkeley, CA) to obtain 50% and 95% lethal concentration (LC<sub>50</sub> and LC<sub>95</sub>). Significant differences in the LC<sub>50</sub> and LC<sub>95</sub> values were based on non-overlap of 95% confidence intervals. Abbott's formula (Abbott, 1925) was applied to correct percentage mortality if control mortality was more than 5%. In case the control mortality ranged from 5-20%, the observed percentage mortality (%M) was corrected by Abbott's formula 1:

$$\%Mortality = \frac{\% \text{ test mortality} - \% \text{ control mortality}}{100 - \% \text{ control mortality}} \times 100$$

### 4.4 RESULTS

#### 4.4.1 Preliminary Test

Insecticidal effects of crude extract from aerial part and root crude extract of *P. sarmentosum* were evaluated for their toxicity potential against storage pests. Results of hexane (HE), dichloromethane (DI) and methanol (ME) extracts are presented in Table 4.1. One mg/ml (1000 ppm) of aerial part HE and ME showed high toxicity on *Sitophilus oryzae* and led to 100% mortality after 72 hours of exposure. *Rhizopertha dominica* and *Plodia interpunctella* were not as susceptible as compared to *S. oryzae*

(Table 4.1). Mean mortality of *R. dominica* at the 1mg/ml of aerial part extracts reached 100% after 96 hours of exposure. *P. interpunctella* was more resistant against HE and ME aerial part extracts with 76.8 and 64.1% mortality on tested larvae. DI extracts of aerial part only exhibited less than 13% mortality in all tested insects after 96 hours of exposure. *P. sarmentosum* DI aerial part extracts showed very low toxicity against all tested insects.

**Table 4.1:** Percentage mortality of *S. oryzae*, *R. dominica* adults and *P. interpunctella* larvae after exposure to 1mg/ml hexane, methanol and dichloromethane of *P. sarmentosum* aerial part extracts.

Storage pests	Extract	Mortality (mean±SE, %)			
		24h	48h	72h	96h
<i>S. oryzae</i> (Adults)	HE	12.3±0.7a	47.1±1.7a	100±0.0a	100±0.0a
	ME	4.6±0.6b	32.8±1.4b	100±0.0a	100±0.0a
	DI	0	0	9±3.93d	13±3.1c
<i>R. dominica</i> (Adults)	HE	3.3±0.7b	18.0±1.8c	74.6±1.9b	100±0.0a
	ME	7.3±0.3b	21.8±1.6c	67.6±1.8b	100±0.0a
	DI	0	0	10±3.1d	13±2.6c
<i>P. interpunctella</i> (3 <sup>rd</sup> instar larvae)	HE	0	10.6±1.4d	33±2.9c	76.8±2.6b
	ME	1.1±0.4c	11.2±1.3d	24.1±2.7c	64.1±3.4b
	DI	0	0	0	0

<sup>a</sup>HE (Hexane extracts), DI (dichloromethane extracts), ME (Methanol extracts).

<sup>b</sup> Mean (± SE) followed by the same letters in a row indicate no significant difference ( $p < 0.05$ ) according to the Tukey test.

<sup>c</sup> Fractions with no activity were not presented.

On the other hand root HE, Me and DI extracts had similar effects as aerial part extracts. Root HE and ME extracts caused 100% mortality of *P. sarmentosum* HE and ME extracts showed reliable toxicity to all tested insects (Table 4.2). The aerial part and

roots HE and ME extract were further fractionated using column chromatography (silica) which led to nine fractions from each extract.

**Table 4.2:** Percentage mortality of *S. oryzae*, *R. dominica* adults and *P. interpunctella* larvae after exposure to 1mg/ml of hexane, methanol and dichloromethane *P. sarmentosum* roots extract.

Storage pests	Extract	Mortality (mean±SE, %)			
		24h	48h	72h	96h
<i>S. oryzae</i> (Adults)	HE	32.3±1.9a	56.1±2.6a	100±0.0a	100±0.0a
	ME	27.5±1.2a	46.7±2.5a	100±0.0a	100±0.0a
	DI	0	0	11±2.6e	18±2.1d
<i>R. dominica</i> (Adults)	HE	4.2±0.9b	28.0±2.8b	84.2±2.9b	100±0.0a
	ME	6.7±0.4b	28.8±2.4b	71.6±2.5c	100±0.0a
	DI	0	0	16.2±2.1e	19.7±3.3d
<i>P. interpunctella</i> (3 <sup>rd</sup> instar larvae)	HE	0	15.6±3.4c	41±2.4d	88.8±3.6b
	ME	4.2±0.3b	13.2±1.1c	36.2±0.7d	62.1±4.1c
	DI	0	0	0	0

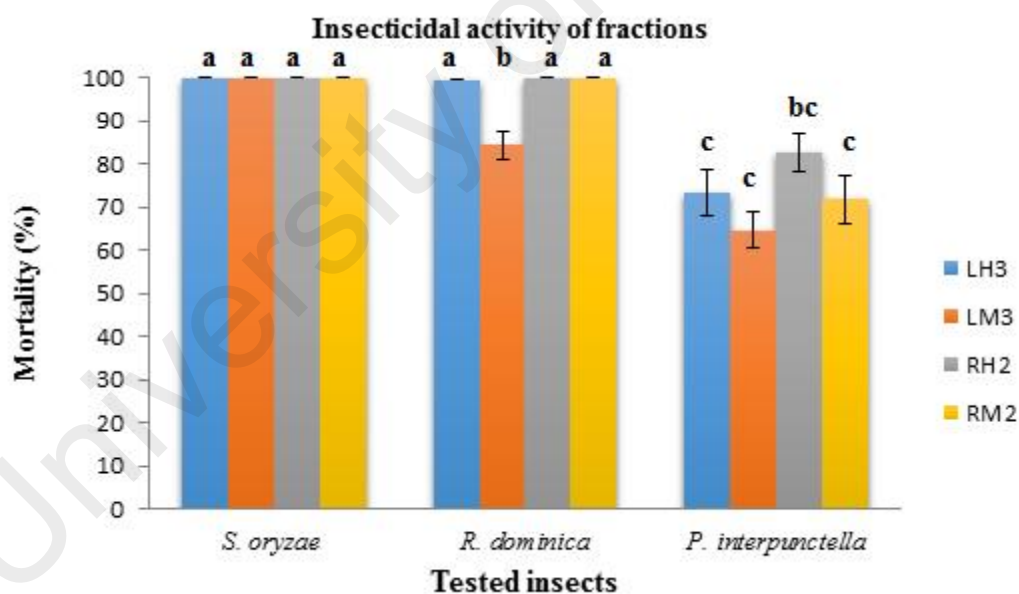
<sup>a</sup>HE (Hexane extracts), DI (dichloromethane extracts), ME (Methanol extracts).

<sup>b</sup> Mean (± SE) followed by the same letters in a row indicate no significant difference ( $p<0.05$ ) according to the Tukey test.

<sup>c</sup> Fractions with no activity were not presented.

Insecticidal activity of hexane and methanol fractions, after fractionizing the extracts, LH3 (Aerial part hexane fraction 3; hexane: CH<sub>2</sub>CL<sub>2</sub>; 60: 40), LM3 (aerial part methanol fraction 3; hexane: CH<sub>2</sub>CL<sub>2</sub> 40:60), RH2 (roots hexane extracts hexane: CH<sub>2</sub>CL<sub>2</sub> 40:60) and RM2 (roots methanol extracts hexane: CH<sub>2</sub>CL<sub>2</sub> 40:60) were highly toxic against *S. oryzae*, *R. dominica* adults and larvae of *P. interpunctella*. LH3, RH2 and RM2 at 0.5mg/ml concentration caused 100% mortality to *S. oryzae* and *R. dominica*. As shown in Figure 4.5, it also caused 50 to 80% mortality to *P.*

*interpunctella* (Figure 4.5). LM2 toxicity effect was slightly lower on *R. dominica* and *P. interpunctella*. Since the early fraction of both extracts was active and solvent system used was highly non polar relevantly the nature of active compounds must be very non polar. More over all four fraction in Figure 4.5 were strongly active against tested insect pests. LH3, LM2, RH2 and RM2 were submitted for thin layer chromatography to purify and isolate the active compounds (Chapter 3). The active fractions from hexane and methanol extraction contained similar components except compound 4 in methanol fraction which was not toxic. Yield of both active compounds 1 and 2 were more in hexane fraction. This may one of the reasons that activity of hexane was significantly higher than methanol fraction on *R. dominica* and *P. interpunctella*. Structures of compounds 1-3 used in the bioassays for the LC values are shown in Chapter 3.



**Figure 4.5:** Mortality percentage caused by exposing the *S. oryzae*, *R. dominica* adults and *P. interpunctella* larvae to active fractions (LH3: third fraction of aerial part hexane extracts), (LM3: third fraction of aerial part methanol extracts), (RH2: second fraction of aerial part hexane extracts) (RM2: second fraction of aerial part methanol extracts)



#### 4.4.2 Evaluation of Lethal Concentration Dose

Compounds **1** and **2** showed high toxicity against all three tested insects (Table 4.3). The  $LC_{50}$  of *S. oryzae* was estimated to be 4.7  $\mu\text{g/ml}$  for **1** and 5.6  $\mu\text{g/ml}$  for **2** which considered the lowest  $LC_{50}$  among the tested insects. Similarly lowest  $LC_{95}$  value (13.6  $\mu\text{g/ml}$ ) was observed from *S. oryzae* in respond to compound **1** followed by compound **2** with value of 14.3  $\mu\text{g/ml}$ . *R. dominica* had higher resistance to **1**, **2** and **3** compared to *S. oryzae* with relative **1** and **2**  $LC_{50}$  value of  $\geq 10.6$   $\mu\text{g/ml}$  and  $LC_{95}$  of  $\geq 18.6$   $\mu\text{g/ml}$ . Finally *P. interpunctella* larvae was the most resistance insects with the relative significant higher  $LC_{50}$  and  $LC_{95}$  value of **1** and **2** based on 95% confidence comparison of ( $LC_{50}$  value of  $\geq 15.7$   $\mu\text{g/ml}$  and  $LC_{95}$  of  $\geq 35.9$   $\mu\text{g/ml}$ ).

Based on the  $LC_{50}$  values and their respective 95% confidence intervals, **3** was found to be significantly less effective towards *S. oryzae*, *R. dominica* adults and *P. interpunctella* larvae. The  $LC_{50}$  and  $LC_{95}$  values for all three species were variable and species specific. This result is supported by other reports, which showed great variations in the susceptibility of insects to other biopesticide product (Nattudurai, Paulraj, & Ignacimuthu, 2012). The percentage of variation in the sensitivity of stored grain insect pests is high in response to toxicity of compounds and susceptibility of insect pests itself. Similar results have been reported by several researches on insecticidal activities of other plant extracts (Huang, Tan, Kini, & Ho, 1997).

**Table 4.3:** Toxicity of **1**, **2** and **3** against *S. oryzae* and *R. dominica* and *P. interpunctella* using treated rice, 72 h.

Compounds	Tested Insects					
	<i>S. oryzae</i> <sup>b</sup>		<i>R. dominica</i>		<i>P. interpunctella</i>	
	LC <sub>50</sub> (µg/ml) (95%C.I.) <sup>c</sup>	LC <sub>95</sub> (µg/ml) (95%C.I.)	LC <sub>50</sub> (µg/ml) (95%C.I.)	LC <sub>95</sub> (µg/ml) (95%C.I.)	LC <sub>50</sub> (µg/ml) (95%C.I.)	LC <sub>95</sub> (µg/ml) (95%C.I.)
<b>1</b> <sup>a</sup>	4.7 (3.6 to 5.6)	13.6 (10.5 to 21.6)	10.6 (8.1 to 11.9)	18.6 (15.8 to 30.3)	17.37 (13.4 to 20.3)	35.9 (29.1 to 57.1)
<b>2</b>	5.6 (4.5 to 6.6)	14.3 (11.5 to 20.8)	8.7 (7.16 to 10.6)	28.1 (20.3 to 49.2)	15.7 (12.4 to 19.1)	37.7 (29.1 to 61.5)
<b>3</b>	258.9 (218.7 to 303.1)	670.2 (531.1 to 967.7)	396.4 (328.1 to 474.7)	1283.56 (959.6 to 2072.3)	432.4 (387.3 to 485.2)	1795.7 (1425.1 to 2444.6)

<sup>a</sup>Compounds **1-3** represent phenylpropanoids isolated for *P. sarmentosum* hexane and methanol active fractions.

<sup>b</sup>Test performed on *S. oryzae* and *R. dominica* two weeks old adults and *P. interpunctella* their instar larvae.

<sup>c</sup>LC<sub>50</sub> and LC<sub>95</sub> values significant difference ( $p < 0.05$ ) was based on non-overlap of the 95% CL.

#### 4.4.3 Contact Toxicity

The results clearly indicated that compounds **1**, **2** and **3** did not show potent contact toxicity against stored grain insect pest even at the highest concentration of 200 µg/ml. This is most probably due to the poor absorption through the insects' cuticle. The efficacy in respect to the toxicity of **1**, **2** and **3** towards *S. oryzae* and *R. dominica* adults and *P. interpunctella* larvae were relatively weak up to 48 h monitoring after the treatment. According to previous research some natural compounds express toxicity against stored product pests through consumption, such as citrus peel, eucalyptus, but not all are active against insect pests through contact toxicity (Bernier, Furman, Kline, Allan, & Barnard, 2005; Kim, Park, Ohh, Cho, & Ahn, 2003).

#### 4.4.4 Repellency bioassay

The first repellency assay which involved in contaminating the rice grains with the isolated compounds was successful. Compounds **1** and **2** showed similar repellency activity at 10 µg/ml towards *S. oryzae*, *R. dominica* and *P. interpunctella* (Table 4.4). *S. oryzae* and *R. dominica* were susceptible to repellence activity caused by compounds **1** and **2** with overall mean repellency of 70-78% after 20 h, while *P. interpunctella* was less susceptible with overall 40% repellency. Compound **3** was less effective in repellency with less than 20% repellency for all tested insects.

The result of second repellency test with procedure of dissolving the isolated compounds in acetone as solvent and disperse it on the filter paper was moderate. The repellency was below 10%.

**Table 4.4:** Repellency activity of compounds **1**, **2** and **3** against *S. oryzae*, *R. dominica* and *P. interpunctella*.

Compounds	Insects	% Repellency (mean±SE)				Mean repellency (%)
		5 h <sup>b</sup>	10 h	15 h	20 h	
<b>1</b>	<i>S. oryzae</i>	51.2 ±10.3 a	67.2 ±8.5 a	73.7±8.5 a	82.5±6.4 a	68.6±6.6 a
	<i>R. dominica</i>	60 ±7.1 a	74.5± 6.4a	81.2±8.5 a	85±7.8 a	75.1±5.5 a
	<i>P. interpunctella</i>	33.7±4.7 b	38.7 ±7.5 b	48.7 ±6.2 b	50±7.1 b	42.7±3.9 b
<b>2</b>	<i>S. oryzae</i>	51.2±7.5 a	60±7.8 a	69.5±6.2 a	79.2±8.3 a	68.1±6.2 a
	<i>R. dominica</i>	58.7±13.1 a	65±7.9 ab	72.5±5.5 a	79.2±5.1 a	68.8±4.4 a
	<i>P. interpunctella</i>	31.2± 2.5 b	37.5± 6.5 b	41.2± 2.5 b	58.7± 4.8 b	42.1±5.8 b
<b>3</b>	<i>S. oryzae</i>	NA <sup>c</sup>	NA	NA	NA	NA
	<i>R. dominica</i>	NA	NA	NA	NA	NA
	<i>P. interpunctella</i>	NA	NA	NA	NA	NA

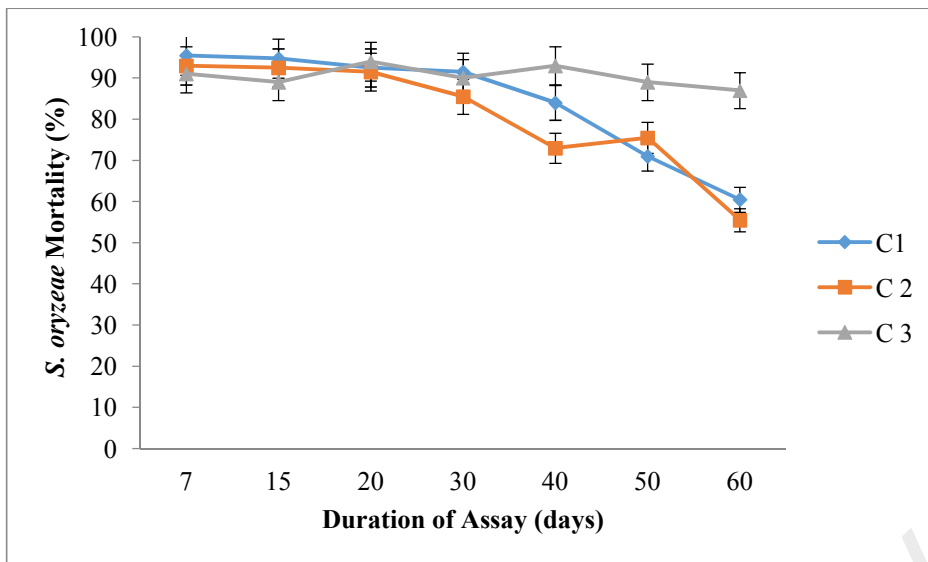
<sup>a</sup> Each datum represents the mean of four replicates, values followed by the same letter are not significantly different according to the Tukey test ( $p<0.05$ ).

<sup>b</sup> h: hours after treatment.

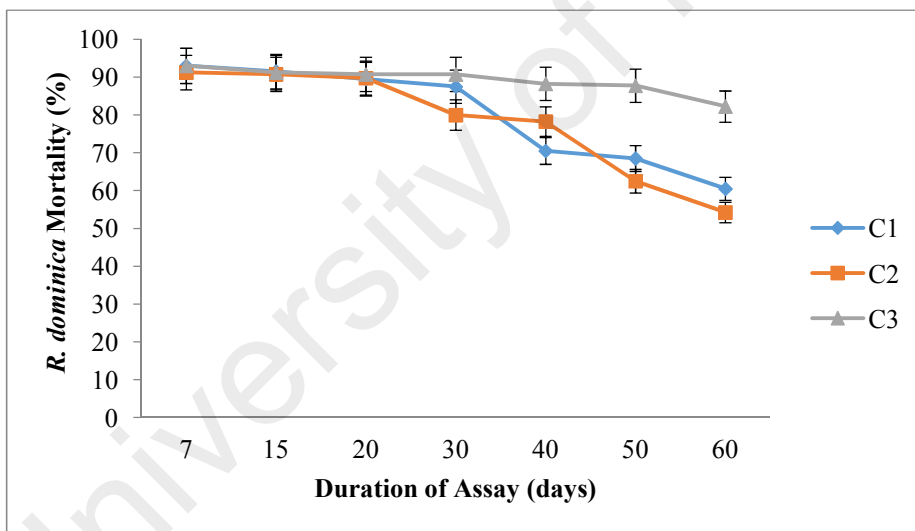
<sup>c</sup>NA: below 10% repellency.

#### 4.4.5 Residual Toxicity Using LC<sub>95</sub> Value of Each Active Compound

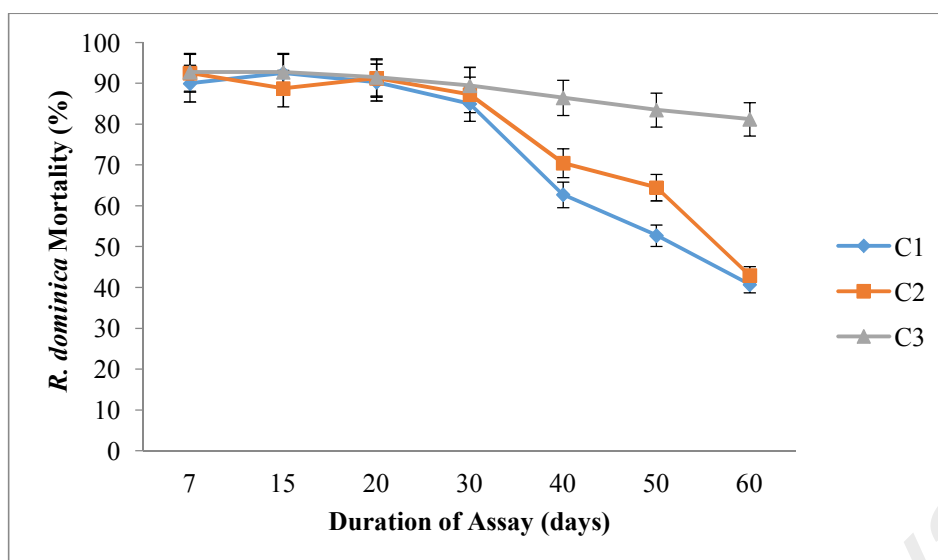
The mean mortality of *S. oryzae*, *R. dominica* and *P. interpunctella* were recorded during the 60 days of bioassay (Figures 4.6, 4.7 and 4.8). The efficacy of compounds **1** and **2** with their relative LC<sub>95</sub> against *S. oryzae* was consistent during the first 30 days and slowly decreased and this pattern continued until the last day of assay. On the other hand, toxicity efficacy of compound **3** with high LC<sub>95</sub> value of 670.2 µg/ml was consistent till the 60<sup>th</sup> day. *R. dominica* and *P. interpunctella* response to residual effects of compounds **1**, **2** and **3** were similar and followed the same pattern as *S. oryzae*. Compounds **1** and **2** showed consistent toxicity against tested insects during the first 30 days according to the mortality rate. The insecticidal activity reduced and by the 60<sup>th</sup> day, it was at the lowest point (Figures 4.6, 4.7 and 4.8). Insecticidal activity of compound **3** showed consistent toxicity throughout the 60 days although it was less toxic compared to compounds **1** and **2** (Table 4.3). This observation suggested that **3** has a longer half-life compared to **1** and **2**. This result may be due to the level of degradation of these three compounds. Both compounds **1** and **2** were fast acting toxin while **3** needed longer time to be effective.



**Figure 4.6:** Mean ( $\pm$ SEM) mortality percentage of *S. oryzae* adults on rice grain treated with compounds 1-3 relative LC<sub>95</sub> value, exposed from 0 to 60 days after treatment.



**Figure 4.7:** Mean ( $\pm$ SEM) mortality percentage of *R. dominica* adults on rice grain treated with compounds 1-3 relative LC<sub>95</sub> value, exposed from 0 to 60 days after treatment.



**Figure 4.8:** Mean ( $\pm$ SEM) mortality percentage of *P. interpunctella* larvae on rice grain treated with compounds **1-3** relative  $LC_{95}$  value, exposed from 0 to 60 days after treatment.

#### 4.5 DISCUSSION

The experimental work presented here is based on bioassays guided procedure in which the insecticidal activity of *P. sarmentosum* tested on three major storage insects pests. The bioassay guided study lead to isolated four phenylpropanoids. Compounds **1** and **2** had reliable toxic effect against *S. oryzae*, *R. dominica* adults and *P. interpunctella* third instar larvae. In the work done by Vassilakos et al., (2014), toxicants were active by direct contact to *S. oryzae*. In this study, several assays were conducted to study the mode of action of phenylpropanoids isolated from active fractions of *P. sarmentosum*. The contact toxicity assay revealed that none of isolated phenylpropanoids had contact toxicity. While the assay with treated grain showed high toxicity of compounds **1** and **2**. These results suggested that compounds **1** and **2** are stomach poison which after consumption can kill the target insect pest. On other hand long lasting activity of compound **3** same time low toxicity of it make it a candidate for further investigation. Among the three tested insects, *S. oryzae* was more susceptible than *R. dominica* and *P. interpunctella*. The sensitivity differences between tested

insects may be due to inherent variation in the susceptibility of these insects to compounds **1**, **2** and **3**. This result is in line with many reports that show great variations in the susceptibility between insects responds to insecticides, irrespective of size, genera or species (Gahukar, 2014; Huang, Tan, Kini, & Ho, 1997; Nattudurai, Paulraj, & Ignacimuthu, 2012). As expected, there was a strong difference in efficacy of compounds between *S. oryzae*, *R. dominica* and *P. interpunctella*. Compound **3** had low toxicity but it showed consistent activity during 60 days. In general between the tested storage insect pests, *P. interpunctella* was the most resistant tested subject towards **1**, **2** and **3**.

The present studies showed that compounds **1** and **2** have high potential to be used as botanical insecticide. Both compounds **1** and **2** can be considered as effective alternatives in insect pests control for stored grains. Effects against *S. oryzae*, *R. dominica* adults and *P. interpunctella* larva shows **1** and **2** potential toxicity against several storage pests in their different life cycle. On the other hand, compounds **1** and **2** had a strong repellence activity when applied on the grains against *S. oryzae*, *R. dominica* and *P. interpunctella*. These results are in agreement with the previous studies on essential oils from *Piper* family reported by Yadav et al., (2009). Compounds **3** and **4** did not show any repellence activity. *Piper sarmentosum* leaves and roots have been used as medicine and consumed as food for decades (Burkill, 1966; Nair & Burke, 1990). *P. sarmentosum* has been used as food and beverage till the current day and there are no records on its toxicity activity towards humans (Burkill, 1966; Nair & Burke, 1990). This research reveals the potential of the active compounds isolated from *P. sarmentosum* in storage pest control. Nevertheless, more researches on formulation and in vivo assays are needed in order to commercialize compounds **1**, **2** and **3**.



#### 4.6 CONCLUSION

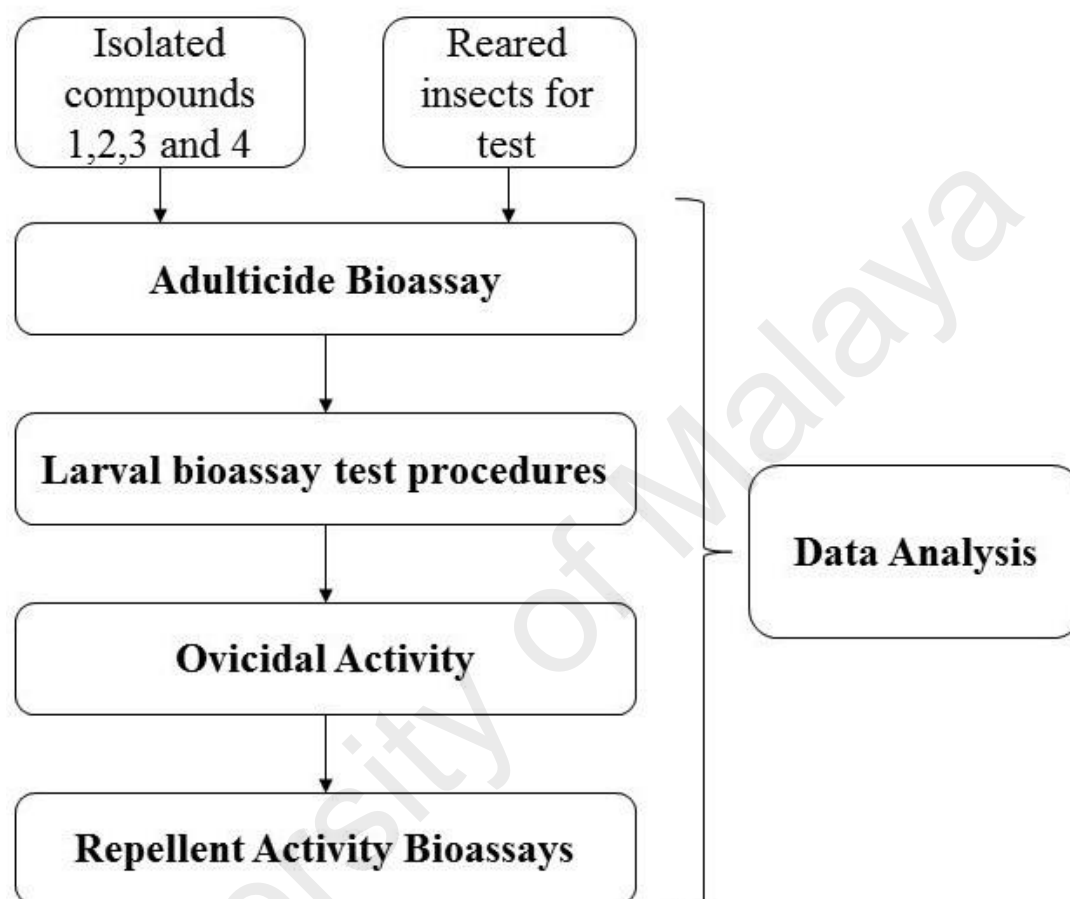
This is the first study conducted on the insecticidal activity of the compounds isolated from *P. sarmentosum* on storage insect pest control. Compounds **1** and **2** were exhibited potent insecticidal and repellency activities against three major storage pests *S. oryzae*, *R. dominica* and *P. interpunctella*. While results suggested that all three compounds acted as stomach poison and they were effective after being consumed by the insect pests. The residual toxicity test revealed that **1**, **2** and **3** can give protection against *S. oryzae*, *R. dominica* and *P. interpunctella* over a relatively long period (over 30 days), making their activity more significant. Although the insecticidal activity of compound **3** was very low but its long lasting toxicity effect was consistent for 60 days, it may be a good option to be used in mixtures with compounds **1** and **2** in future study. The results obtained from this study and the fact that *P. sarmentosum* has been consumed as vegetable during long period of time shows the potential use of its extracts and its active components in storage pest management.

## CHAPTER 5: INSECTICIDAL ACTIVITY OF ISOLATED PHENYLPROPANOIDS ON THREE MOSQUITO VECTORS

### 5.1 INTRODUCTION

The earlier chapters described in detail the investigation on *Piper sarmentosum* which had led to the isolation of several phenylpropanoids compounds. Compounds **1**, **2** and **3** asaricin **1**, isoasarone **2** and *trans*-asarone **3** were active against three main storage pest (*S. oryzae*, *R. dominica* and *P. interpunctella*). This result showed the potential of these phenylpropanoids for further investigation. Mosquitoes are pests of medical importance which have shown high resistance to the current insecticides including pyrethroids and organophosphate family. Their resistance combine with their ability of spreading fatal disease make the interesting subject for investigation. Therefore, the effects of compounds asaricin **1**, isoasarone **2**, *trans*-asarone **3** and asaraldehyde **4** were investigated on *Ae. aegypti*, *Ae. albopictus* and *Cx quinquefasciatus*. These compounds were tested at different life stages of each vectors and monitored for susceptibility to the insecticide in order to determine its toxicity against mosquitoes. Test methods for both larval and adult mosquitoes have been standardized by WHO. Full instructions have been published by the WHO (1970) and the American Mosquito Control Association (Uejio et al., 2014). These tests are essential for routine surveillance of control operations, even when resistance is not yet expected. The detection and measurement of insecticide susceptibility in each insect were done by standardized methods upon comparison of LC<sub>50</sub> and LC<sub>95</sub> value of each tested compounds. To investigate the mode of each isolated compound against the mosquito the following assays were conducted; 1) adulticide bioassay, 2) larval bioassay test procedures, 3) ovicidal activity and 4) repellent activity. With screening

the results of mentioned bioassays the study could come up with brief understanding of level of toxicity and possible mode of action of each toxic compound (Figure 5.1).



**Figure 5.1:** General procedures of bioassay and activity tests of compounds 1, 2, 3 and 4 against mosquito vectors.

## 5.2 LITERATURE REVIEW

*Aedes* and *Culex* mosquitoes are vectors of several diseases such as dengue fever, chikungunya fever, Eastern Equine Encephalitis (EEE), Rift Valley Fever (RVF) and filariasis (Zoubiri, Baaliouamer, Seba, & Chamouni, 2014; Edriss, Satti, & Alabjar, 2013). Dengue fever becomes endemic in South East Asia, Africa and America (Hostettmann, Marston, Ndjoko, & Wolfender, 2000). *Aedes aegypti* and *Aedes*

*albopictus* are the most important vectors for dengue fever and dengue haemorrhagic fever in many regions, including Malaysia. *Culex quinquefasciatus* is common in tropics and subtropics. It is also found in temperate regions of North and South Hemisphere (Edriss, Satti, & Alabjar, 2013). Since mosquitoes transmit parasites and viruses that cause diseases worldwide, it is important to control the mosquito populations.

Synthetic insecticide such as Dichloro diphenyl trichloro ethane (DDT) was used as the main insecticide in the first half of the last century (Kalimuthu et al., 2013), later replaced with organophosphates due to the problem of insecticide resistance and human hazard. Nevertheless, this substitution didn't last long due to development of resistance in mosquitoes to the whole organophosphates family (Hemingway & Georghiou, 1983; Ffrench-Constant & Bonning, 1989; Campbell et al, 1998). Pyrethroids and synthetic version of natural pyrethrins seemed to be the best answer for controlling these vectors. However, pyrethroids resistance was soon reported in *Ae. aegypti* (Shukor, Wajidi, Avicor, & Jaal, 2014; Saeaeue, Morales, Komalamisra, & Vargas, 2011). These facts created the need for stronger bioinsecticides which are more environmentally safe, degradable and target specific agents for pest control purposes. Plant extracts become more important in insect control, being considered as environmentally safe, less hazardous to non-target insects, simply inexpensive and can be applied effectively (Shukor, Wajidi, Avicor, & Jaal, 2014). Several plant extracts have been evaluated as potential acute or chronic insecticides against a variety of insect species (Naik, Vaidya, & Namjoshi, 2013; Bachrouch, Ferjani, Haouel, & Jemâa, 2015). Several limonoids, for instance azadirachtin from *Azadirachta indica* and toosendanin from *Melia toosendan* have insecticide activity (Adel & Sehnal, 2000; Maciel et al., 2006). Both of these compounds are used as insecticides and have been commercialized

in USA and China. *Piper sarmentosum* essential oil has been tested and it shows some larvicidal activity against *Aedes aegypti* but active components have not been isolated for this specific activity (Silvia et al., 2008; Anees, 2008).

## **5.3 MATERIALS AND METHODS**

### **5.3.1 Test Mosquitoes**

To determine the insecticidal activity of the isolated phenylpropanoids, three species of *Aedes aegypti*, *Aedes albopictus* and *Culex quinquefasciatus* were used for their susceptibility over a period of one year (year 2012 to 2013) in this study. Each different species was categorized and reared in laboratory as laboratory strains. *Ae. aegypti*, *Ae. albopictus* and *Cx. quinquefasciatus* collected from cemetery near Kuala Lumpur International Airport (KLIA) in year 2011. Mosquito larvae were kept in the plastic containers located in cages (23cm x 23cm x 23cm) at temperature  $27\pm 2^{\circ}\text{C}$  and relative humidity (RH) 80% with a photoperiod of 13 h of daylight and 11 h of darkness for emergence. Male and female adults of each mosquito (*Ae. aegypti*, *Ae. albopictus* and *Cx. quinquefasciatus*) were transferred to separate cages and provided 10% sucrose solution on cotton pads, over four days to allow for mating. All colonies were reared simultaneously in laboratory handled in the same manner through all manipulations. The temperature of each colony was maintained at  $27^{\circ}\text{C}\pm 2^{\circ}\text{C}$  and relative humidity (R.H.) was maintained at 80% (Division of Medical Entomology, IMR, Kuala Lumpur, 2000). Emerged adults were fed with 10% sucrose supplemented soaked on tissue paper or cloth and placed inside a small plastic container.

### **5.3.2 Egg Hatching and Mosquito rearing**

The eggs of all test mosquitoes were collected on the filter papers (Whatman no. 1) and locked in a plastic rearing tray (24 cm x 35 cm x 6 cm) containing 1000 ml of

chlorine-free tap water. Hatching occurs within 48 to 72 h. Cow liver powder was added as larval food for better growth of larvae and uniform population of mosquito larvae. The larvae were reared in the laboratory condition at temperature of  $27 \pm 2^\circ\text{C}$  and RH 80% with a photoperiod of 13 h of daylight and 11 h of darkness. *Ae. aegypti* and *Ae. albopictus* larvae were fed with liver powder while *Cx. quinquefasciatus* larvae were fed with ground mice pellets which were cleaned dried in powder form (Figure 5.2). About 0.5 g larval food was added in alternative days. Immature stages of larvae in plastic trays were covered to prevent contamination with other mosquitoes (Figure 5.3). Late third and early fourth instar larvae were used for larval bioassay test.



**Figure 5.2:** Top left liver powder for rearing larvae of *Aedes aegypti* and *Aedes albopictus*, top right grind mice pellets for *Culex quinquefasciatus* larvae.



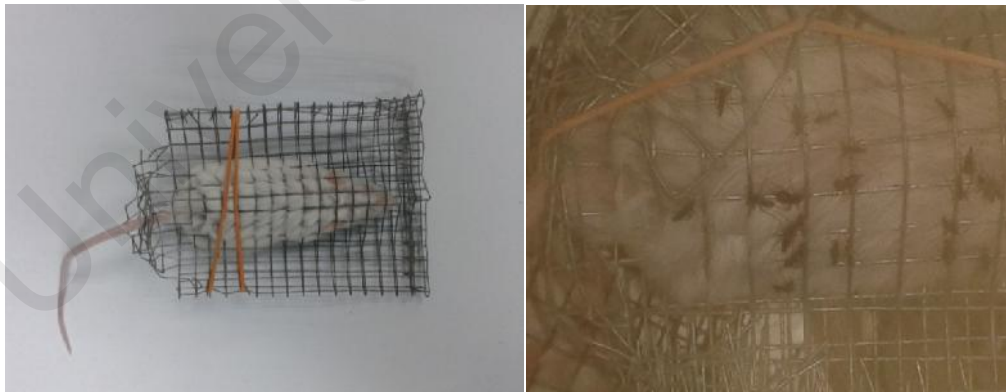
**Figure 5.3:** Introducing mosquito eggs of *Ae. aegypti* to plastic containers containing liver powder and dechlorinated tap water.

After seven days, *Ae. aegypti*, *Ae. albopictus* and *Cx. quinquefasciatus* larvae were started to pupate. Large size pipette suitable with the size of pupae were used to remove pupae daily from the larvae trays to other plastic tray. Later, the tray with about 400 pupae was placed in a mosquito cage measuring (30 cm x 30 cm x 30 cm) for emergence. Rearing cages for adult mosquitoes were made up of wood frames covered with netted screen on the back and the top. The front of the cage was covered with netted sleeves about 50 cm in length. This area was used for transferring the mosquitoes, introduction of tray of pupae and for introduction of mice on which the adult female mosquitoes fed. After adults were emerged, they were fed with 10% sucrose solution soaked in lint cloth and placed inside a small glass bottle (Figure 5.4).



**Figure 5.4:** Feeding mosquitoes with a 10% sucrose solution soaked in lint cloth.

About five days after emergence, female adult mosquitoes were transferred for adult bioassay. The remained females were used for rearing by permitted to feed on mice. This was accomplished by placing a wire caged white mouse into the mosquito cage for overnight feeding (Figure 5.5).



**Figure 5.5:** Top left wire caged mouse, Top right mosquitoes blood feeding on the caged mouse.

After four days, adults were allowed to feed on a mouse (Figure 5.5). Three day after blood feeding, engorged females deposited their eggs in the 50 ml vial filled with



distilled water. Eggs are deposited about 3-4 days after the blood meal. Then, water containing the eggs was filtered round filter paper (Whatman No 1, 10 cm in diameter) and the remaining water was drained. The filter paper containing the eggs was then dried in room temperature (27 °C) for further use (Figure 5.6).



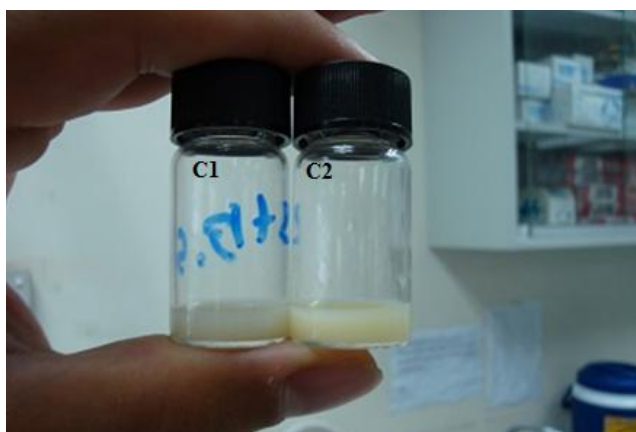
**Figure 5.6:** *Aedes aegypti* eggs collected on filter paper.

### 5.3.3 Tested Compounds

Compounds **1**, **2**, **3** and **4** were used in this study. Different concentrations were prepared by using absolute acetone as a solvent. For mosquito adult bioassay, serial dilution was prepared with distilled water (Figure 5.7). Compounds **1**, **2** and **3** were dissolved in the water to make a uniform milky combination. The amount of insecticide and diluents required were computed using the formula:

$$C_1V_1=C_2V_2$$

Where C is concentration in µg/ml and V is volume.



**Figure 5.7:** Pure compounds **1** and **2** uniform dispersion in water.

#### 5.3.4 Adulticide Bioassay

The adulticide activity of the *Piper sarmentosum* isolated compounds were determined by topical application to the adult female mosquitoes (Figure 5.9 and 5.10), following slightly modified versions of the WHO Standard Protocols 32 (WHO, 1995). Each isolated compound was dissolved in acetone yielding a graded series of concentrations.



**Figure 5.8:** Female mosquito captured in vials.

To test compounds **1**, **2** and **3**, non blood-fed females were captured in separated vial (Figure 5.8). Adult mosquitoes were briefly anaesthetized by keeping them in  $-3\text{ }^{\circ}\text{C}$  for 2 min. The anaesthetized mosquitoes were then placed on an ice cold plate (Figure

5.9). This procedure gave the enough time for application of the tested compounds on each subjects before they become active again. During this procedure there was no mortality due to the temperature.



**Figure 5.9:** Vials of anaesthetized mosquitoes on cold plate.

Treatment was performed with the aid of a magnifier. A 0.1  $\mu\text{L}$  droplet of each compound dissolved in acetone was applied onto the upper part of the immobilized mosquito's pronotum micro pipet. Since the droplet was bigger than Hamilton's digital syringe which idealistic use for this assay the control group death was measured carefully not to exceed 20% mortality (Figure 5.10 and 5.11). Dosages were expressed in  $\mu\text{g}$  per ml of each compound. A total of twenty-five individuals were used at each concentration, with 4 to 6 concentrations.



**Figure 5.10:** WHO standard protocols of topical application female mosquito using digital syringe (WHO, 1995).

The application was done very carefully right aim to place the drop on pronotum without physical damage to the tested mosquitoes. Similarly procedure carried out for the control group.



**Figure 5.11:** Application of active compounds on the female mosquito thorax.

Controls were divided into two groups: acetone-treated and untreated groups. Both groups were treated in a similar manner to that of isolated compounds treated

groups. After application, the females in all groups were maintained at  $27 \pm 2$  °C and 80% RH in plastic cups, with 10% sucrose. At the end of a 24-h recovery period, the mosquitoes were considered dead if they showed no sign of movement such as lying on the bottom of the plastic cup and not responding to mechanical stimulation. Eight replicates were studied for each plant extract with mosquitoes from different rearing batches, and the results were pooled.

### 5.3.5 Larval Bioassay Test Procedures

The larval bioassay test was conducted according to WHO (1981) larval susceptibility bioassay procedure. Twenty-five early 4<sup>th</sup> instar larvae were selected and transferred into a small plastic cups using wide-mouthed pipette (Figure 5.12).



**Figure 5.12:** Late 3<sup>rd</sup> and early 4<sup>th</sup> instar larvae of *Aedes albopictus* transferred into a small plastic cups.

Any larvae showing abnormalities, for example a fuzzy appearance were discarded. Twenty-five larvae were distributed into each disposable 250 ml plastic-cup containing 150 ml dechlorinated tap water which was kept overnight. Average temperature of the water was 25 °C. The test concentrations were prepared by pipetting the appropriate standard insecticide solution just above the surface of the water in each

of the plastic cups and stirring vigorously for 30 sec with a glass rod. Each test concentration and control was replicated 4 times. Within 15 to 30 min of the preparation of the test concentrations, the mosquito larvae were added by using pipette into 150 ml tap water. Then, water was added up to 200 ml in each plastic cup (Figure 5.13). For control, acetone was added and tested with tap water alone.



**Figure 5.13:** Mosquitoes larvae bioassay using disposable plastic cups.

After a period of 24 h, mortality counts on dead or moribund larvae were recorded. Dead larvae were those that could not be induced to move when probed. Moribund larvae were those with characteristics of incapable of rising to the surface or did not show normal diving when probed with tips of a needle on the siphon or abdominal region, or larvae with unnatural positions, tremors and incoordination (WHO 1970). The entire tests were conducted at  $27 \pm 2$  °C and were replicated three times. Percentage of mortality for all the insecticides tested was assessed 24 h after the treatment. The  $LC_{50}$  and  $LC_{95}$  value were calculated by probit analysis using Polo plus.

Six concentrations of the insecticides were used to determine lethal concentration (LC) value. In all cases, the bioassay data were pooled and analysed as it described in WHO (1970). If the mortality exceeded 20% in control, test was discarded and repeated (WHO, 1970). If mortality in control was between 5% and 20%, the percentage mortalities were corrected by Abbot's (1925).

Formula:

$$x = \frac{\% \text{ test mortality} - \% \text{ control mortality}}{100 - \% \text{ control mortality}} \times 100$$

### 5.3.6 Ovicidal Activity

Ovicidal activity was assessed by modifying the method of Kamaraj and co-worker (2010). The eggs of *Ae. aegypti*, *Ae. albopictus* and *Cx. quinquefasciatus*, were collected from the laboratory batch. Pure compounds; asaricin **1**, isoasarone **2** and transasarone **3**, were separately dissolved in 1 ml acetone and further diluted with distilled water to achieve various concentrations ranging from 5 to 25 µg/ml inside the disposable testing plastic cups with 250 ml capacity. Twenty five eggs of each tested mosquito species were individually exposed to each concentration in the cups. The tests for each concentration were performed in 4 replicates. After 48 hours of treatment, the number of dead eggs from each concentration were individually recorded under microscope. The percentage of ovicidal activity was calculated by using the following formula:

$$\% \text{ of egg mortality} = \frac{\text{No. of hatched larvae}}{\text{Total No of eggs}} \times 100$$

### 5.3.7 Repellent Activity

This study was carried out according to method described by WHO (1996). Three-day-old blood-starved female of *Ae. aegypti*, *Ae. albopictus* and *Cx. quinquefasciatus* (100 each ) were kept in a net cage (23 cm × 23 cm × 23 cm). Hands that had no contact with lotions, perfumes or perfumed soaps were exposed to the mosquito cage. Only 25 cm<sup>2</sup> dorsal side of the skin on each arms was exposed and the remaining area covered by rubber gloves (Figure 5.14).



**Figure 5.14:** Repellency assay on female mosquitoes.

The tested substance was applied at several dosages, separately in the exposed area of the forearm. Only acetone was served as control. The timing of the test depended on whether the tested mosquitoes were day or night biters. *Ae. aegypti* and *Ae. albopictus* were tested from 6 am to 9 am hours, while *Cx. quinquefasciatus* was tested from 8 am to 7 pm hours. Both control and treated arms were introduced simultaneously into the mosquito cage, and followed by gentle tapping on the sides of the experimental cages to activate the mosquitoes.





**Figure 5.15:** Number of bites received from the mosquitoes.

Each test concentration was repeated four times. The volunteers conducted test for each concentration by inserting the treated and control arms into the same cage for one minute and repeated this action every five minutes. The mosquitoes that landed on the hand were recorded and then shaken off before imbibing any blood (Figure 5.15). If the mosquito landed successfully and deliver a bite the place of bite were marked by marker to prevent any complication in the test.

The percentage of repellency was calculated by the following formula,

$$\% \text{ Repellency} = [(T_a - T_b) / T_a] \times 100, \text{ where}$$

T<sub>a</sub>: is the number of mosquitoes in the control group.

T<sub>b</sub>: is the number of mosquitoes in the treated group.

### 5.3.8 Data Management and Statistical Analysis

It was essential to obtain not less than three mortality counts of between 10% and 90%. Experimental tests that demonstrated more than 20% control mortality were discarded and repeated. However, when the control mortality ranged from 5-20%, the observed percentage mortality (%M) was corrected by Abbott's formula1:

$$\%M = \frac{\% \text{ test mortality} - \% \text{ control mortality}}{100 - \% \text{ control mortality}} \times 100$$

The 95% confidence intervals (CI) of the lethal dosage of 50 and 95% (LC<sub>50</sub> and LC<sub>95</sub>, respectively) calculated by a probit analysis.

## 5.4 RESULTS

### 5.4.1 Mosquito Larvicidal Activity of Isolated Compound

The isolated phenylpropanoids from *P. sarmentosum* were tested against three species of mosquitoes in the search for potential bioinsecticides. The larvicidal activity of isolated compounds at different concentrations was evaluated against the late third and early 4<sup>th</sup> instar larvae of *Ae. aegypti*, *Ae. albopictus* and *Cx. quinquefasciatus*.

The LC<sub>50</sub> and LC<sub>95</sub> values of compounds **1**, **2**, **3** and **4** against *Ae. aegypti*, *Ae. albopictus* and *Cx. quinquefasciatus* larvae is shown in Table 5.1. As presented in Table 6.1, all three mosquito larvae were highly susceptible to compounds **1** and **2**. The LC<sub>50</sub> value of compounds **1** and **2** against *Ae. aegypti* larvae was 4.3 and 4.1 µg/ml respectively, which were not significantly lower than *Ae. albopictus* and *Cx. quinquefasciatus*. Relatively compounds **1** and **2** had lowest LC<sub>95</sub> (8.3 and 7.1 µg/ml) against *Ae. aegypti*. The LC<sub>95</sub> values of compounds **1** and **2** for *Ae. albopictus* and *Cx. quinquefasciatus* were similarly higher than *Ae. aegypti*. Compound **3** toxicity against

*Ae. aegypti*, *Ae. albopictus* and *Cx. quinquefasciatus* was moderate with  $LC_{50} > 500$  and  $LC_{95} > 1000$ . Compound **4** did not show any significant toxicity even at very high concentration (800  $\mu\text{g}/\text{ml}$ ).

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**Table 5.1:** Larvicidal activity of compounds **1**, **2** and **3** from *P. sarmentosum* against 3<sup>rd</sup> and early 4<sup>th</sup> instar mosquito larvae.

Compounds	Tested Insects third instar					
	<i>Aedes aegypti</i> <sup>b</sup>		<i>Aedes albopictus</i>		<i>Culex quinquefasciatus</i>	
	LC <sub>50</sub> (µg/ml) (95% C.I.)	LC <sub>95</sub> (µg/ml) (95% C.I.)	LC <sub>50</sub> (µg/ml) (95% C.I.)	LC <sub>95</sub> (µg/ml) (95% C.I.)	LC <sub>50</sub> (µg/ml) (95% C.I.)	LC <sub>95</sub> (µg/ml) (95% C.I.)
1 <sup>a</sup>	4.3 (3.1 to 5.3)	8.3 (6.7 to 12.8)	6.1 (4.8 to 7.1)	14.4 (13.0 to 17.2)	6.3 (5.0 to 10.3)	15.2 (11.5 to 19.6)
2	4.1 (3.4 to 5.7)	7.12 (6.4 to 10.3)	6.5 (4.6 to 8.1)	15.12 (12.6 to 18.3)	5.8 (4.2 to 9.7)	12.46 (9.8 to 22.7)
3	576 (341 to 769)	1340 (919 to 1763)	648 (487 to 987)	1453 (1224 to 1982)	662 (413 to 887)	1374 (1192 to 2192)
4	NA	NA	NA	NA	NA	NA

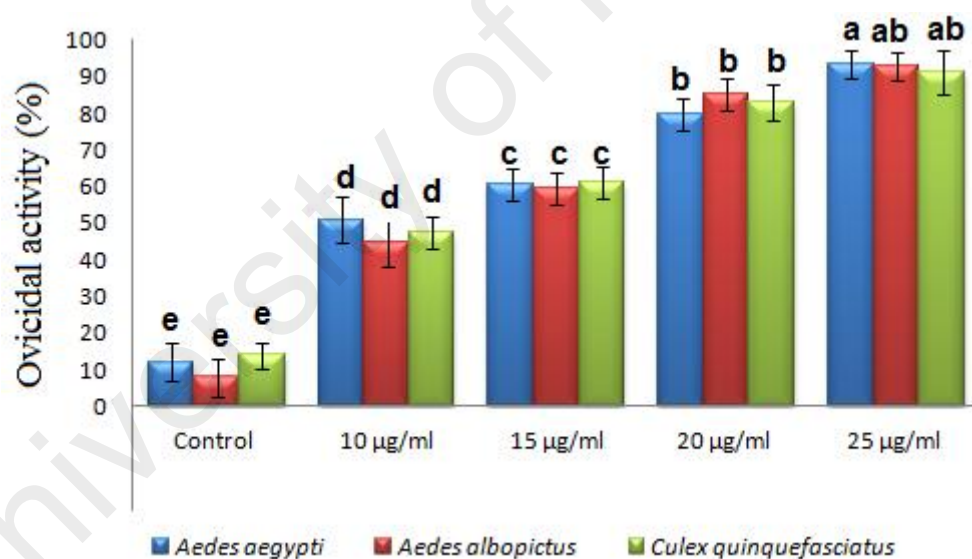
<sup>a</sup> Compounds **1-3** represent phenylpropanoids isolated for *P. sarmentosum* hexane and methanol active fraction (H2).

<sup>b</sup> Test performed on *Ae. aegypti*, *Ae. albopictus* and *Cx. quinquefasciatus* late their and early fourth instar larvae.

<sup>c</sup> LC<sub>50</sub> and LC<sub>95</sub> values significant difference ( $p < 0.05$ ) was based on non-overlap of the 95% CL.

### 5.4.2 Ovicidal Activity

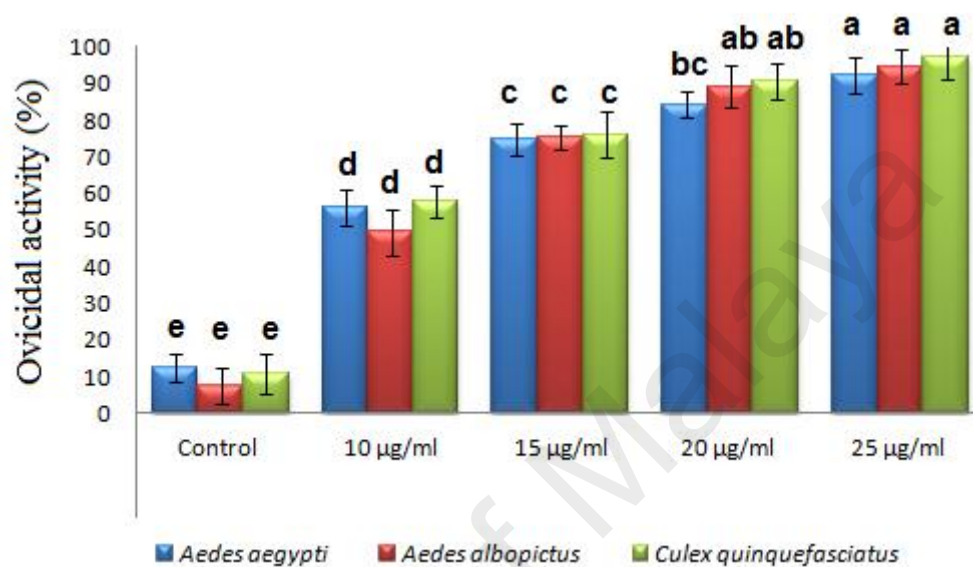
In this part, compounds **1**, **2**, **3** and **4** were studied for use as natural insecticide to inhibit the mosquito eggs from hatching. Statistical differences were observed ( $P < 0.05$ ) among the efficacy averages of percentage for each compound. As shown in Fig 5.14 and 5.15, compounds **1** and **2** showed potent ovicidal activity on *Ae. aegypti*, *Ae. albopictus* and *Cx. quinquefasciatus* at dosage of 25  $\mu\text{g/ml}$ . More than 90% of inhibition was observed at 25  $\mu\text{g/ml}$ . The ovicidal response of *Ae. aegypti*, *Ae. albopictus* and *Cx. quinquefasciatus* towards compound **1** at 25  $\mu\text{g/ml}$  were similar by comparing the mean percentage of the ovicidal activity (Figures 5.16, 5.17). Compounds **3** and **4** had no effect on egg hatchability of the three mosquitoes species tested.



**Figure 5.16:** Ovicidal activity of compound **1** against *Ae. aegypti*, *Ae. albopictus* and *Cx. quinquefasciatus*. Mean ( $\pm$  SE) followed by the same letters in a row indicate no significant difference ( $p < 0.05$ ) according to the Tukey test.

The highest inhibition was observed at the highest dosage (25  $\mu\text{g/ml}$ ). Ovicidal effect of compound **2** on *Ae. aegypti*, *Cx. quinquefasciatus* and *Ae. albopictus* was relatively similar. The inhibition range was 80 to 90% at 20  $\mu\text{g/ml}$ .

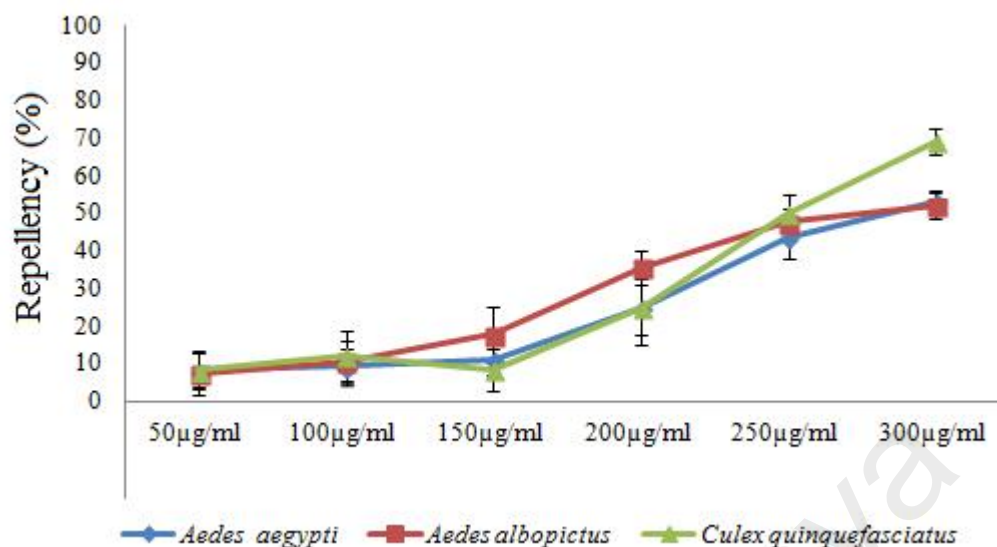
Ovicidal test of compounds **1** and **2** at range of 10 to 25  $\mu\text{g/ml}$  showed similar inhibition on development of egg to larvae. **3** had no effect on egg hatchability of the three mosquito species tested even at higher dosage of 100  $\mu\text{g/ml}$ .



**Figure 5.17:** Ovicidal activity of compound **2** against *Ae. aegypti*, *Ae. albopictus* against *Cx. quinquefasciatus*. Mean ( $\pm$  SE) followed by the same letters in a row indicate no significant difference ( $p < 0.05$ ) according to the Tukey test.

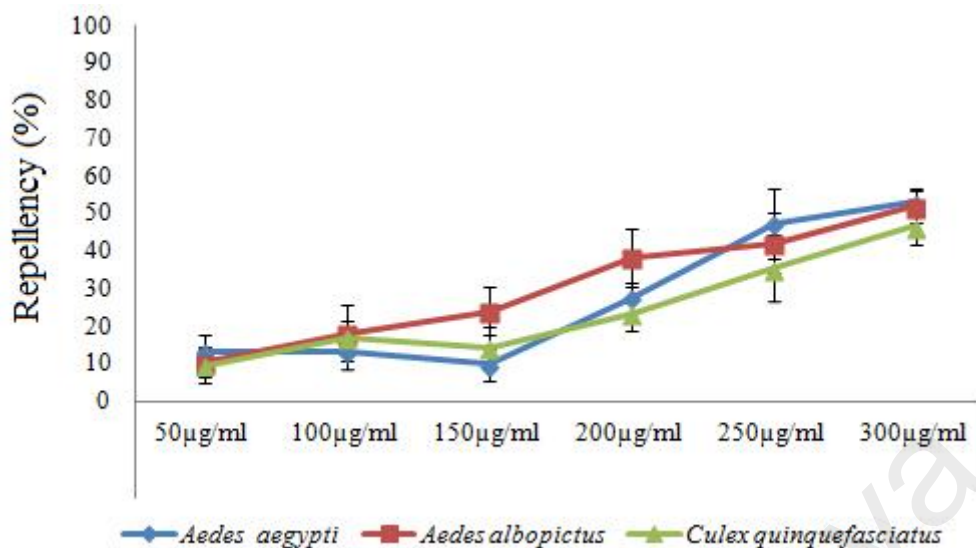
#### 5.4.3 Repellent Activity

Compound **1** was found to be very weak as a repellent agent. Even at high dosage of 300  $\mu\text{g/ml}$ , its repellence activity was below 65%. As shown in Figures 5.16, the repellent activity was very low at the initial stage of exposure.



**Figure 5.18:** Repellent activity of compound 1 on *Ae. Aegypti*, *Ae. albopictus* and *Cx. quinquefasciatus*.

Compound 2 was slightly weaker than compound 1; however, at high dosage of 300 µg/ml, the percentage of repellency was below 55% for *Ae. aegypti* and *Ae. albopictus*. *Cx. quinquefasciatus* showed more sensitivity in this test and it was more susceptible than *Ae. aegypti* and *Ae. albopictus*. (Figure 5.17). Repellent activity of compounds 3 and 4 were not significant and they didn't show any sign of repellency activity. Repellent activity of compounds 1 and 2 between the concentration ranges of 50 to 150 µg/ml was very weak. Only at concentration of 300 µg/ml, it showed some repellent activity which was considered moderate activity.



**Figure 5.19:** Repellent activity of compound 2 on *Ae. Aegypti*, *Ae. albopictus* and *Cx. quinquefasciatus*.

#### 5.4.4 Adulticide Activity

The LD<sub>50</sub> and LD<sub>95</sub> values of *Ae. aegypti*, *Ae. albopictus* and *Cx. quinquefasciatus* adults laboratory strains exposed to various dosage of compounds **1**, **2**, **3** and **4** is shown in Table 5.2. The initial mortality occurred after 24 h of exposure. Compounds **1** and **2** had toxicity effect against *Ae. aegypti*, *Ae. albopictus* and *Cx. quinquefasciatus* adults only at a very high dosage. The LD<sub>50</sub> value range for compounds **1** and **2** for all three mosquitoes was  $\geq 0.3 \mu\text{g/ml}$  (300 ppm). Compounds **3** and **4** did not show any potency even at high dosage of 1000  $\mu\text{g/ml}$ .



**Table 5.2:** Adulticide activity of compounds **1**, **2** and **3** from *P. sarmentosum* against adult female mosquitoes.

Compounds	Adulticide test					
	<i>Aedes aegypti</i>		<i>Aedes albopictus</i>		<i>Culex quinquefasciatus</i>	
	LD <sub>50</sub> (µg /ml) (95% C.I.)	LD <sub>95</sub> (µg /ml) (95% C.I.)	LD <sub>50</sub> (µg /ml) (95% C.I.)	LD <sub>95</sub> (µg /ml) (95% C.I.)	LD <sub>50</sub> (µg /ml) (95% C.I.)	LD <sub>95</sub> (µg /ml) (95% C.I.)
1	380.9 202.6 to 517.6	1033 728.7 to 2968.5	427.7 229.9 to 558.4	1080.4 792.7 to 2724.0	403.1 261.4 to 514.2	1041.6 773.7 to 2113.1
2	354.5 602 to 1146	1012 713.6 to 6841.6	398.6 239.7 to 521.2	1099.585 794.2 to 2514.2	417.2 271.3 to 542.0	1078.4 773.6 to 2591.5
3	NT	NT	NT	NT	NT	NT
4	NT	NT	NT	NT	NT	NT

<sup>a</sup>Compounds **1-3** represent phenylpropanoids isolated for *P. sarmentosum* hexane and methanol active fractions.

<sup>b</sup>Test performed on *Aedes aegypti*, *Aedes albopictus* and *Culex quinquefasciatus* adults.

<sup>c</sup>LC<sub>50</sub> and LC<sub>95</sub> values significant difference ( $p < 0.05$ ) was based on non-overlap of the 95% CL.

Based on the results obtained, compounds **3** and **4** did not have any adulticide activity against mosquitoes. In WHO adulticide assay, all compounds did not show potent adult killing effects towards adults of three tested mosquito species (WHO, 1970). Results of direct application of on mosquito thorax showed that all compounds were not highly potent in its pure form.

## 5.5 DISCUSSION

Pesticides have significant role in public health and agriculture. Nevertheless, misuse and abuse of pesticide have resulted in many negative effects such insecticide resistance and environmental pollution. Botanical pesticides can be a suitable alternative to synthetic insecticides in future as they relatively less toxic and degradable (Senthil Nathan et al., 2007). Extracts of many medicinal plants have toxic effects on different species of vectors such as mosquitoes (Korayem, Hasabo, & Ameen, 1993). Use of locally medicinal plants extracts for mosquito control will reduce dependence on expensive imported products and maximize the local efforts to enhance public health (Korayem, Hasabo, & Ameen, 1993). Although many extracts has been proven to have insecticidal activity, additional research in natural products is needed to investigate main component of these plants for vector control program. *Piper sarmentosum* has been used as spice and vegetable in local cousin and has consum for its medicinal activities as well there for its not toxic to human (Singh, Kaur, Mittal, Batish, & Kohli, 2009). In search for potent bioinsecticide against mosquitoes, compounds **1**, **2**, **3** and **4** were isolated from the *P. sarmentosum* active hexane and methanol extracts. *In vitro* bioassay against *Ae. aegypti*, *Ae. albopictus* and *Cx. quinquefasciatus* larvae proved that compounds **1** and **2** had the strong toxic effect against tested mosquitoes larvae. LC<sub>50</sub> and LC<sub>95</sub> value of compounds **1** and **2** were lower on *Ae. aegypti* compared to two other species. Toxic action of these compounds could be result of unspecified toxicity related

to hydrophobicity and the generation of organic radicals and reactive oxygen species (Aradottir et al., 2014). To clarify the mechanism of action of compounds **1** and **2**, acetylcholinesterase enzyme inhibition activity of both compounds were investigated in Chapter 6. Compounds **1** and **2** showed reliable ovicidal activity in this study against three mosquito species *Ae. aegypti*, *Ae. albopictus* and *Cx. quinquefasciatus*. The toxicity against the adults was at 0.5 mg/ml (500 ppm). This was very low as compared to other natural insecticides and essential oils reported in previous studies (Rajkumar & Jebanesan, 2009). Compounds **1** and **2** did not exhibit strong adulticide activities. More than 90% inhibition of tested mosquito eggs hatchability showed the potential of **1** and **2**. This result supported by Kamaraj et al. (2010) research on ovicidal activity of several extracts and essential oils of Piper family with different chemical constitution. Based on literature review, this is the first report of highly effective larvicidal, ovicidal activities of specific phenylpropanoids compounds from *P. sarmentosum*. Extracts from *P. sarmentosum* plants could thus represent a promising tool in the management and control of represented vectors. *P. sarmentosum*-derived extracts may not cause any hazard to other beneficial organism based on its food usage (Zainal Ariffin et al., 2009; Hussain, Ismail, Sadikun, & Ibrahim, 2010). Each of these compounds has the potential to be used and formulated for generation of novel biopesticide. Further studies on the active compounds including mode of action and interaction of the activity under field condition are needed.

## 5.6 CONCLUSION

Mosquito vectors population control has been a big challenge facing all over the world especially in tropical country. Using plants bioactive compounds has the potential to enhance current control measures, and possibly contribute towards long term control of mosquito populations. The experiments shown in Chapters 4 illustrate the potential of

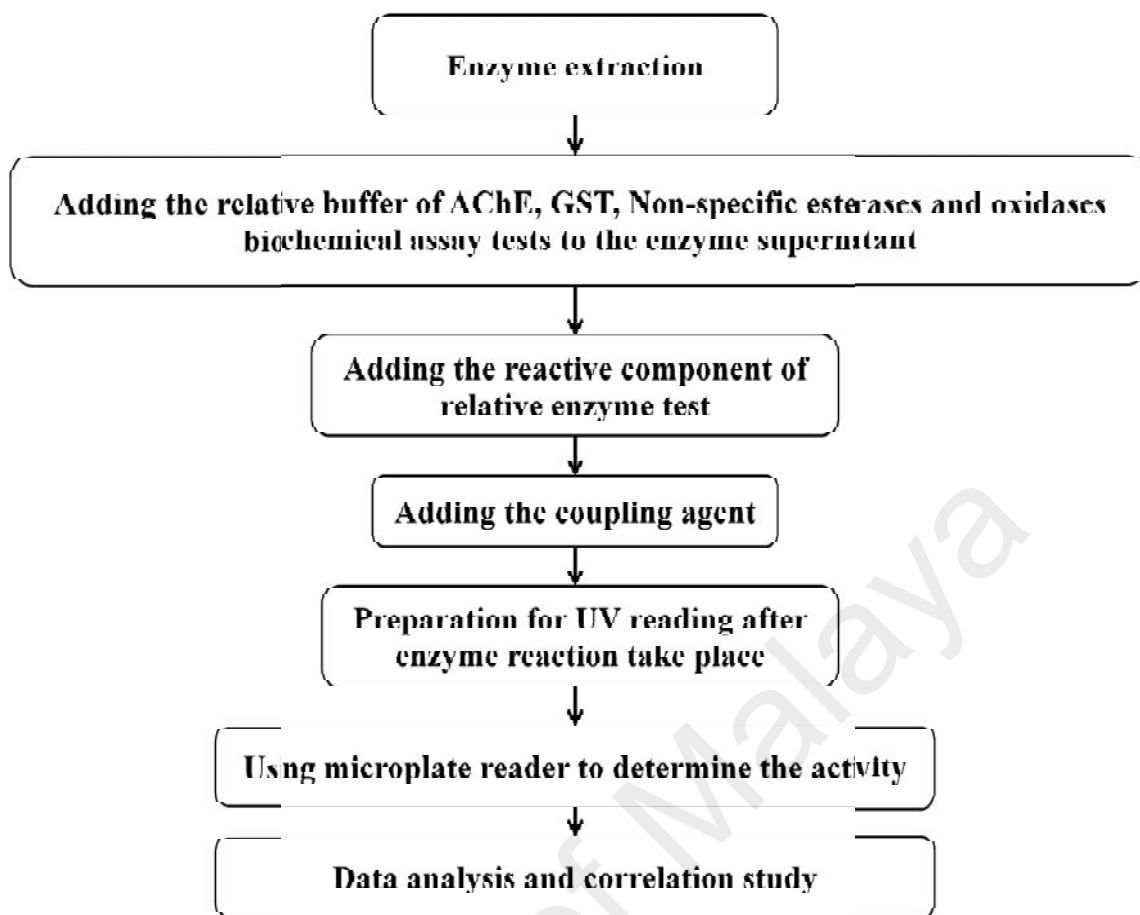
*P. sarmentosum* bioactive agents to have chronic effects upon mosquito larvae. These chronic effects could be used to establish long term mosquito control by using integrated pest management (IPM) techniques through direct application (Skinner, Parker & Kim, 2014; Abrol, 2014). Compounds **1** and **2** were the most potent component with strong larvicidal activity in this study against *Ae. aegypti*, *Ae. albopictus* and *Cx. quinquefasciatus* larvae. The ever increasing emphasis on developing environmentally friendly pest control agents can bring these two compounds for further investigation. Its possible that compound **3** be useful in mixture for additive or synergistic activity because its molecular structure. Results of this chapter suggested that compounds **1** and **2** from hexane and methanol extracts are promising as larvicide and ovicidal agents against *Ae. aegypti*, *Ae. albopictus* and *Cx. quinquefasciatus*. The rapid mortality as indicated by the LC<sub>50</sub> and LC<sub>95</sub> value of the mosquito larvae and their ovicidal activity has proved that these compounds have the potential to be used as control agents for vector-borne diseases especially from the mosquitoes. However, further research is needed before a new botanical pesticide can be implemented. Besides, studies regarding stability and their impacts on human health and non-target organisms in mosquito feeding habitats are also needed.

## CHAPTER 6: STUDY ON THE EFFECT OF ACTIVE PHENYLPROPANOIDS ON ESTERASE ACTIVITY

### 6.1 INTRODUCTION

The isolated phenylpropanoids: asaricin **1** and isoasarone **2** had strong insecticidal activity against storage pests and mosquito vectors and *trans*-asarone **3** activity was moderate (Chapters 4 and 5). Although their activity was potent but the level of their toxicity were varied through the tested insects. This may due to their mechanism of action and the insets response. In this chapter several biochemical assays were conducted to help on the better understanding of their mechanism of action. Investigation on the esterase enzymes involved in toxicity of toxicants and insects responds. This will give wider vision for future research to help controlling the storage products and mosquito vectors.

The purpose of this biochemical study was to study the mechanism of action of isolated toxic phenylpropanoids **1**, **2** and **3** on tested insects. In addition, this test also elucidated the enzymology of the level of resistance related to enzymes of the species studied. Statistical correlation study was conducted to understand the relation between acetylcholinesterase, glutathione S-transferases, oxidases and none specific esterases activity and lethal concentration dose of **1**, **2** and **3** (Figure 6.1).



**Figure 6.1:** General procedures of biochemical assay for enzymes activity.

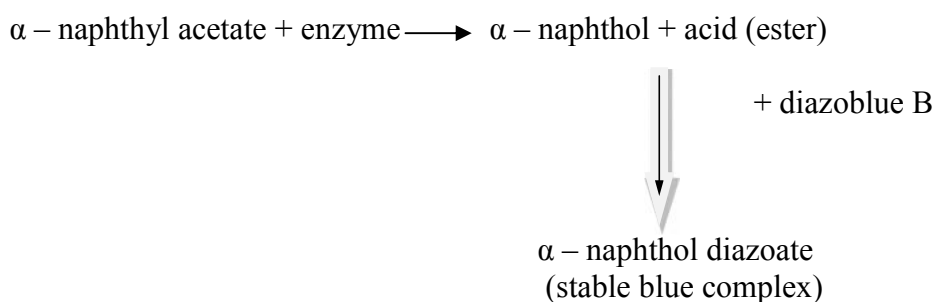
## 6.2 LITERATURE REVIEW

Esterase enzymes have many functions in insects including proteolysis, nervous system function, hormone metabolism and xenobiotic metabolism sequestration (Prapanthadara, Promtet, Koottathep, Somboon, & Ketterman, 2000). Studies on the insecticides mechanisms of action have indicated the specific relevance of esterases with regard to xenobiotic metabolism in several insect species (Malcolm, 1988). The esterases (Cholinesterase and Carboxylesterase) constitute a large group of enzymes which occur in insects. These enzymes are involved in the detoxification and resistance mechanism towards organophosphate and carbamates, for example in the *Aedes aegypti* (Malone, Gatehouse, & Barratt, 2008), spider mites (Zhu & He, 2000), *Ceratitidis capitata* (Wiedemann) and *Musca domestica* (Ugaki et al., 1985). Generally, any

change in level of enzymes involved in detoxification in insects can lower or mutual the toxic effect of the substance. Present trends in biochemical genetics and the impetus provided by molecular biology emphasize the nature of genes and proteins as dynamic process undergoing constant evolutionary changes (Chen, Han, Qiao, & Qu, 2007). For example Acetylcholinesterase (AChE) belongs to the class of enzymes in the nervous systems and most of organophosphate family activity is by inhibiting it (Kotze et al., 2014.). In addition to these effects, insecticides can also trigger a response from enzymes such as glutathione S-transferases (GST) oxidases and non-specific esterases for detoxification in insects. It has been proved that resistance to some insecticides is associated with a higher level of glutathione S-transferases (GST) oxidases and non-specific. The assumption is that the insecticide is inactivated by hydrolysis with higher level of enzyme produced by resistant strain compared to the susceptible strain (Kotze et al., 2014; Neufeind et al., 1997).

The enzymes can be studied by following their action on substrate. For example, ( $\alpha$  – naphthyl acetate) is split into an ester ( $\alpha$  – naphthol) and acid which may be coupled with a variety of diazonium salts to form highly stable diazoate blue complex (Hemingway et al 1983). Spectrophotometric methods can be used to measure the activity of esterases, estimated by measuring these insoluble dyes.

The reaction of the substrate towards the enzyme is as follows:



Thus, more sensitive and definitive methods of detection must be used. Several biochemical assay techniques especially those based on microassay have been developed. Biochemical techniques are essential based on the detection and quantification of enzymes known to have interaction with insecticide and responsible for resistance (Rajendran et al., 2001).

Researchers developed such test using microplate system to measure acetylcholinesterase (AChE), glutathione S-transferases (GST), oxidases and non-specific esterases activities in insects as small biological samples. The more recently developed biochemical microassay technique can detect the enzyme reaction with insecticides (Lee et al., 1990; Scemes et al., 1982) This method has been used to study the insecticides mechanisms of action in individual insects and this facilitates confirmation of resistance in the few survivors from bioassay test (Liu et al., 2013; Maestre-Serrano, Gomez-Camargo, Ponce-Garcia, & Flores, 2014). Several other microassay works have been conducted on enzyme using microplate technique. Detection of metabolic-based on mechanism of action with this technique is very accurate; however, it requires a microplate reader (Hemingway, 2000).

## **6.3 MATERIALS AND METHODS**

### **6.3.1 Strains**

The mechanism of action of isolated toxic compounds on non-specific esterase present in adults of storage pests (*S. oryzae* and *R. dominica*) and mosquito vectors larvae (*Ae. aegypti*, *Ae. albopictus* and *Cx. quinquefasciatus*) were determined using biochemical microplate assay. Esterase assay was conducted as described by (Hemingway, 2000; Barry & Polavarapu, 2004; Lee et al., 1990). All strains were collected from the laboratory strain which were used in bioassay studies. Insects were



subjected for enzyme microassays study to detect the toxic compound mechanism of action on each insect. Adults of *S oryzae* and *R. dominica* and larvae of *Ae. aegypti*, *Ae. albopictus* and *Cx. quinquefasciatus* were used in this study. Each insect was studied for its non-specific esterases, glutathione S-transferases, acetylcholinesterase and oxidases activity. The assay was carried out according to the methods described by (Hemingway, 2000; Barry & Polavarapu, 2004; Bass et al., 2014). Biochemical tests were conducted on fresh material prepared for assays. For each assay, 30 individual insects from each group were used in 4 replicates.

### **6.3.2 Preparation of Enzyme Test Solution for Acetylcholinesterase**

To prepare buffer for Acetylcholinesterase (AChE), 4.735g of  $\text{Na}_2\text{HPO}_4$  dissolved in 500 ml distilled  $\text{H}_2\text{O}$  then 4.540 g of  $\text{KH}_2\text{PO}_4$  dissolved in 500 ml distilled  $\text{H}_2\text{O}$  to form potassium phosphate buffer (pH 6.8). Substrate was formed using 75 mg Acetylcholine iodine (ACTHI) (Sigma, United States) then dissolved in 10 ml acetone and further added 90 ml of 0.5 M potassium phosphate buffer (pH 6.8). Coupling Reagent was formed using 13 mg of 5, 5- dithiobis 2-nitrobenzoic acid (DTNB) (Sigma, United States) dissolved in 100 ml of potassium phosphate buffer (pH 6.8). asaricin **1**, isoasarone **2** and *trans*-asarone **3** dissolved in acetone with their relative  $\text{LC}_{95}$  values were prepared as inhibitors. The inhibitors were prepared – ACTHI concentration.

### **6.3.3 Preparation of Enzyme Test Solution for Glutathione S-transferases**

Buffer for GST enzyme activity assay was prepared using 1.816 g of  $\text{KH}_2\text{PO}_4$  dissolved in 200 ml distilled  $\text{H}_2\text{O}$  as solution (A) and 0.947 g of  $\text{Na}_2\text{HPO}_4$  dissolved in 100 ml distilled  $\text{H}_2\text{O}$  as solution (B). Finally, 160.8 ml of solution A was added to 39.2 ml of solution B to form 200 mL of 0.5M potassium phosphate buffer (pH 7.4).

Substrate for GST assay was prepared using 0.03 g glutathione (Sigma, United States) which was dissolved in 50 ml of 0.5M potassium phosphate buffer (pH 7.4). The Coupling Reagent was prepared in 0.01 g of 1- chloro-2,4-dinitrobenzene (CDNB) (Sigma, United States) then dissolved in 0.5 ml of 0.5M potassium phosphate buffer (pH 7.4) (Figure 6.2).



**Figure 6.2:** *S. oryzae* acetylcholinesterase biochemical detection before adding compounds **1**, **2** and **3** as inhibitors.

#### 6.3.4 Preparation of Enzyme Test Solution for Non-specific esterases (EST)

Non-specific esterases was performed by adding 4.5 g of  $\text{Na}_2\text{HPO}_4$  (Sigma, United States) to 1.70 g of  $\text{KH}_2\text{PO}_4$  (Sigma, United States) and dissolved them in 500 ml of distilled  $\text{H}_2\text{O}$  to give 2.0 M potassium phosphate buffer (pH 7.6). Substrate was assisted using 0.06 g  $\alpha$ - naphthyl acetate (Sigma, United States) which dissolved in 10 ml acetone to form 0.5 ml of stock substrate, then diluted in 50 ml of 0.2 M potassium phosphate buffer (pH 7.0). Coupling Reagent was prepared from 0.875 g of sodium dodecyl sulphate (SDS) (Sigma, United States) and 0.075 g of fast blue salt (FBS) (tetrazotised *o* – dianisidine) (Sigma, United States) which was dissolved in 50 ml of distilled  $\text{H}_2\text{O}$ . Non-specific esterases biochemical assay need stopping solution which made from 10 ml of 10% acetic acid diluted in 90 ml distilled  $\text{H}_2\text{O}$ .

### 6.3.5 Preparation of Enzyme Test Solution for Oxidases

Buffer for Oxidases biochemical assay was prepared by dissolving 10.25 g of  $\text{CH}_3\text{COONa}$  (0.25) (Sigma, United States) in 500 ml distilled  $\text{H}_2\text{O}$  with pH 5.0 and the pH was adjusted using acetic acid. Substrate was prepared using 3,3',5, 5'-tetramethylbenzidine (TMBZ) (Sigma, United States). The substrate was prepared fresh by dissolving 50 mg TMBZ in 25 ml absolute methanol and adding 75 ml 0.25 M sodium acetate buffer (pH5.0). Coupling Reagent was prepared from 3% hydrogen peroxide (commercial grade) (Sigma, United States).

### 6.3.6 Preparation of Enzyme Homogenates

Individual insects of their relative life stages used in bioassay in Chapter 4 and 5 were homogenized in 100  $\mu\text{l}$  of respective enzyme in wells porceline plate using a glass rod. Microplate assay test was carried out on surface of smashed ice because pigments in the mosquito homogenates oxidizes rapidly (within minutes) to produce a black-coloured solution in which the esterase quickly lost its activity. For the larvae, samples were transferred onto several layers of tissue paper in order to drain out excess water from samples body surface before homogenizing in micro centrifuge tube.

The homogenates solution were diluted into a final volume of 500  $\mu\text{l}$  with same respective buffer and transferred to microcentrifuge tubes. The mixture centrifuged at 14 000 rpm for 10 min at 4 °C. The supernatant served as the enzyme source. In the case of oxidase, tested insects were homogenized in 100  $\mu\text{l}$  sodium acetate buffer in microcentrifuge tubes using plastic pestles. Homogenates were diluted further to 1 ml with additional buffer centrifuged at 14 000 rpm for 10 min and supernatant used as enzyme source.

### **6.3.7 Acetylcholinesterase**

Fifty  $\mu\text{L}$  of the respective supernatant for the enzyme AChE were aliquot into each microplates well. Fifty  $\mu\text{L}$  aliquot mixture of 10% acetone-buffer solution of acetylthiocholine iodide (ACTHI) plus each of active compound with their relative  $\text{LC}_{95}$  were added in to test well in separated rows (Table 4.2 and 5.3). This was followed by adding of 50  $\mu\text{L}$  aliquot of DTNB using micropipette. Yellowish or colourless solution was observed and reaction for 30 min at room temperature. The reading was done immediately by (Synergy H1 Hybrid Multi-Mode Microplate Reader) at 410 nm optical density value were obtained. Another set of supernatant was loaded with 50  $\mu\text{L}$  of ACTHI and DTNB solution only which served as control. The inhibition of acetylcholinesterase activity by isolated compound was determined by the method of Hemingway et al (1983) on individual insects.

### **6.3.8 Glutathione S- transferases**

Fifty  $\mu\text{L}$  of respective supernatant for enzyme GST was added to microplate well. Fifty  $\mu\text{L}$  aliquot of glutathione (substrate and 50 $\mu\text{L}$  1-choloro-2, 4- dinitrobenzene (CDNB- coupling reagent) were added using single channel micropipetter. Yellowish colour was observed and the reaction was incubated at room temperature for 30 min. The colour intensities were read in a microplate reading spectrophotometer at 410 nm and optical density value were obtained.

### **6.3.9 Non-specific Esterases Procedures**

A total of four replicate aliquots of 50 ml of the supernatant from each sample was transferred into a microplates well. Using the four replicate aliquots were made available from each single tested insects. Fifty microliter of  $\alpha$ - naphthyl acetate (substrate) was added into each individual sample and left for 60 sec. This procedure

was followed by adding 50 µl coupling agent Fast Blue salt (FBS) and sodium dodecyl sulphate (SDS) for colour indication. Immediately dark pinkish colour developed which will turn to blue after incubating at room temperature ( $27 \pm 2$  °C). This was due to hydrolysis of  $\alpha$ - naphthyl acetate into of  $\alpha$ - naphthol which reacted with FBS, thus producing a change in absorbance of the solution. The enzyme reaction was then allowed to run for 10 min. The intensity of the final colour indicated the esterase activity. As soon as appearance of colour reaction, the colour intensity result was expressed quantitatively as an absorbance of optical density (O.D.) using Synergy H1 Hybrid Multi-Mode Microplate Reader. For stopping the reaction 50 ml of 10% acetic acid added to each well to stop the reaction and preserve it for colour documentation optical density readings were pooled together and analyzed by using computer programs. The activity was measured spectrophotometrically after 10 min using an immunoassay reader. The intensity of the final colour was indicative of the presence level on non- specific esterases.

To study the mechanism of action, 100 µg of each toxic compound (compounds **1** and **2**) was prepared in methanol. Each reaction well in microplates containing enzyme was incubated with 50 µl of each inhibitor for 10 min to allow the inhibitors to react before the addition of substrate and coupling reagent. The activity was measured after 10 min as it described with immunoassay reader.

### **6.3.10 Oxidases**

To 50 µL of each insects homogenate, 200 µL of 3,3',5,5'-tetramethylbenzidine (TMBZ) solution were added in assay wells followed by 25 µL of 3% hydrogen peroxide. TMBZ act as a substrate and hydrogen peroxide was added to initiate heme peroxidation reaction. The reactions were incubated for 5 min at room temperature (27

°C). The colour intensities were read in microplate reader spectrophotometer (Synergy H1 Hybrid Multi-Mode Microplate Reader) at test wavelength of 630 nm. The results were expressed as optical density reading.

### 6.3.11 Data Analysis and Interpretation

Enzyme assays were performed to estimate the relative mechanism of activity or quantity of suspected enzymatic reaction of *P. sarmentosum* isolated toxic compounds. To determine level of non-specific esterase present in storage pests and mosquito vectors microassay technique used which developed by Lee (1990). Percentage of inhibition calculated based on the mean optical density of enzyme as below:

$$\text{Inhibition\%} = \left| 1 - \left( \frac{\text{Absorbance sample} - \text{Absorbance of background}}{\text{Absorbance of blank} - \text{Absorbance of background}} \right) \right| \times 100$$

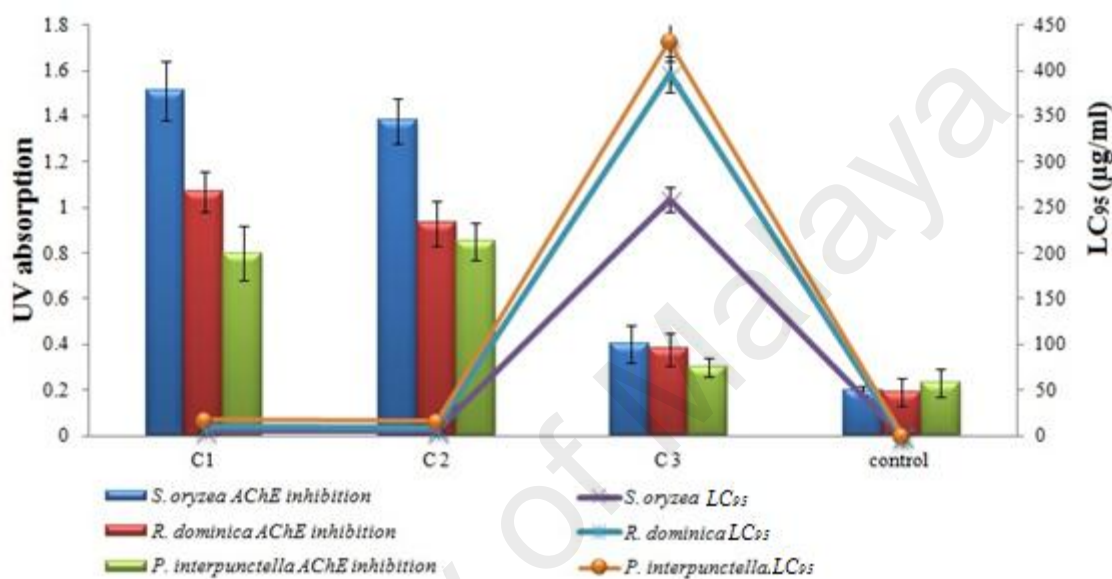
Significance of results was evaluated by one-way ANOVA with Tukey test. The resulting data for each selected insects were analyzed using correlation study compare the enzyme expression levels between different insects in respond to **1**, **2** and **3**. All levels of statistical significant were determined at  $P < 0.05$ .

## 6.4 RESULTS

### 6.4.1 Acetylcholinesterase Assay on Storage Pests

The results of *S. oryzae*, *R. dominica* and *P. interpunctella* acetylcholinesterase (AChE) inhibition assay by compounds **1**, **2** and **3** are presented in Figure 6.3. Biochemical assay on the AChE inhibition of **1**, **2** and **3** between the tested subjects were significantly different ( $p < 0.05$ ). Level of AChE inhibition by compounds **1** and **2** were relatively high compared to compound **3** in all tested subjects (Figure 6.4). The mean percentage AChE inhibition of **1** and **2** in *S. oryzae* was 216% and 176%

respectively which were significantly higher than *R. dominica* and *P. interpunctella*. The *R. dominica* AChE mean inhibition activity by compounds **1** and **2** were 167.5% and 132.5%, relatively high compare to control but lower than *S. oryzae*. The results for *P. interpunctella* mean percentage of AChE inhibition was not significantly different from *R. dominica*.



**Figure 6.3:** Inhibition on acetylcholinesterase enzyme by compounds **1**, **2** and **3** in comparison with their relative LC<sub>95</sub> value on *S. oryzae*, *R. dominica* and *P. interpunctella*.

The correlation study between the LC<sub>95</sub> and mean percentage of AChE activity was significant ( $p > 0.05$ ). The results of correlation coefficient is presented in Table 6.1. The correlation study on *S. oryzae* AChE enzyme was meaningful and negative for **1** and **2** (correlation coefficient ( $r$ ) of **1**: - 0.920 and **2**: -0.946) (Table 6.2). *Rhizopertha dominica* correlation coefficient of compound **1** was  $r = -0.859$  and compound **2** was  $r = -0.910$ , both had negative and significant correlation. Compound **3** correlation was negative and significant as well ( $r = -0.833$ ). *Plodia interpunctella* correlation study was similar to *S. oryzae* and *R. dominica* negative and significant. These results suggested

that there was significant and negative relationship between AChE inhibition and LC value in *S. oryzae* and *R. dominica* adults and *P. interpunctella* larvae.

**Table 6.1:** Correlation coefficients between *S. oryzae*, *R. dominica* and *P. interpunctella* AChE inhibition and the relative LC<sub>95</sub> of compounds **1**, **2** and **3**.

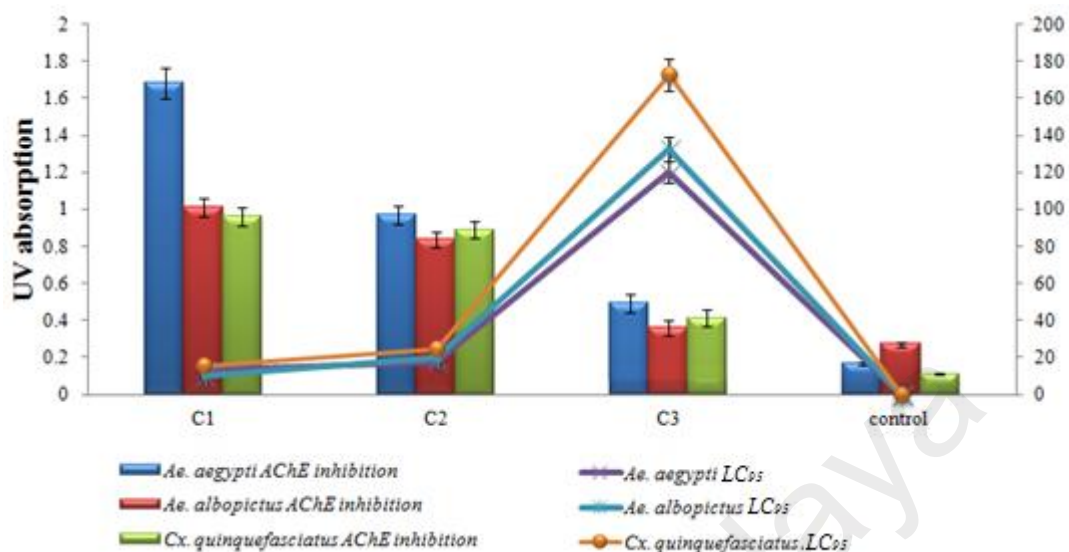
AChE enzyme	Correlation coefficient		
	1	2	3
<i>S. oryzae</i>	- 0.920	-0.946	-0.866
<i>R. dominica</i>	-0.859	-0.910	-0.833
<i>P. interpunctella</i>	-0.953	-0.938	-0.863

The de-attenuated correlation coefficient was calculated using  $r = \frac{n(\sum xy) - (\sum x)(\sum y)}{[\sum x^2 - (\sum x)^2/n][\sum y^2 - (\sum y)^2/n]}$  x represent percentage of AChE mean percentage activity, y represent relative LC<sub>95</sub> of compounds **1**, **2** and **3** against *S. oryzae*, *R. dominica* and *P. interpunctella*.

#### 6.4.2 Acetylcholinesterase Assay on Mosquito Vectors Larvae

The *Ae. aegypti*, *Ae. albopictus* and *Cx. quinquefasciatus* larvae AChE inhibition in contrast with their relative **1**, **2** and **3** LC<sub>95</sub> value represented in Figure 6.4. The absorbance results showed high percentage of inhibition in larvae of *Ae. aegypti*, *Ae. albopictus* and *Cx. quinquefasciatus* AChE enzyme by compounds **1** and **2**. However, it was noted that compounds **3** exhibited lower inhibition of AChE at the same dosage (Figure 6.4). Compounds **1** and **2** mean AChE inhibition on *Ae. aegypti* enzyme in comparison with control was 433% and 426% higher than control. The *Ae. albopictus* and *Cx. quinquefasciatus* AChE enzyme inhibition percentage was similar to *Ae. aegypti* without any significant difference. Compound **3** mean AChE enzyme inhibition was lowest between the tested compounds with 60% on *Ae. aegypti*, 53% on *Ae. albopictus* and 32% on *Cx. quinquefasciatus*. Correlation study confirmed significant and negative correlation between the LC value and the AChE inhibition by **1**, **2** and **3**. *Ae. aegypti* correlation of compound **1** LC<sub>95</sub> and AChE mean activity was significant and negative ( $p > 0.05$ ,  $r = -0.961$ ). Similarly, compound **2** had meaningful negative correlation ( $r = -0.814$ ) (Table 6.2).





**Figure 6.4:** Inhibition on acetylcholinesterase enzyme by compounds **1**, **2** and **3** in comparison with their relative LC<sub>95</sub> value on *Ae. aegypti*, *Ae. albopictus* and *Cx. quinquefasciatus*.

Correlation study on *Ae. albopictus* and *Cx. quinquefasciatus* AChE interaction with **1**, **2** and **3** were similar (Table 6.2). Correlation of **1**, **2** and **3** with AChE inhibition was negative and significant against all three tested vectors larvae. The AChE assay result for all three compounds was similar to storage pests. This result indicated that there was meaningful and negative correlation between LC<sub>95</sub> value and AChE inhibition mean activity in *Ae. aegypti*, *Ae. albopictus* and *Cx. quinquefasciatus* larval stage (Figure 6.4)

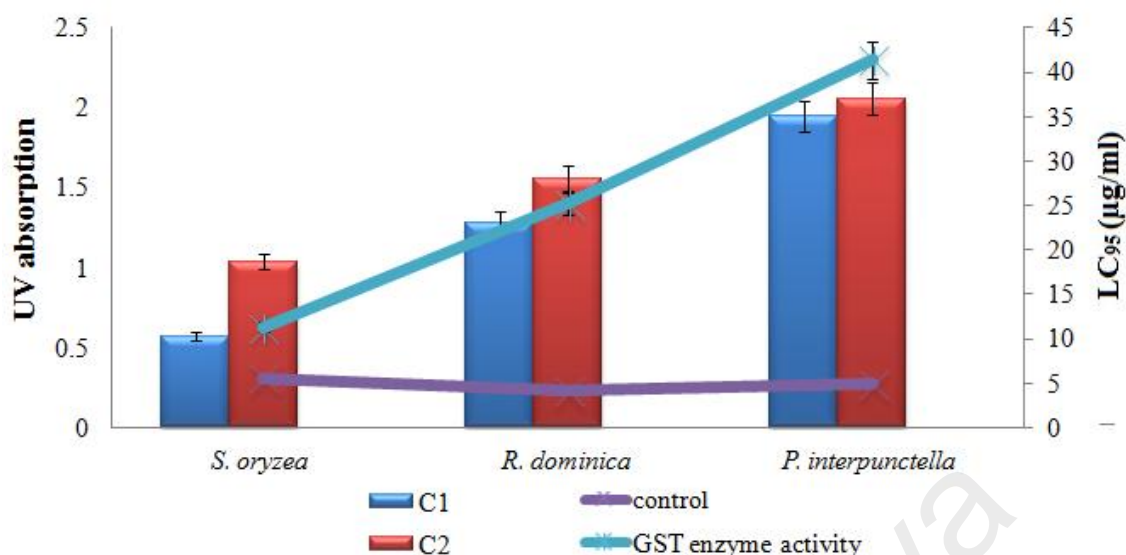
**Table 6.2:** Correlation coefficients between *Ae. aegypti*, *Ae. albopictus* and *Cx. quinquefasciatus* AChE inhibition and the relative LC<sub>95</sub> of compounds **1**, **2** and **3**.

AChE enzyme	Correlation coefficient		
	1	2	3
<i>Ae. aegypti</i>	-0.961	-0.814	-0.887
<i>Ae. albopictus</i>	-0.859	-0.943	-0.933
<i>Cx. quinquefasciatus</i>	-0.983	-0.974	-0.863

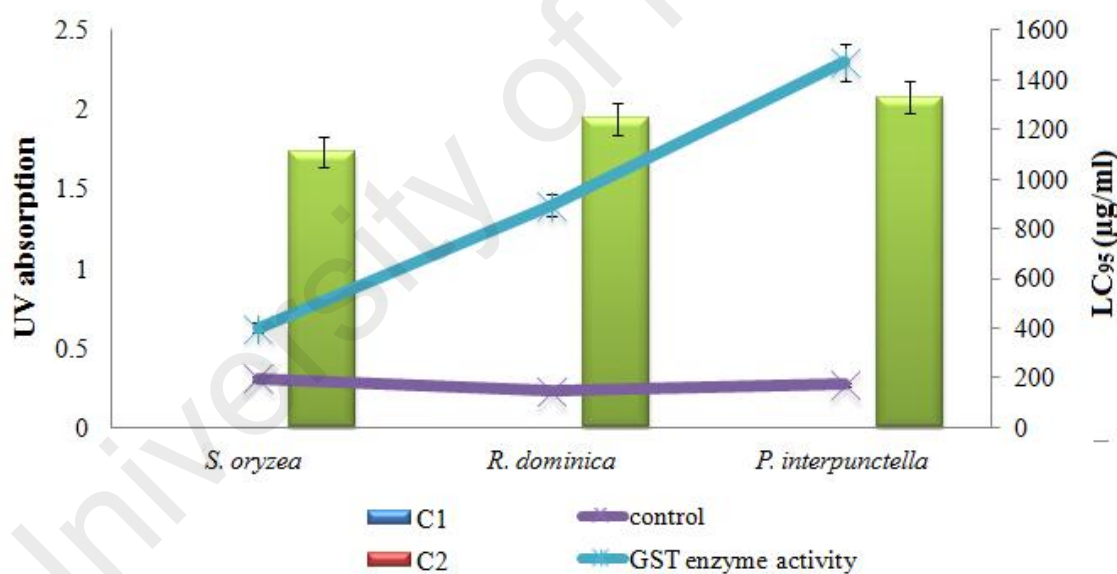
The de-attenuated correlation coefficient was calculated using  $r = \frac{n(\sum xy) - (\sum x)(\sum y)}{[\sum x^2 - (\sum x)^2/n][\sum y^2 - (\sum y)^2/n]}$  x represent percentage of AChE mean percentage activity, y represent relative LC<sub>95</sub> of compounds **1**, **2** and **3** against *S. oryzae*, *R. dominica* and *P. interpunctella*.

#### 6.4.3 Glutathione S-transferases in Storage Pests

The mechanism of insects resistance and enzyme detoxification upon the activity of glutathione S-transferase (GST) to compounds **1**, **2** and **3** as an bioinsecticide were studied by determining the percentage of GST activity with biochemical enzyme microassay. The GST activity in each tested insects enzyme (*S. oryzae*, *R. dominica* and *P. interpunctella*) was determined using microplates reader 10 min after treatment. Results showed significant alteration of the GST activity in contrast to toxicity of tested compounds. However, the GST mean percentage enzyme's activity in *R. dominica* was significantly higher than *S. oryzae* with 34%. *P. interpunctella* had the highest mean GST enzyme activity with 24.6% higher activity than *R. dominica* and 67% higher than *S. oryzae*. UV absorbance of the homogenate enzyme in *S. oryzae*, *R. dominica* and *P. interpunctella* in contrast with LC<sub>95</sub> value is shown in Figure 6.5 and 6.6. The correlation study for GST activity showed high involvement of GST in resistance ratio of LC<sub>95</sub> activity.



**Figure 6.5:** Glutathione S-transferase enzyme activities of *S. oryzae*, *R. dominica* and *P. interpunctella* in comparison of their relative 1 and 2 relative LC<sub>95</sub> value on.



**Figure 6.6:** Glutathione S-transferase enzyme activities of *S. oryzae*, *R. dominica* and *P. interpunctella* in comparison of their relative 3 relative LC<sub>95</sub> value on.

The correlation study for GST activity showed high involvement of GST in resistance ratio of LC<sub>95</sub> activity. Correlation activity of GST showed significant ( $p > 0.05$ ) positive correlation (higher GST activity resulted higher LC<sub>95</sub>). Correlation of GST enzyme activity with compounds 1, 2 and 3 are calculated as r values presented in

Table 6.3 respectively. Similarly, *S. oryzae*, *R. dominica* and *P. interpunctella* showed relatively significant correlation of their level of GST activity with their resistance to the toxicant. *S. oryzae* correlation of **1**, **2** and **3** relative LC<sub>95</sub> with GST mean activity was significant and positive ( $p > 0.05$ ,  $r = 0.972$ ,  $r = 0.833$  and  $r = 0.852$ ) similar to *R. dominica* and *P. interpunctella* (Table 6.3). On other hand the elevated GST activity showed that the more susceptible individuals of this stage had lower GST enzyme activity (Figures 6.5 and 6.6).

**Table 6.3:** Correlation coefficients between *S. oryzae*, *R. dominica* and *P. interpunctella* GST activity and the relative LC<sub>95</sub> of compounds **1**, **2** and **3**.

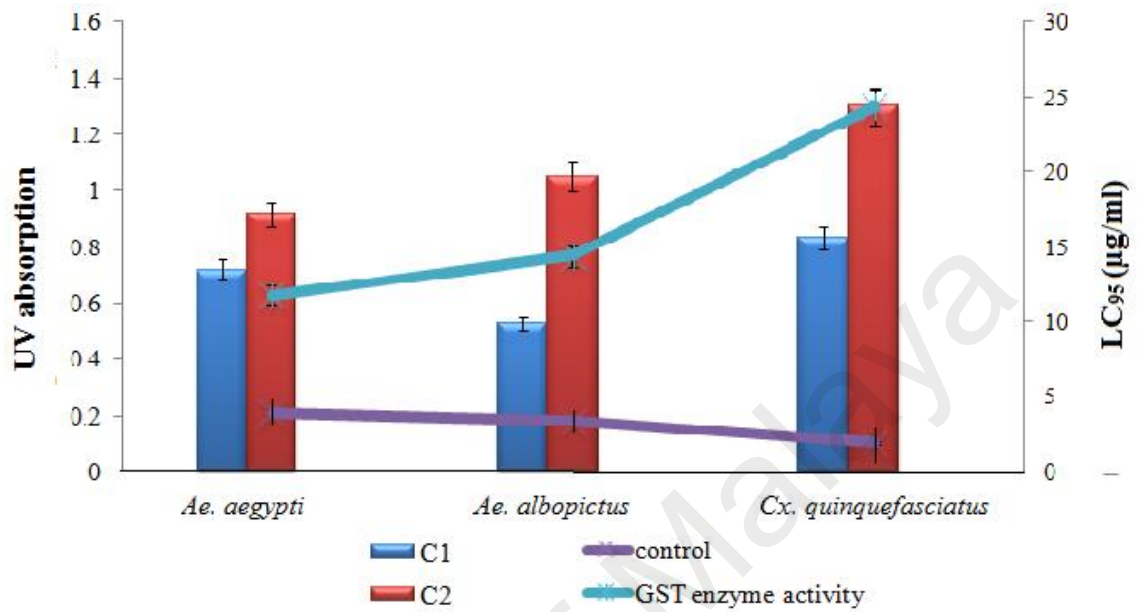
GST enzyme	Correlation coefficient		
	1	2	3
<i>S. oryzae</i>	0.972	0.833	0.852
<i>R. dominica</i>	0.853	0.990	0.975
<i>P. interpunctella</i>	0.830	0.846	0.942

The de-attenuated correlation coefficient was calculated using  $r = \frac{n(\sum xy) - (\sum x)(\sum y)}{[\sum x^2 - (\sum x)^2/n][\sum y^2 - (\sum y)^2/n]}$ , x represent percentage of GST mean percentage activity, y represent relative LC<sub>95</sub> of compounds **1**, **2** and **3** against *S. oryzae*, *R. dominica* and *P. interpunctella*.

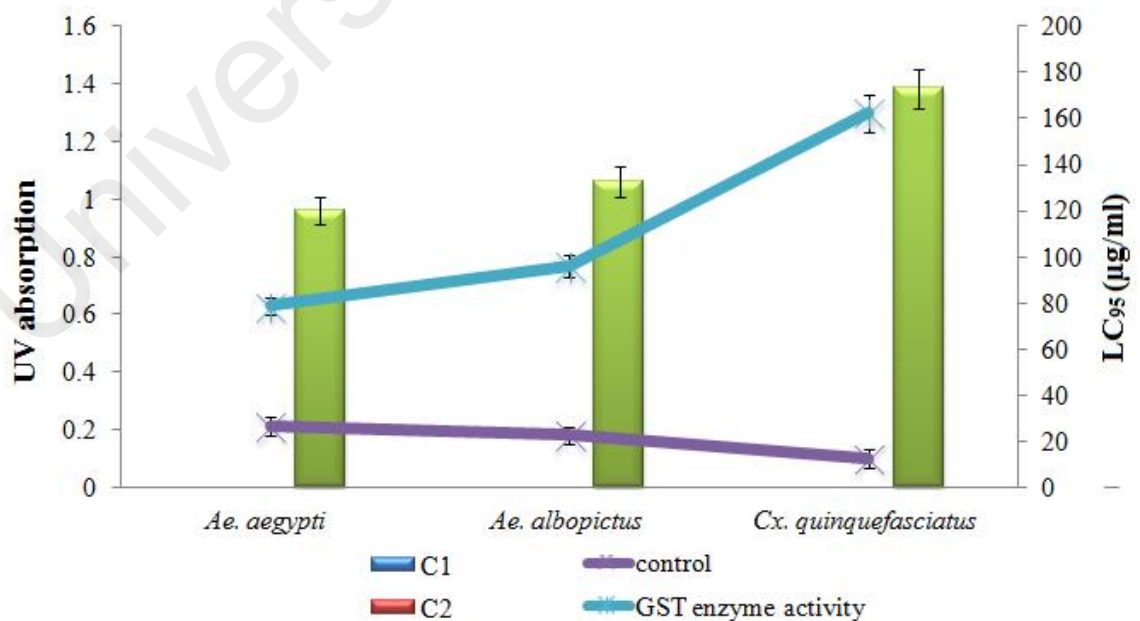
#### 6.4.4 Glutathione S-transferases in Mosquito Larvae

The glutathione S-transferase (GST) activities during the larvae stage of the mosquitoes were studied in same way as storage pest and results shown in Figure 6.7 and 6.8. The data were expressed as mean percentage activities due their specific absorption level in comparison with the control. Previous assay on storage pest showed relative effect of GST on resistance to toxicity of each compound. The specific activities between mosquitoes larvae were relatively high. There was no significant difference between the levels of GST activity between *Ae. aegypti* and *Ae. albopictus*. *Cx. quinquefasciatus* mean GST activity was higher than *Ae. aegypti* and *Ae. albopictus* up

to 35%. This result could explain the higher LC value in *Cx. quinquefasciatus* compare to *Ae. aegypti* and *Ae. albopictus*.



**Figure 6.7:** Glutathione S-transferase enzyme activities of *Ae. aegypti*, *Ae. albopictus* and *Cx. quinquefasciatus* in comparison of their relative 1 and 2 relative LC<sub>95</sub> value.



**Figure 6.8:** Glutathione S-transferase enzyme activities of *Ae. aegypti*, *Ae. albopictus* and *Cx. quinquefasciatus* in comparison of their relative 3 relative LC<sub>95</sub> value

The correlation study for mean GST activity confirmed the involvement of GST in resistance ratio. Correlation study of mean activity of GST of *Ae. aegypti* and *Ae. albopictus*. *Cx. quinquefasciatus* with their relative LC<sub>95</sub> was significantly ( $p > 0.05$ ) positive. Correlation coefficient of *Ae. aegypti*, *Ae. albopictus* and *Cx. quinquefasciatus* with compounds **1**, **2** and **3** are demonstrated in Table 6.4. *Ae. aegypti* correlation of **1**, **2** and **3** relative LC<sub>95</sub> with GST mean activity was significant and positive ( $p > 0.05$ ,  $r = 0.840$ ,  $r = 0.947$  and  $r = 0.930$ ) similar to *Ae. albopictus* and *Cx. quinquefasciatus*.

**Table 6.4:** Correlation coefficients between *Ae. aegypti*, *Ae. albopictus* and *Cx. quinquefasciatus* GST activity and the relative LC<sub>95</sub> of compounds **1**, **2** and **3**.

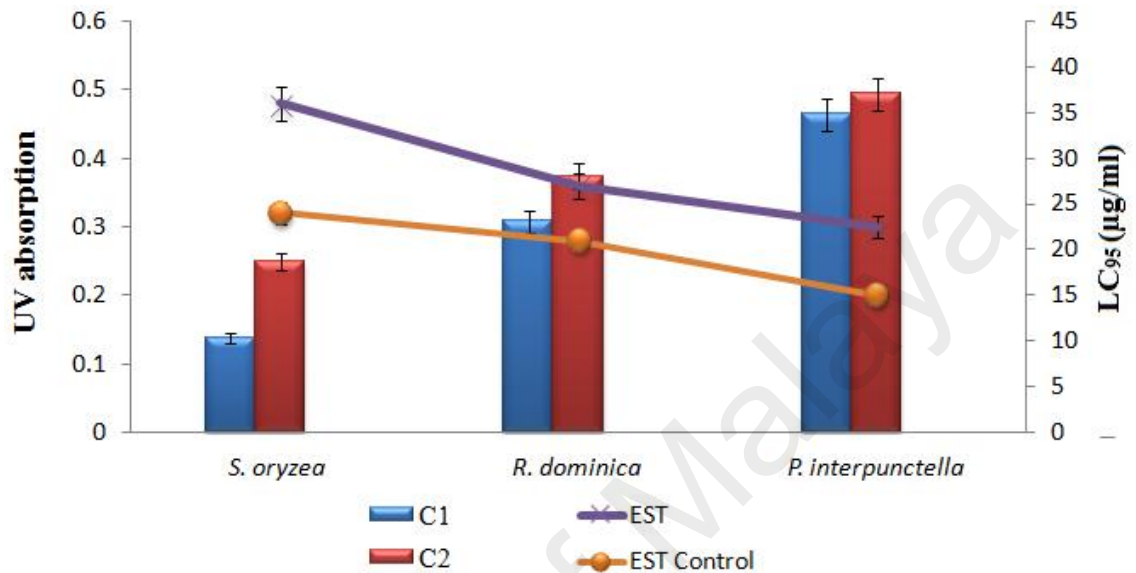
GST enzyme	Correlation coefficient		
	1	2	3
<i>Ae. aegypti</i>	0.840	0.947	0.930
<i>Ae. albopictus</i>	0.902	0.976	0.984
<i>Cx. quinquefasciatus</i>	0.830	0.935	0.897

The de-attenuated correlation coefficient was calculated using  $r = \frac{n(\sum xy) - (\sum x)(\sum y)}{[\sum x^2 - (\sum x)^2/n][\sum y^2 - (\sum y)^2/n]}$ , x represent percentage of GST mean percentage activity, y represent relative LC<sub>95</sub> of compounds **1**, **2** and **3** against *Ae. aegypti*, *Ae. albopictus* and *Cx. quinquefasciatus*

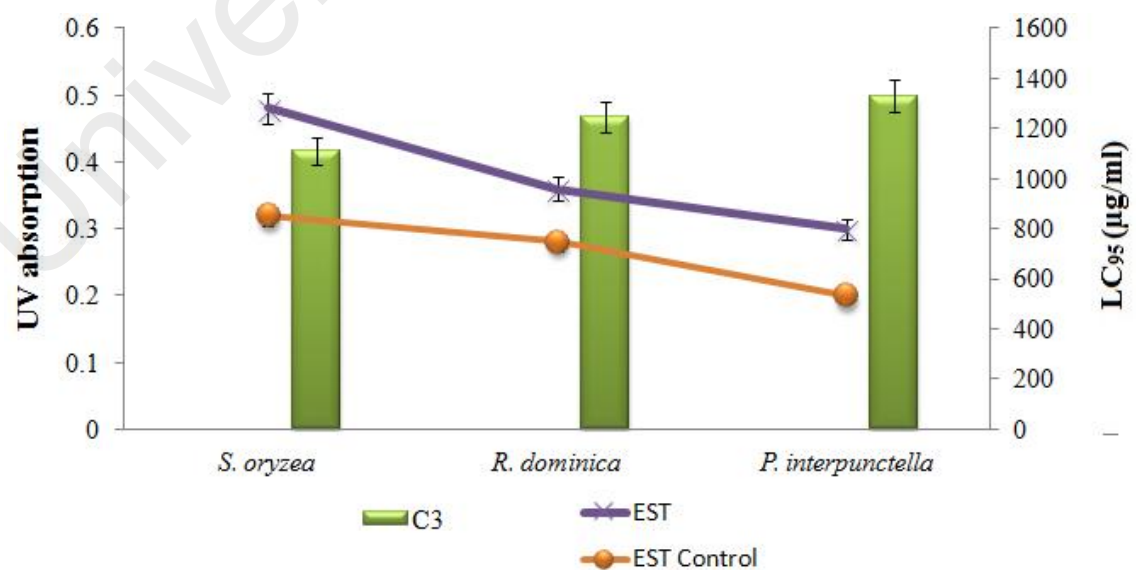
#### 6.4.5 Non-specific Esterases on Storage Pests

The level of non-specific esterases were measured in homogenate samples of *S. oryzae*, *R. dominica* from adults stages and larvae of *P. interpunctella*. In the experiment, each well of microtiter plate was evaluated quantitatively for optical density reading. The enzyme assay of non-specific esterases for compounds **1**, **2** and **3** were studied. The mean esterase activity expressed the level of the absorption of UV. The highest UV absorption was belonging to *P. interpunctella*  $< 0.5$  OD (optical density). All tested storage pests inhibition percentage was below 0.1% relatively highly resistant. Interestingly, the mean value of the esterase expression did not show any dramatic increase or decrease in contrast with the LC<sub>95</sub>. The results did not show any

significant increase ( $p < 0.05$ ) in esterase levels which could be correlated with  $LC_{95}$  in tested insects (Figures 6.9 and 6.10). From the results, the quantitative scoring showed that color did not change and mean of absorption was 0.48 for *S. oryzae*, 0.36 for *R. dominica* and 0.45 for *P. interpunctella*.



**Figure 6.9:** Non-specific esterases enzyme activities of *S. oryzae*, *R. dominica* and *P. interpunctella* in comparison of their relative 1 and 2  $LC_{95}$  value activity.



**Figure 6.10:** Non-specific esterases enzyme activities of *S. oryzae*, *R. dominica* and *P. interpunctella* in comparison of their relative 3  $LC_{95}$  value activity.

There was no meaningful correlation between the LC<sub>95</sub> values of compounds **1**, **2** and **3** and non-specific esterases in *S. oryzae* and *R. dominica* adults, and *P. interpunctella* larvae. *S. oryzae* correlation of **1**, **2** and **3** relative LC<sub>95</sub> with GST mean activity was significant and positive ( $p > 0.05$ ,  $r = -0.361$ ,  $r = -0.214$  and  $r = -0.208$ ). *R. dominica* adults, and *P. interpunctella*  $r$  value were similarly not meaningful in comparison with LC<sub>95</sub>.

Thus, non-specific esterase activity in of *S. oryzae*, *R. dominica* and *P. interpunctella* fell in the range of  $< 0.5$  OD with mild purple colour. The frequencies of the replicates were all distributed below the resistance threshold and were not significantly higher than control. These result showed that non-specific esterase was not associated in resistance in tested insects (Figure 6.9 and 6.10) (Table 6.5).

**Table 6.5:** Correlation coefficients between *S. oryzae*, *R. dominica* and *P. interpunctella* Non-specific esterases activity and the relative LC<sub>95</sub> of compounds **1**, **2** and **3**.

Non-specific esterases	Correlation coefficient		
	1	2	3
<i>S. oryzae</i>	-0.361	-0.214	-0.208
<i>R. dominica</i>	-0.734	-0.393	-0.129
<i>P. interpunctella</i>	-0.669	-0.337	-0.108

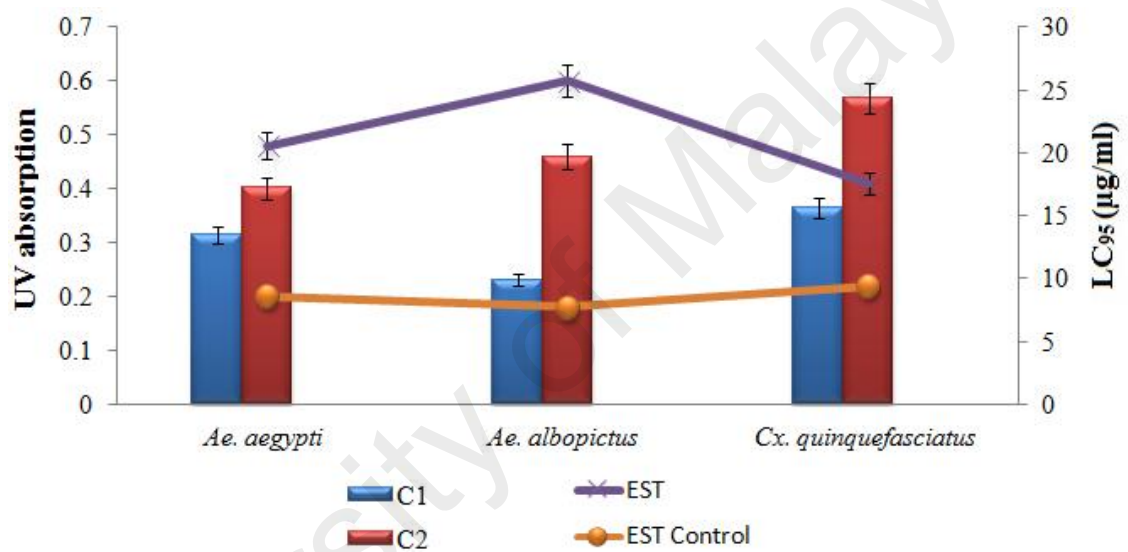
The de-attenuated correlation coefficient was calculated using  $r = \frac{n(\sum xy) - (\sum x)(\sum y)}{[\sum x^2 - (\sum x)^2/n][\sum y^2 - (\sum y)^2/n]}$ ,  $x$  represent percentage of Non-specific esterases mean percentage activity,  $y$  represent relative LC<sub>95</sub> of compounds **1**, **2** and **3** against *S. oryzae*, *R. dominica* and *P. interpunctella*

#### 6.4.6 Non-specific Esterases on Mosquito Vectors Larvae

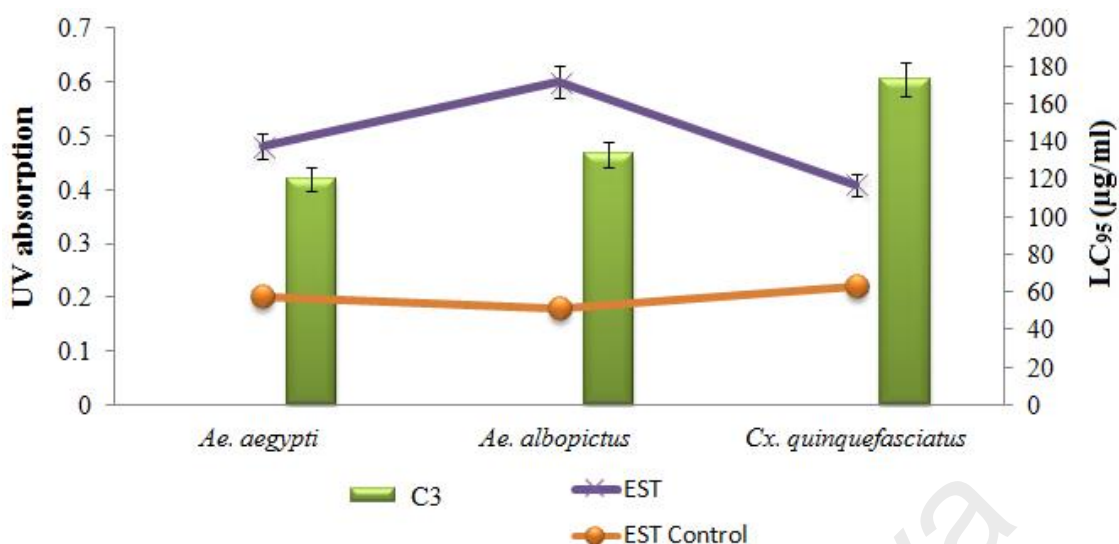
Resistance ratio of the levels of non-specific esterases in *Ae.aegypti*, *Ae. albopictus* and *Cx. quinquefasciatus* in comparison with compounds **1**, **2** and **3** LC<sub>95</sub> value represented at (Figures 6.11 and 6.12). The comparison showed varied non-specific esterases activity in comparison to lethal concentration dose. Mean percentage



of non-specific esterases activity in *Ae. aegypti*, *Ae. albopictus* and *Cx. quinquefasciatus* were moderate in comparison to control (Figures 6.11 and 6.12). The OD of mosquito larvae was the results did not show any significant increase ( $p < 0.05$ ) in esterase levels between tested larvae. From the results, the quantitative scoring showed moderate change in color and mean of absorption was 0.38 for *Ae. aegypti*, 0.46 for *Ae. albopictus* 0.40 for *Cx. quinquefasciatus*. The highest  $LC_{95}$  value belonged to *Cx. quinquefasciatus* and highest absorption of UV belonged to *Ae. albopictus*.



**Figure 6.11:** Non-specific esterases enzyme activities of *Ae. aegypti*, *Ae. albopictus* and *Cx. quinquefasciatus* in comparison of their relative 1 and 2  $LC_{95}$  value activity.



**Figure 6.12:** Non-specific esterases enzyme activities of *Ae. aegypti*, *Ae. albopictus* and *Cx. quinquefasciatus* in comparison of their relative **3** LC<sub>95</sub> value activity.

Correlation coefficient for non-specific esterase mean activity of *Ae. aegypti*, *Ae. albopictus* and *Cx. quinquefasciatus* showed insignificant correlation for compounds **1**, **2** and **3** ( $p > 0.05$ ). *Ae. aegypti*, *Ae. albopictus* and *Cx. quinquefasciatus* correlation coefficient of non-specific esterases enzyme with their relative LC<sub>95</sub> of compounds **1**, **2** and **3** were not significant (there was no meaningful relationship between the mean percentage of the enzyme activity with the relative lethal concentration dosage) (Table 6.6).

**Table 6.6:** Correlation coefficients between *Ae.aegypti*, *Ae. albopictus* and *Cx. quinquefasciatus* Non-specific esterases activity and the relative LC<sub>95</sub> of compounds **1**, **2** and **3**.

Non-specific esterases	Correlation coefficient		
	1	2	3
<i>Ae. aegypti</i>	-0.277	0.214	0.154
<i>Ae. albopictus</i>	0.271	-0.257	0.054
<i>Cx. quinquefasciatus</i>	0.130	0.143	0.042

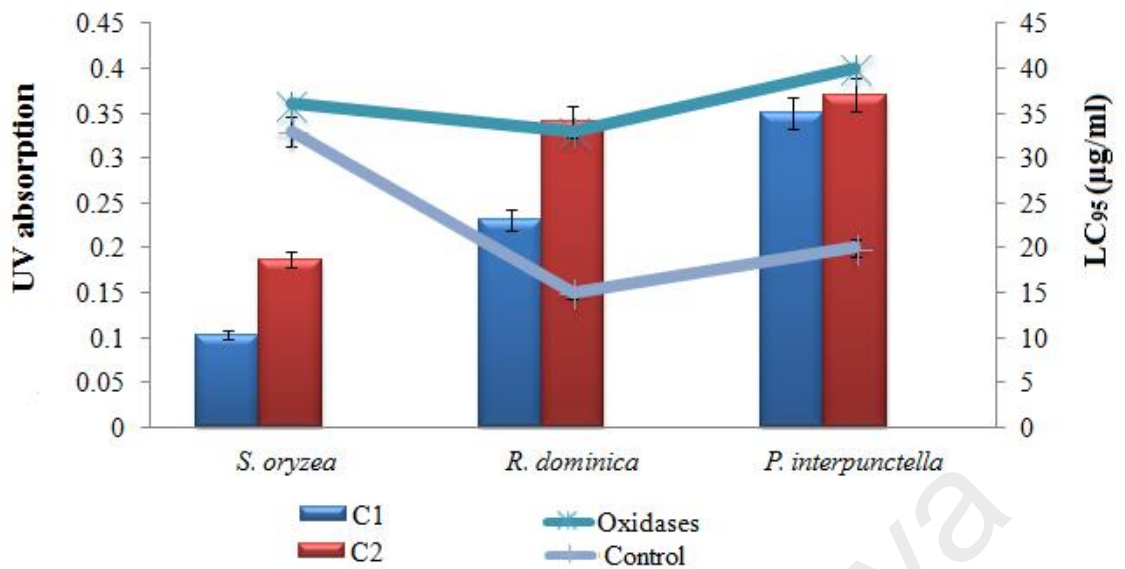
The de-attenuated correlation coefficient was calculated using  $r = \frac{n(\sum xy) - (\sum x)(\sum y)}{[\sum x^2 - (\sum x)^2/n][\sum y^2 - (\sum y)^2/n]}$ , x represent percentage of Non-specific esterases mean percentage activity, y represent relative LC<sub>95</sub> of compounds **1**, **2** and **3** against *Ae. aegypti*, *Ae. albopictus* and *Cx. quinquefasciatus*

#### 6.4.7 Oxidases Enzyme Microassay in Storage Pests

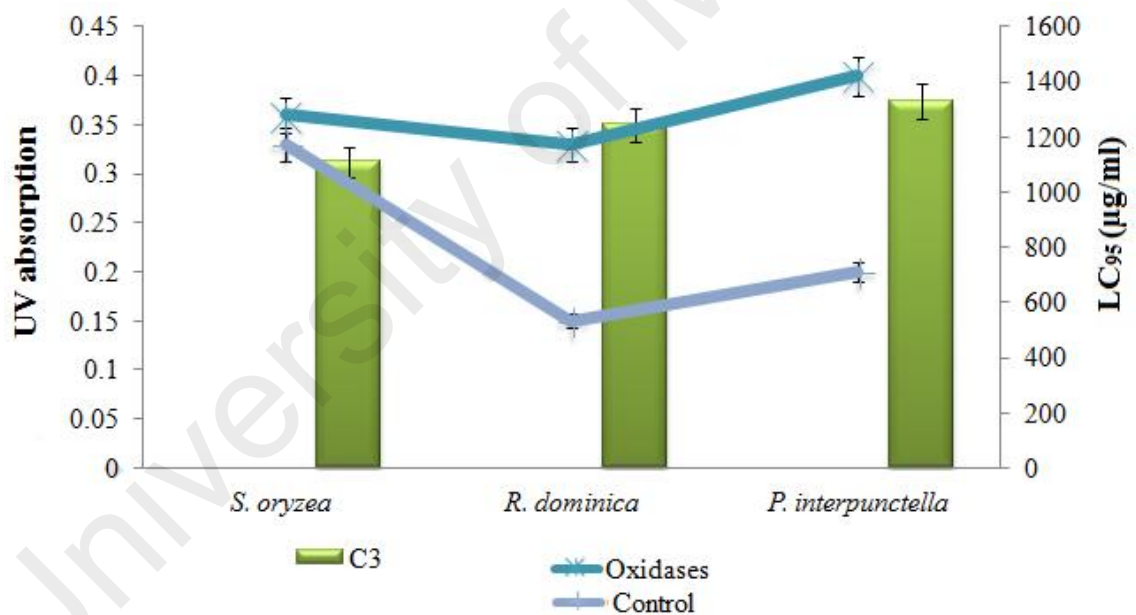
The oxidases activity of *S. oryzae*, *R. dominica* and *P. interpunctella* were not significantly different from the control. In all three assays, the mean absorption was  $\leq 0.31$  which was 3% higher than control. The oxidases mean percentage activity of *S. oryzae*, *R. dominica* and *P. interpunctella* were not significant compare to the control. On the other hand correlation study of oxidases enzyme did not show any meaningful relation between the oxidases enzymes and the relative LC<sub>95</sub> value of **1**, **2** and **3** ( $p > 0.05$ ).

Biochemical resistance detection indicated that there was no significant difference ( $p < 0.05$ ) in oxidase level between *S. oryzae*, *R. dominica* and *P. interpunctella* (Figures 6.13 and 6.14). All the tested insects exhibited insignificant oxidase activity. *S. oryzae* with 13%, *R. dominica* with 9% and *P. interpunctella* with 11 % mean percentage activity.

*S. oryzae* of oxidases activity correlation with **1**, **2** and **3** relative LC<sub>95</sub> with activity was not significant ( $p > 0.05$ ,  $r = - 0.261$ ,  $r = - 0.347$  and  $r = 0.052$ ). Results of correlation study on *R. dominica* adults, and *P. interpunctella* were similarly not meaningful in comparison with LC<sub>95</sub>. These results proved that oxidase enzyme had no effects in detoxification of **1**, **2** and **3** in this study (Table 6.7).



**Figure 6.13:** Oxidases enzyme activity of *S. oryzae*, *R. dominica* and *P. interpunctella* in comparison of their relative 1 and 2 using their relative LC<sub>95</sub>.



**Figure 6.14:** Oxidases enzyme activity of *S. oryzae*, *R. dominica* and *P. interpunctella* in comparison of their relative 3 using their relative LC<sub>95</sub>.

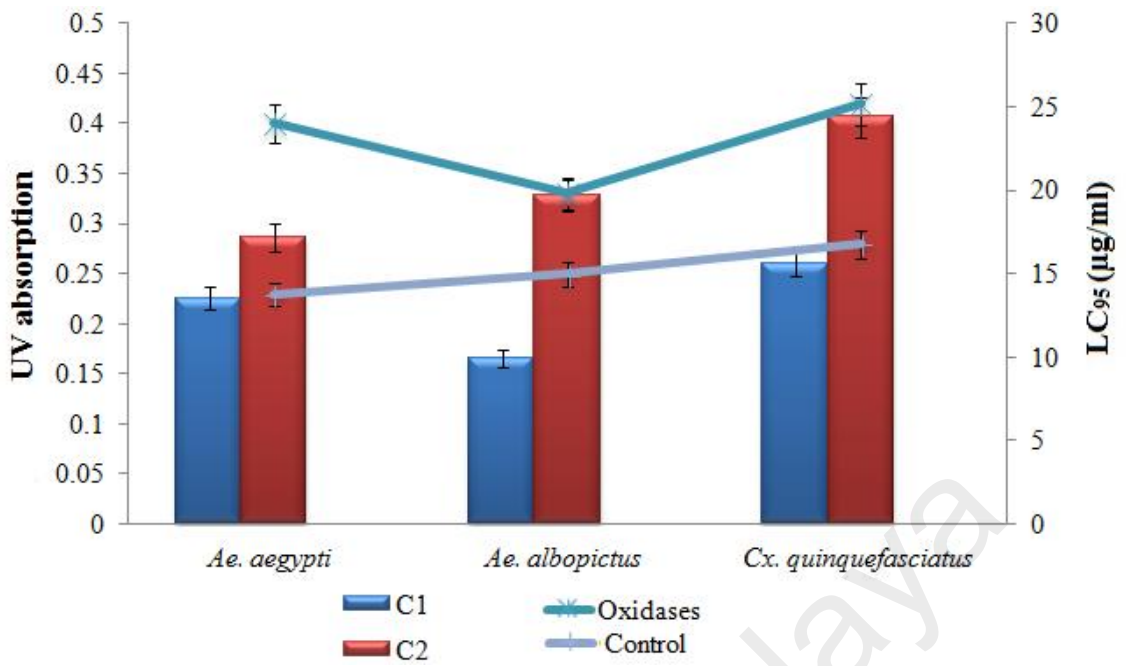
**Table 6.7:** Correlation coefficients between *S. oryzae*, *R. dominica* and *P. interpunctella* oxidases activity and the relative LC<sub>95</sub> of compounds **1**, **2** and **3**.

oxidase enzyme	Correlation coefficient		
	1	2	3
<i>S. oryzae</i>	-0.261	-0.347	0.052
<i>R. dominica</i>	-0.170	0.204	0.051
<i>P. interpunctella</i>	0.504	0.189	0.062

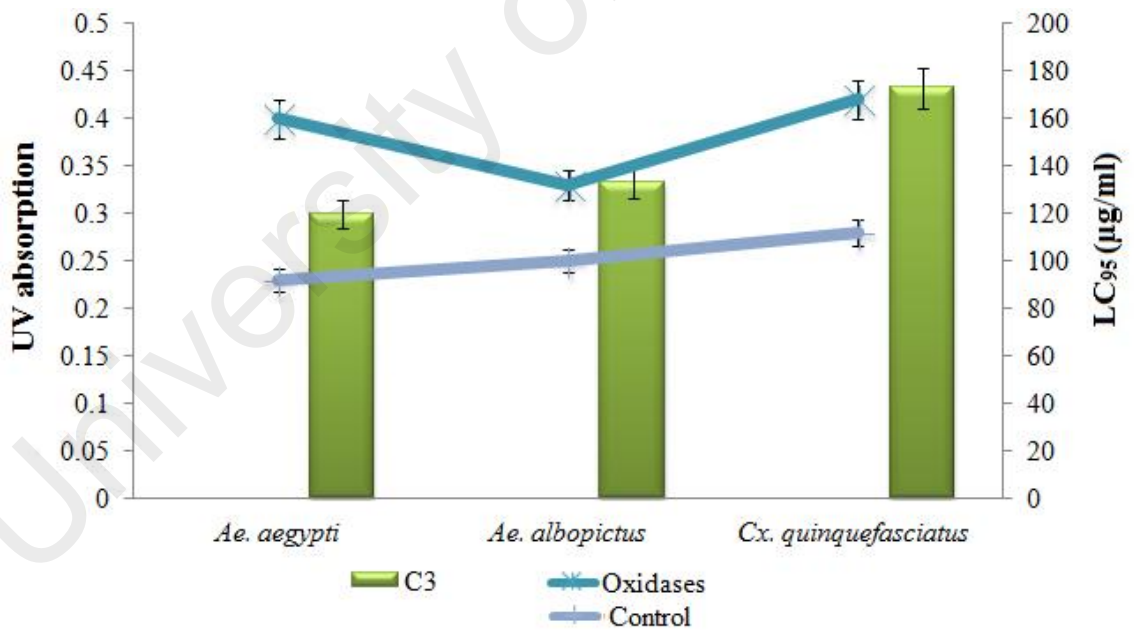
The de-attenuated correlation coefficient was calculated using  $r = \frac{n(\sum xy) - (\sum x)(\sum y)}{[\sum x^2 - (\sum x)^2/n][\sum y^2 - (\sum y)^2/n]}$ , x represent percentage of oxidases mean percentage activity, y represent relative LC<sub>95</sub> of compounds **1**, **2** and **3** against *S. oryzae*, *R. dominica* and *P. interpunctella*

#### 6.4.8 Oxidases enzyme assay on mosquito vectors Larvae

The oxidases activity of *Ae. aegypti*, *Ae. albopictus* and *Cx. quinquefasciatus* were relatively moderate. In all three assays, the mean absorption was  $\leq 0.42$  which was 6% higher than control (Figures 6.15 and 6.16). The oxidases mean percentage activity of *Ae. aegypti*, *Ae. albopictus* and *Cx. quinquefasciatus* were not significant compare to the control. The correlation study of oxidases enzyme showed insignificant ( $p > 0.05$ ) correlation between the enzyme and the LC<sub>95</sub> value. These results similarly did not meaningful correlation between oxidases activity in detoxifying compound **1**, **2** and **3**. (Table 6.8).



**Figure 6.15:** Oxidases enzyme activity of *Ae. aegypti*, *Ae. albopictus* and *Cx. quinquefasciatus* in comparison of their relative **3** using their relative LC<sub>95</sub>



**Figure 6.16:** Oxidases enzyme activity of *Ae. aegypti*, *Ae. albopictus* and *Cx. quinquefasciatus* in comparison of their relative **3** using their relative LC<sub>95</sub>.

*Ae. aegypti* correlation of **1**, **2** and **3** relative LC<sub>95</sub> with oxidases enzyme mean activity was not significant ( $p > 0.05$ ,  $r = -0.261$ ,  $r = -0.347$  and  $r = 0.052$ ). Results of

correlation study on *Ae. albopictus* and *Cx. quinquefasciatus* were similarly not meaningful in comparison with LC<sub>95</sub>.

However there was no meaningful correlation between the LC<sub>95</sub> values of active compounds with oxidases activity in tested mosquitoes in this study (Table 6.8).

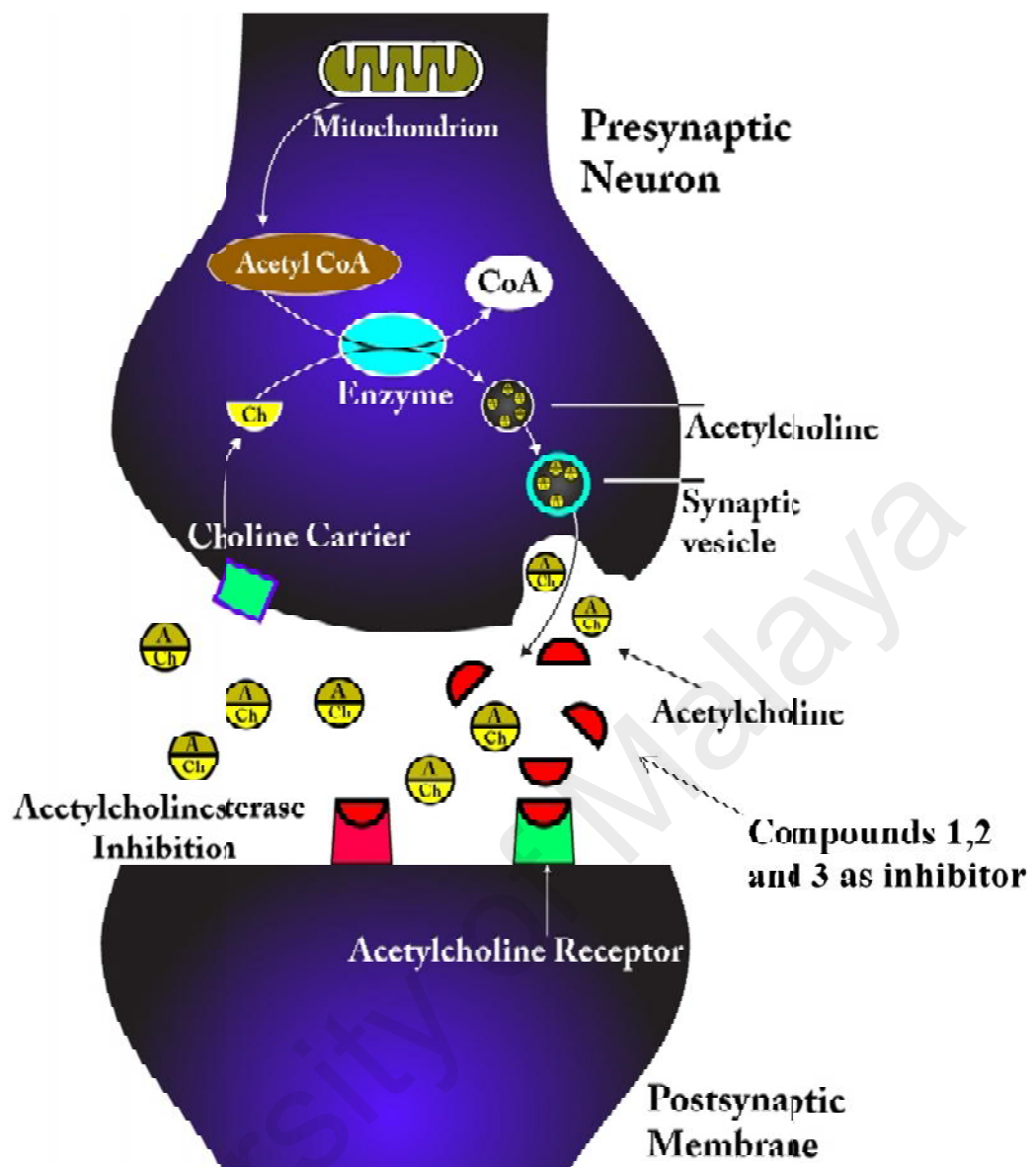
**Table 6.8:** Correlation coefficients between *Ae. aegypti*, *Ae. albopictus* and *Cx. quinquefasciatus* oxidases activity and the relative LC<sub>95</sub> of compounds **1**, **2** and **3**.

Oxidases enzyme	Correlation coefficient		
	1	2	3
<i>Ae. aegypti</i>	-0.063	-0.114	-0.065
<i>Ae. albopictus</i>	-0.088	-0.773	-0.044
<i>Cx. quinquefasciatus</i>	-0.331	-0.231	-0.565

The de-attenuated correlation coefficient was calculated using  $r = \frac{n(\sum xy) - (\sum x)(\sum y)}{[\sum x^2 - (\sum x)^2][\sum y^2 - (\sum y)^2]}$ , x represent percentage of oxidases mean percentage activity, y represent relative LC<sub>95</sub> of compounds **1**, **2** and **3** against *Ae. aegypti*, *Ae. albopictus* and *Cx. quinquefasciatus*

## 6.5 DISCUSSION

Acetylcholinesterase (AChE) biochemical test revealed the inhibition of this enzyme in counter with compounds **1**, **2** and **3**. Compound **3** AChE inhibition was mild compare to **2** and **3**. In AChE inhibition assay absorbance  $\geq 1.00$  revile the similar susceptibility in storage pest and mosquitoes. In all tested insects, AChE inhibition was high. This result concluded that compounds **1** and **2** had direct effect on nervous system of the adult insects and larvae. Similar inhibition from *P. sarmentosum* extracts have been reported (Chandar & Ganguly, 2013; Lee et al., 1990; Bijlsma et al., 2014). Inhibition of AChE by toxicant leads to the accumulation of acetylcholine in nerve synapses causing interruption in continuous and excessive stimulation of the nervous system. The interruption in nerves system potentially leading to the death of the insect pest (Mohamed et al., 2017; Pham et al., 2017).



**Figure 6.17:** Schematic procedure of AChE enzyme inhibition by 1, 2 and 3 at molecular level.

This indicates that increased level of AChE inhibition plays an important role in toxic mechanism in tested insects. Han et al., (2006) have reported that acetylcholinesterase played an important role in response of mosquitoes to malathion. In comparison, *P. interpunctella* and *R. dominica* GST activity was significantly ( $p < 0.05$ ) higher than *S. oryzae*. Correlation study on interaction of LC<sub>95</sub> of 1, 2 and 3 and GST activity on storage pest was significant and positive with their level of resistance. GST levels were present in high proportions and revealed a clear pattern in relation to resistance (Almli et al., 2002). Among three mosquitoes species, *Cx. quinquefasciatus*



showed higher GST. Since glutathione S-transferase has direct effect on resistance mechanisms in individual insects, therefore these activities can confirm higher level of resistance between mosquitoes and storage pests. Apart from that, there was not any significant difference ( $p < 0.05$ ) in non-specific esterase level. Similarly, level of oxidases in mosquitoes and storage pests were not significantly different. The correlation study on storage pest did not show the significant relationship between the level of oxidases and non-specific esterase enzyme activity with the level of insects resistance. In mosquito vectors, correlation study also did not show any significant relationship between oxidases and non-specific esterase to their response to active compounds. Lee (1990) revealed the role of elevated levels of non-specific esterase activity which were correlated with degree of malathion resistance (Hemingway & Georghiou, 1983). Since the nature of active compound in this study is different from malathion (Organothiophosphate) this could be the fact that non-specific esterase did not correlate with the resistance to toxic compounds (Osta, Rizk, Labbé, Weill, & Knio, 2012). On the other hand, since the tested insects were lab strain, their level of resistance and detoxifying enzyme may be reduced (Williamson, Denholm, Bell, & Devonshire, 1993). The role of esterase and GST in detoxifying the insects also depended upon the structure and concentration of insecticide and possibly the age of the insect under test (Thom, Dixon, Edwards, Cole, & Laphorn, 2001). Pyrethroid resistance research on Puerto Rican strain of *Ae. aegypti* suggested that non-specific esterase did not associate with a quantitative change in esterase activity (Hemingway & Georghiou, 1983). The present study strongly supported that GST enzyme was higher in the insects with higher resistance and tolerance to active compounds (Hussey & Hayes, 1993). *Ae. aegypti* and *Ae. albopictus* had lower GST activity compared to *Cx. quinquefasciatus*. The result of correlation study indicated positive relationship between the GST activity and resistance to compounds **1** and **2**. There could be other enzyme

detoxification mechanism involved involve in resistance such as insensitive AChE as in the case of *P. interpunctella*. In general the results of AChE inhibition and GST activity were in agreement with the insecticide toxicity test conducted earlier in Chapter 4 and 5.

## 6.6 CONCLUSION

Mechanism of action of compounds **1** and **2** may similar to organophosphate insecticides due to their strong AChE inhibition. All the three compounds inhibited AChE but in the different rates. Compound **3** had the lowest inhibition and relatively was the weakest in toxicity against tested insects. On the other hand, GST activity significantly revealed its importance in insects resistance. *P. interpunctella* with the highest resistance to **1**, **2** and **3** had the highest GST. *Ae. aegypti*, *Ae. albopictus* and *Cx. quinquefasciatus* had similar response to compounds **1** and **2**, and their GST activity was relatively high. Correlation study showed significant and negative interaction between the GST and LC<sub>95</sub>. Non-specific esterases and oxidases did not show any significant correlation with the level of the resistance of each tested pest. This may be due to use the lab strain which due to long time raring has become more susceptible. These results may help to understand the nature of action of **1**, **2** and **3** and for better use in insect pests control in future.

## CHAPTER 7: STRUCTURE-BASED INHIBITOR DOCKING VALIDATION ON ACETYLCHOLINESTERASE AND GLUTATHIONE S- TRANSFERASE

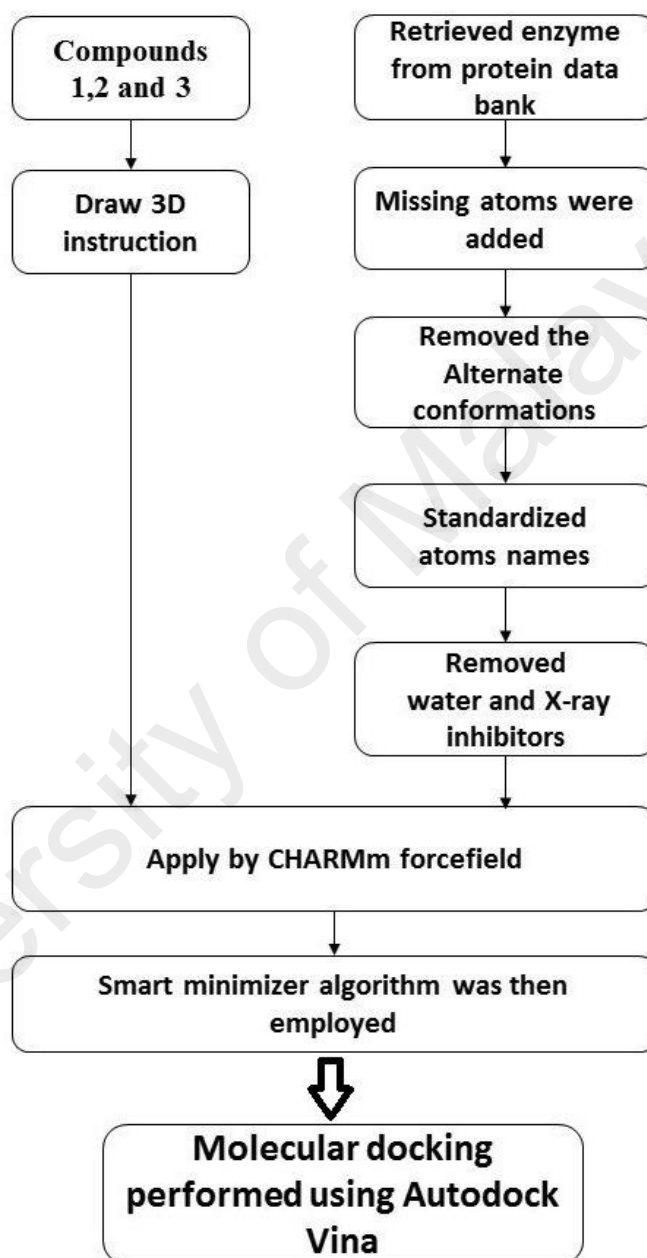
### 7.1 INTRODUCTION

In Chapter 6 the correlation of acetylcholinesterase (AChE) inhibition and response of insects to the toxicant by glutathione S-transferase (GST) with Asaricin **1**, isoasarone **2**, *trans*-asarone **3** and asaraldehyde were studied. In this chapter the relationship of AChE and GST enzyme with toxicant were studied at their molecular level to see the interaction of each compounds with the active site of the enzymes. This will give broad perspective for better understanding of their activity and will help in future work to compare their activity with current commercialised insecticides with similar mechanism of action.

The enzyme assays as presented in the previous chapter revealed the mode of action of the active phenylpropanoids; asaricin **1**, isoasarone **2** and *trans*-asarone **3** and their correlation with AChE and GST enzyme. The possible bonding pathways of each compound with AChE and GST were investigated in this chapter to understand the impact of toxic compound on the enzymes. C-docker program facilitated further characterization the efflux pathway. Molecular docking as computation method could evaluate how the compounds and the target enzyme fit together. Hence, this chapter hypothesis focuses on analyze the interaction study of **1**, **2** and **3** with AChE and GST enzyme using online database and Autodock/Vina program. The study focus on interaction of **1**, **2** and **3** with target enzymes and the stability of their interaction by determination of the level of their bonding energy between ligand and the enzymes.

The AChE and GST junction interaction with active sites were studied by docking each compound as ligand to 3D structure of each enzyme (Vitorović-Todorović, Koukoulitsa, Juranić, Mandić, & Drakulić, 2014). This work is

computational recreating of similar reaction in insects in respond to AChE inhibition by toxins and detoxification process by GST.



**Figure 7.1:** General procedure of computation work on compounds 1, 2 and 3 with AChE and GST enzyme 3D structures.

## 7.2 LITERATURE REVIEW

Over the past three decades, the use of synthetic pesticides has significantly increased. This is particularly more significant in developing countries. Tropical countries in Asia and South America with rapid increasing population are in need for higher volume of agricultural products (Tavares et al., 2010; Tripathi, 2014). The agriculture industry uses pesticides such as organophosphates to combat insect pests. Because of broad spectrum use of insecticides, these chemicals poses threat to non-target insects and animals. The toxicity mode of action of insecticides has been demonstrated by many field and laboratory studies (Vassilakos, Athanassiou, Chloridis, & Dripps, 2014). The modes of action of organophosphates, carbamates and some bioinsecticides are mostly targeting acetylcholinesterase (AChE) enzyme. Enzymes are the most important proteins catalysing chemical reactions in all living organisms (Trott & Olson, 2010; Basiri et al., 2014).

These enzymes such as AChE have an essential role from transmitting the pulses between the neurons. For this matter computation analysis has developed for simulating the interaction of the micro molecules such as insecticides with an enzyme as macromolecule. The process is involve with docking process of the toxicant as ligand conformation and orientation (or posing) within a targeted active sites of enzyme (Vitorović-Todorović, Koukoulitsa, Juranić, Mandić, & Drakulić, 2014). In general, there result of docking studies will lead to prediction of activity of ligands. In view of these challenges, docking is generally devised as a multi-step process in which each step introduces one or more additional degrees of complexity (Renuga Parameswari et al., 2015). The process begins with the application of docking the ligand in the active site. The docking process is challenging, as even simple molecules can have several degrees of freedom. Each docking process must be performed with sufficient accuracy

to the specific site identified as the pocket (Musilek et al., 2011). The best matches to the receptor structure will identify by comparison of the energy level of the binding. AutoDock/Vina analysis is complemented by evaluating the binding energy  $e$  through valuation of interactions between compounds and potential targets (Kalimuthu et al., 2013). Evaluating the energy level of the binding energy conformers are often further evaluated using more complex with hydrogen binding or van der Waals interactions (Vitorović-Todorović, Koukoulitsa, Juranić, Mandić, & Drakulić, 2014). It should also be noted that ligand-binding with 3D structure of the enzyme sometime only gives the rough idea about the events since not all the 3D structures of different insects enzymes are available ((Yang et al., 2013; Xu et al., 2008). In such cases the closest species will chose for the best simulation (Basiri et al., 2014).

## **7.3 MATERIALS AND METHODS**

### **7.3.1 Molecular Docking Study on AChE**

The initial structure of *Drosophila melanogaster* acetylcholinesterase (AChE) was retrieved from protein data bank (<http://www.pdb.org>) with PDB ID:1QON. The enzyme was then prepared under the protein preparation protocol implemented in Discovery Studio (Accelry Inc, 2.5.5) (Discovery Studio. Version 2.5.5 ed. San Diego, CA, USA: Accelrys Inc.; 2009) in which missing atoms in the incomplete residues were added, alternate conformations were removed and the atom names were standardized. The missing residues from 103–135, 574–585 were not included in this model as the residues were located very far from the investigated binding sites. Prior to minimization using smart minimizer algorithm, molecular properties of both compounds and the enzyme were described by CHARMM forcefield (Brooks et al., 1983) with Momany-Rone for the partial charge setting. The complex of enzyme and compound was generated using Autodock/Vina (Trott & Olson, 2010) by allowing the ligand to be

flexible. The input site sphere was set at the three binding sites a) catalytic binding site which include three catalytic triads (CAS:S238,E367,H480), b) the peripheral site (PAS:E72,W271) which is above the active site and close to the mouth of the gorge on the protein surfaced, and c) anionic binding site (W83) (Leonetti et al., 2008). The docked complexes would be further minimized and calculated for their respective interaction energy per residue.

### **7.3.2 Molecular docking study on GST**

The initial structure of *Drosophila melanogaster* for GST, the X-ray crystal structure from *Anopheles dirus* species B. (PDB ID: 1JLV) was used and the molecular docking was performed where the binding site was defined from the center of the ligand found in the X-ray structure. The enzyme was then prepared under the protein preparation protocol implemented in Discovery Studio 2.5 (Accelry Inc, 2.5.5) suite of program. The input protein underwent a series of processes including the missing atoms were added in incomplete residues, alternate conformations were removed, the atom names were standardized, and the water and X-ray inhibitors was removed. Prior to minimization, molecular properties both of the compound and the enzyme were described by CHARMM forcefield with Momany-Rone<sup>47</sup> for the partial charge setting. Smart minimizer algorithm was then employed for the minimization purpose. The molecular docking was performed using Autodock/Vina<sup>48</sup> where the compound was flexible. The input site sphere was set at GSH-701 as its active site (Figure 7.1).

## **7.4 RESULTS**

### **7.4.1 Computational investigation for phenylpropanoids compounds toward the three binding sites of AChE**

Binding energy evaluation provided a correlation to the activity performed at the experimental stage. The best docked pose with the lowest binding energy was selected from series of poses generated after calculating their binding energy. The binding

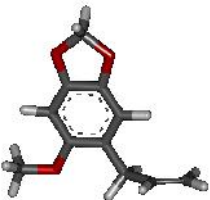

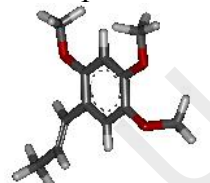
energy follows the equation as Binding Energy = Energy of Complex—Energy of Ligand Energy of Receptor. The more negative the binding energy, the better the binding activity. In another word, the binding between the compound and the target will be more favourable. Binding affinity calculated from Autodock/Vina revealed that asaricin **1**, isoasarone **2** and trans-*asarone* **3** bound in the anionic site stronger with -6.7 kcal/mol and -6.0 kcal/mol respectively as compared to CAS (asaricin **1**: -4.7, isoasarone **2**: -3.9 kcal/mol, trans-*asarone* **3**: -3.9) and PAS (asaricin **1**: -5.4, isoasarone **2**: -5.2 kcal/mol, trans-*asarone* **3**: -5.4) site (Table 7.1). Apart from the interaction energy of the enzyme and compounds at the active sites, interaction energy for the residues within 3 Å region was also calculated as a more precise indication on the binding strength of asaricin **1**, isoasarone **2** and trans-*asarone* **3**. Summation of the interaction energy of each residue with asaricin **1**, isoasarone **2** and trans-*asarone* **3** was compared in Tables 7.2 to 7.4. 3Å IE of asaricin **1**, isoasarone **2** and trans-*asarone* **3** in anionic site is -31.43 kcal/mol and -36.49 kcal/mol, respectively which is stronger than CAS (asaricin **1**: -14.26, isoasarone **2**: -16.51 kcal/mol, trans-*asarone* **3**: -25.3) and PAS (asaricin **1**: -20.43, isoasarone **2**: -14.52 kcal/mol, trans-*asarone* **3**: -14.74). Three binding sites of the enzyme AChE were illustrated in Figure 7.3, while Figures 7.4 and 7.5 facilitates visualization of the molecular interaction of compounds bounded in all the three binding pockets. Common residues in binding for both compounds with interaction energy lower than 2 kcal/mol are TYR 71, TRP 83 for CAS site, TYR71, TYR 73, TRP 321, TYR 324 and TYR 374 for PAS site, and TRP 83, TYR 148, GLU 237, SER 238 and TYR 370 for anionic site. Table 7.1 to 7.5, Figures 7.2 (a) and 7.2 (b) illustrates the binding pocket of the enzymes, AChE and GST while Figures 7.3 to 7.7 facilitates visualization of the molecular interaction of compounds bounded in the binding pocket.



#### **7.4.2 Binding affinity of phenylpropanoids towards active site of GST by molecular docking**

GST-ligand complex with the lowest calculated binding energy (Table 7.5) was selected from a series of poses after molecular docking for further analysis. Calculated binding energy would reveal the binding affinity of compounds at the binding site of GST. The more negative the binding energy, the better the binding activity. Compound **1**, **2** and **3** are calculated to have similar binding affinity; -5 kcal/mol, - 4.8 kcal/mol and -4.7 kcal/mol respectively (Table 7.5). The superposition of the compounds **1**, **2** and **3** oriented in the binding pocket of GST were shown in Figures 7.6 and 7.7. Table 7.1 to 7.5 Figure 7.2 (a) and 7.2 (b) illustrates the binding pocket of the enzymes, AChE and GST while Figure 7.3 to 7.6 facilitates visualization of the molecular interaction of compounds bounded in the binding pocket.

**Table 7.1:** Binding interaction energy (kcal/mol) of compounds from Autodock/Vina toward different binding sites and their related experimental activities for AChE and GST.

Inhibitors	CAS site	PAS site	Anionic binding site (W83).	GST	AChE	GST
<p>Compound 1</p> 	-4.7	-5.4	-6.7	-5.0	1.68//1.01/0.96	13.5/9.9/15.6
<p>Compound 2</p>  <p>double bond at end of R-group</p>	-3.9	-5.2	-6.0	-4.8	0.97/0.84/0.89	17.2/19.7/24.4
<p>Compound 3</p> 	-3.9	-5.4	-6.1	-4.7	0.18/0.13/.12	120/133/173

**Table 7.2:** The interaction energy of phenylpropanoids compounds towards AChE peripheral.

Residue	IE (kcal/mol)	VDW (kcal/mol)	Electrostatics (kcal/mol)	Residue	IE (kcal/mol)	VDW (kcal/mol)	Electrostatics (kcal/mol)	Residue	IE (kcal/mol)	VDW (kcal/mol)	Electrostatics (kcal/mol)
AChE											
PAS											
<b>1</b>				<b>2</b>				<b>3</b>			
GLU 69	-8.36	-1.2	-7.16	GLU 69	5.06	-1.41	6.47	GLU 69	-1.07	-1.66	0.58
ARG 70	0.066	-0.28	0.35	ARG 70	-0.89	-0.89	0.0027	ARG 70	1.04	-0.43	1.47
TYR 71	-2.22	-1.53	-0.69	TYR 71	-9.35	-2.98	-6.37	TYR 71	-0.95	-2.79	1.84
TYR 73	-3.76	-0.96	-2.79	TYR 73	-5.42	-1.46	-3.96	TYR 73	-4.31	-1.99	-2.32
VAL 318	1.01	-0.71	1.72	VAL 318	0.082	-1.37	1.45	VAL 318	-1.53	-1.31	-0.22
TRP 321	-4.04	-5.4	1.36	TRP 321	-7.42	-7.85	0.43	TRP 321	-6.88	-5.54	-1.34
TYR 324	-2.85	-1.99	-0.86	TYR 324	-2.89	-2.045	-0.85	TYR 324	-2.19	-1.81	-0.37
TYR 374	-2.05	-0.59	-1.46	TYR 374	-3.36	-0.23	-3.13	TYR 374	-1.94	-0.69	-1.25
3 Å IE	-20.43	-9.68	-10.74	3 Å IE	-14.52	-15.66	1.13	3 Å IE	-14.74	-13.29	-1.46
Surface IE	-22.20	-12.66	-9.53	Surface IE	-24.2	-18.24	-5.96	Surface IE	-17.83	-16.22	-1.61
Total IE	-66.33	-14.82	-51.51	Total IE	-78.41	-21.16	-57.25	Total IE	-84.39	-18.9	-65.5

**Table 7.3:** The interaction energy of phenylpropanoids compounds towards AChE catalytic site.

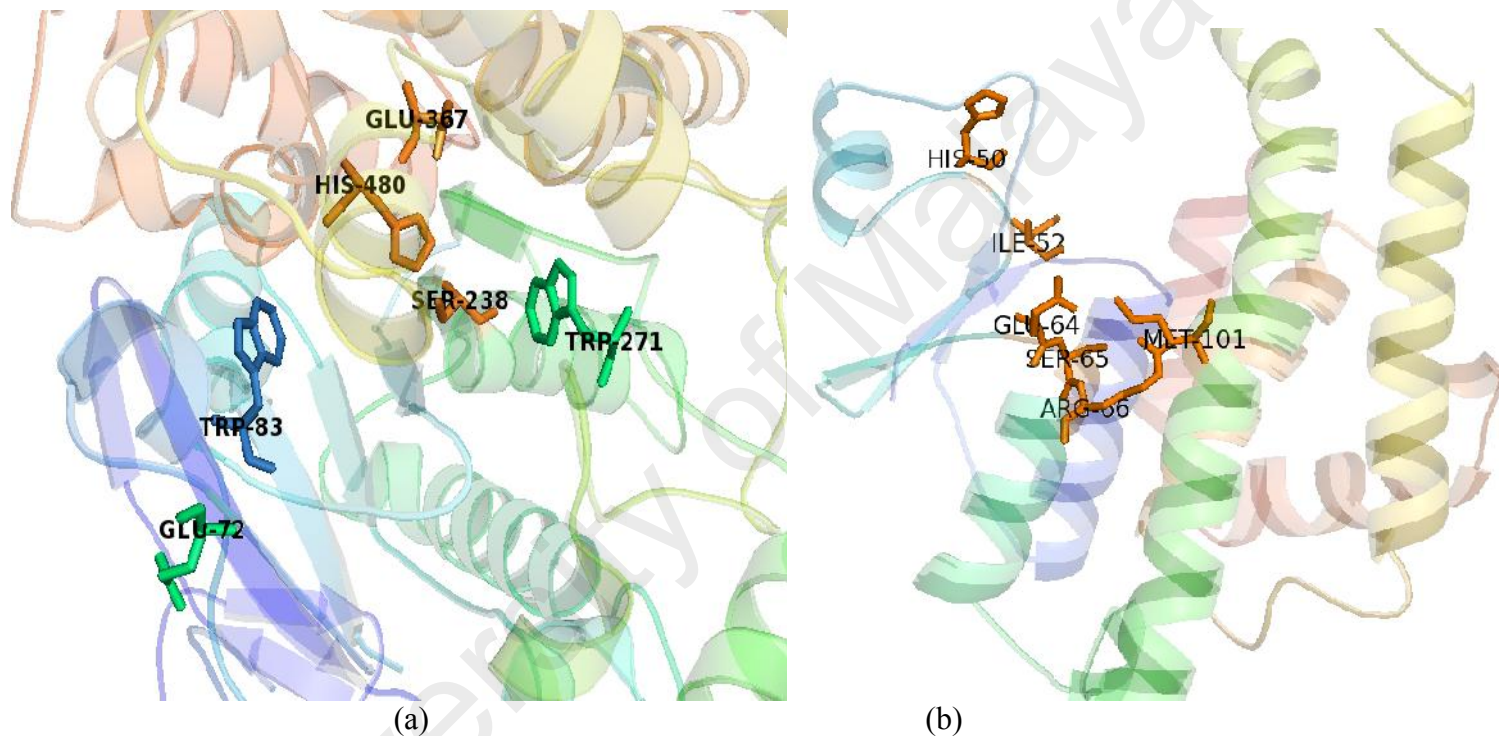
Residue	IE (kcal/mol)	VDW (kcal/mol)	Electrostatics (kcal/mol)	Residue	IE (kcal/mol)	VDW (kcal/mol)	Electrostatics (kcal/mol)	Residue	IE (kcal/mol)	VDW (kcal/mol)	Electrostatics (kcal/mol)
<b>AChE</b>											
<b>CAS site</b>											
<b>1</b>				<b>2</b>				<b>3</b>			
ARG 70	2.02	-0.083	2.1	ARG 70	-1	-0.073	-0.93	ARG 70	1.86	-0.027	1.88
<b>TYR 71</b>	<b>-6.27</b>	<b>-3.34</b>	<b>-2.93</b>	<b>TYR 71</b>	<b>-4.23</b>	<b>-3.39</b>	<b>-0.88</b>	TYR 71	-2.84	-0.62	-2.23
<b>GLU 80</b>	<b>3.29</b>	<b>-1.52</b>	<b>4.81</b>	<b>GLU 80</b>	<b>-1.41</b>	<b>-1.86</b>	<b>0.45</b>	<b>GLU 80</b>	<b>-2.13</b>	<b>-0.99</b>	<b>-1.14</b>
<b>TRP 83</b>	<b>-3.08</b>	<b>-2.72</b>	<b>-0.37</b>	<b>TRP 83</b>	<b>-2</b>	<b>-1.86</b>	<b>-0.14</b>	<b>TRP 83</b>	<b>-4.42</b>	<b>-2.72</b>	<b>-1.7</b>
GLY 149	-1.2	-0.84	-0.36	<b>GLY 149</b>	<b>-1.39</b>	<b>-1.64</b>	<b>0.24</b>	<b>GLY 149</b>	<b>-3.36</b>	<b>-1.8</b>	<b>-1.56</b>
<b>GLY 150</b>	<b>-0.34</b>	<b>-1.16</b>	<b>0.82</b>	<b>GLY 150</b>	<b>2.98</b>	<b>0.51</b>	<b>2.47</b>	<b>GLY 150</b>	<b>1.66</b>	<b>-0.92</b>	<b>2.58</b>
<b>GLY 151</b>	<b>0.14</b>	<b>-0.85</b>	<b>0.99</b>	<b>GLY 151</b>	<b>-0.11</b>	<b>-0.82</b>	<b>0.71</b>	<b>GLY 151</b>	<b>-0.77</b>	<b>-1.03</b>	<b>0.26</b>
THR 154	-0.85	-0.46	-0.38	THR 154	-0.35	-0.54	0.19	THR 154	-0.51	-0.63	0.12
TYR 162	2.22	-0.38	2.6	TYR 162	1.66	-0.47	2.14	<b>TYR 162</b>	<b>-3.91</b>	<b>-1.01</b>	<b>-2.9</b>
GLU 237	-1.32	-0.32	-1	GLU 237	-0.0037	-0.55	0.55	<b>GLU 237</b>	<b>-2.92</b>	<b>0.52</b>	<b>-3.44</b>
SER 238	-2.81	-0.38	-2.43	<b>SER 238</b>	<b>-2.81</b>	<b>-0.82</b>	<b>-1.99</b>	<b>SER 238</b>	<b>-1.62</b>	<b>-1.66</b>	<b>0.038</b>
<b>TYR 370</b>	<b>-1.46</b>	<b>-1.97</b>	<b>0.51</b>	<b>TYR 370</b>	<b>-5.04</b>	<b>-2.18</b>	<b>-2.86</b>	<b>TYR 370</b>	<b>-4.59</b>	<b>-2.49</b>	<b>-2.1</b>
<b>PHE 371</b>	<b>-2.45</b>	<b>-1</b>	<b>-1.45</b>	<b>PHE 371</b>	<b>-0.81</b>	<b>-1.49</b>	<b>0.69</b>	<b>PHE 371</b>	<b>-1.31</b>	<b>-1.22</b>	<b>-0.087</b>
<b>TYR 374</b>	<b>-2.85</b>	<b>-1.4</b>	<b>-1.45</b>	<b>TYR 374</b>	<b>2.08</b>	<b>-1.5</b>	<b>3.58</b>	TYR 374	-4.89	-0.78	-4.11
<b>TRP 472</b>	<b>-1.24</b>	<b>-0.87</b>	<b>-0.37</b>	<b>TRP 472</b>	<b>-1</b>	<b>-0.95</b>	<b>-0.045</b>	<b>TRP 472</b>	<b>-1.45</b>	<b>-1.04</b>	<b>-0.41</b>
HIS 480	-1.05	-0.67	-0.38	<b>HIS 480</b>	<b>-2.77</b>	<b>-1.25</b>	<b>-1.52</b>	HIS 480	-1.72	-1.48	-0.24
GLY 481	-2.05	-0.35	-1.7	GLY 481	-1.57	-0.4	-1.17	<b>GLY 481</b>	<b>-1.72</b>	<b>-1.04</b>	<b>-0.69</b>
ILE 484	-1.32	-0.2	-1.13	ILE 484	-2.47	-0.31	-2.16	<b>ILE 484</b>	<b>1.24</b>	<b>0.22</b>	<b>1.02</b>
Surface IE	<b>-20.62</b>	<b>-18.51</b>	<b>-2.12</b>	Surface IE	<b>-20.24</b>	<b>-19.59</b>	<b>-0.68</b>	Surface IE	<b>-33.4</b>	<b>-18.72</b>	<b>-14.71</b>
3 Å IE	<b>-14.26</b>	<b>-14.83</b>	<b>0.56</b>	3 Å IE	<b>-16.51</b>	<b>-17.25</b>	<b>0.71</b>	3 Å IE	<b>-25.3</b>	<b>-15.18</b>	<b>-10.13</b>
Total IE	<b>-83.21</b>	<b>-21.27</b>	<b>-61.93</b>	Total IE	<b>-100.29</b>	<b>-22.81</b>	<b>-77.48</b>	Total IE	<b>-88.13</b>	<b>-22.92</b>	<b>-65.21</b>

**Table 7.4:** The interaction energy of phenylpropanoids compounds towards AChE anionic site.

Residue	IE (kcal/mol)	VDW (kcal/mol)	Electrostatics (kcal/mol)	Residue	IE (kcal/mol)	VDW (kcal/mol)	Electrostatics (kcal/mol)	Residue	IE (kcal/mol)	VDW (kcal/mol)	Electrostatics (kcal/mol)
<b>AChE</b>											
<b>Anionic site</b>											
<b>1</b>				<b>2</b>				<b>3</b>			
<b>TYR 71</b>	-0.79	-2.41	1.62	<b>TYR 71</b>	0.41	-0.78	1.19	<b>TYR 71</b>	-2.58	-2.13	-0.45
<b>GLU 80</b>	-0.69	-1.75	1.06	<b>GLU 80</b>	-6.62	-1.43	-5.19	<b>GLU 80</b>	-2.52	-3.11	0.59
<b>TRP 83</b>	-5.3	-5.034	-0.27	<b>TRP 83</b>	-6.31	-6.82	0.51	<b>TRP 83</b>	-8.14	-6.94	-1.2
<b>ASN 84</b>	-2.51	-1.53	-0.98	<b>ASN 84</b>	-1.96	-2.35	0.39	<b>ASN 84</b>	-1.27	-2.04	0.77
TYR 148	-4.18	-0.2	-3.99	<b>TYR 148</b>	-3.66	-0.83	-2.83	TYR 148	2.02	-0.19	2.21
<b>GLY 149</b>	-1.8	-1.21	-0.59	<b>GLY 149</b>	-0.075	-1.51	1.43	<b>GLY 149</b>	-2.55	-0.78	-1.77
<b>GLY 150</b>	-2.64	-1.87	-0.76	<b>GLY 150</b>	-1.93	-2.3	0.37	<b>GLY 150</b>	-3.14	-0.49	-2.65
GLY 151	0.68	-0.24	0.91	GLY 151	0.52	-0.23	0.75	GLY 151	-0.063	-0.22	0.16
<b>THR 154</b>	-0.99	-1.42	0.43	<b>THR 154</b>	-2.8	-2.27	-0.53	<b>THR 154</b>	-3.34	-2.38	-0.97
GLY 155	-0.24	-0.36	0.12	<b>GLY 155</b>	-2.42	-0.98	-1.44	<b>GLY 155</b>	-0.48	-0.65	0.18
SER 156	-1.25	-0.096	-1.16	<b>SER 156</b>	-2.86	-0.63	-2.23	SER 156	-1.3	-0.073	-1.24
TYR 162	-0.9	-0.51	-0.38	<b>TYR 162</b>	-4.93	-1.48	-3.45	TYR 162	-1.46	-0.24	-1.21
<b>GLU 237</b>	-6.71	-0.83	-5.88	GLU 237	-2.15	-0.45	-1.7	GLU 237	-0.7	-0.36	-0.34
<b>SER 238</b>	-3.18	-0.85	-2.33	SER 238	-2.24	-0.35	-1.88	SER 238	1.58	-0.61	2.18
<b>TYR 370</b>	-2.13	-1.98	-0.15	<b>TYR 370</b>	-3.33	-1.48	-1.84	<b>TYR 370</b>	-6.43	-3.4	-3.03
PHE 371	-1.26	-0.36	-0.9	PHE 371	0.36	-0.31	0.67	PHE 371	0.26	-0.33	0.59
TRP 472	1.78	-0.22	2	TRP 472	-1.84	-0.31	-1.53	<b>TRP 472</b>	-0.69	0.55	-1.24
<b>HIS 480</b>	-2.19	-1.24	-0.95	HIS 480	1.13	-0.65	1.78	<b>HIS 480</b>	-4.03	-1.03	-3
<b>ILE 484</b>	-2.5	-0.48	-2.03	ILE 484	-0.53	-0.37	-0.16	ILE 484	0.00664	-0.14	0.15
<b>3Å IE</b>	<b>-31.43</b>	<b>-20.60</b>	<b>-10.83</b>	<b>3Å IE</b>	<b>-36.49</b>	<b>-22.86</b>	<b>-13.62</b>	<b>3Å IE</b>	<b>-35.17</b>	<b>-22.4</b>	<b>-12.77</b>
<b>Surface IE</b>	<b>-36.85</b>	<b>-22.59</b>	<b>-14.23</b>	<b>Surface IE</b>	<b>-41.24</b>	<b>-25.53</b>	<b>-15.69</b>	<b>Surface IE</b>	<b>-34.83</b>	<b>-24.56</b>	<b>-10.27</b>
<b>Total IE</b>	<b>-80.74</b>	<b>-26.569</b>	<b>-54.18</b>	<b>Total IE</b>	<b>-118.32</b>	<b>-29.94</b>	<b>-88.38</b>	<b>Total IE</b>	<b>-93.21</b>	<b>-30.03</b>	<b>-63.17311</b>

**Table 7.5:** The interaction energy of phenylpropanoids compounds towards GST.

Residue	IE (kcal/mol)	VDW (kcal/mol)	Electrostatics (kcal/mol)	Residue	IE (kcal/mol)	VDW (kcal/mol)	Electrostatics (kcal/mol)	Residue	IE (kcal/mol)	VDW (kcal/mol)	Electrostatics (kcal/mol)
GST											
<b>1</b>				<b>2</b>				<b>3</b>			
LEU 6	-1.29	-0.0074	-1.28	LEU 6	-3.68	-0.0091	-3.67	LEU 6	0.37	-0.0021	0.37
SER 9	0.46	-0.25	0.71	SER 9	0.19	-0.58	0.77	SER 9	-0.088	-0.17	0.081
ALA 10	-0.34	-0.23	-0.12	ALA 10	-0.74	-1.31	0.57	ALA 10	0.35	-0.75	1.1
PRO 11	-3.15	-3.44	0.28	PRO 11	-4.71	-4.77	0.057	PRO 11	-2.68	-2.32	-0.36
LEU 33	-0.01	-0.024	0.013	LEU 33	0.85	-0.026	0.88	LEU 33	0.67	-0.0095	0.68
GLN 49	-1.34	-0.43	-0.91	GLN 49	0.22	-0.38	0.59	GLN 49	-2.49	-1.15	-1.34
CYS 51	-0.1	-0.94	0.84	CYS 51	-1.71	-0.92	-0.79	CYS 51	-1.39	-0.93	-0.46
PRO 53	-2.38	-1.83	-0.55	PRO 53	-2.77	-0.56	-2.21	PRO 53	-1.94	-1.34	-0.61
GLU 64	-4.44	-2.79	-1.65	GLU 64	-6.49	-0.29	-6.19	GLU 64	-6	-1.91	-4.09
SER 65	-7.93	-2.14	-5.79	SER 65	-5.61	-1.45	-4.16	SER 65	-2.67	-0.87	-1.8
ARG 66	-1.41	-1.48	0.064	ARG 66	-21.36	0.68	-22.04	ARG 66	-7.6	-0.31	-7.29
MET 101	-0.12	-0.95	0.82	MET 101	2.06	-0.48	2.54	MET 101	0.33	-1.43	1.77
TYR 105	-5.54	-2.35	-3.19	TYR 105	-6.09	-3.26	-2.82	TYR 105	-7.64	-3.71	-3.94
GLN 106	-0.4	-0.47	0.066	GLN 106	-1.22	-0.16	-1.06	GLN 106	-1.56	-2.07	0.51
ALA 109	-0.77	-0.17	-0.6	ALA 109	-1.87	-0.17	-1.7	ALA 109	-0.56	-0.56	-0.00051
TYR 113	1.93	-0.23	2.17	TYR 113	0.57	-0.55	1.12	TYR 113	-1.72	-0.13	-1.59
PHE 117	0.019	-0.012	0.03	PHE 117	0.54	-0.024	0.57	PHE 117	-0.67	-0.0015	-0.67
PHE 203	-0.088	-0.045	-0.043	PHE 203	-1.62	-0.15	-1.47	PHE 203	-1.95	-0.03	-1.92
TYR 206	0.24	-0.0065	0.25	TYR 206	-0.18	-0.017	-0.16	TYR 206	0.38	-0.000084	0.34
3Å IE	-25.07	-15.92	-9.18	3Å IE	-46.52	-11.90	-34.60	3Å IE	-32.81	-15.67	-17.15
Surface IE	-26.66	-17.79	-8.89	Surface IE	-53.62	-14.43	-39.17	Surface IE	-36.86	-17.69	-19.22
Total IE	-45.96	-20.43	-25.53	Total IE	-79.69	-17.19	-62.50	Total IE	-69.26	-20.45	-48.81



**Figure 7.2:** Binding residues of (a) three binding sites of AChE enzyme, blue, orange and lime green residues depict the anionic binding site, catalytic binding site and peripheral binding site respectively and (b) binding residues that interact with ligand within 3 Å.

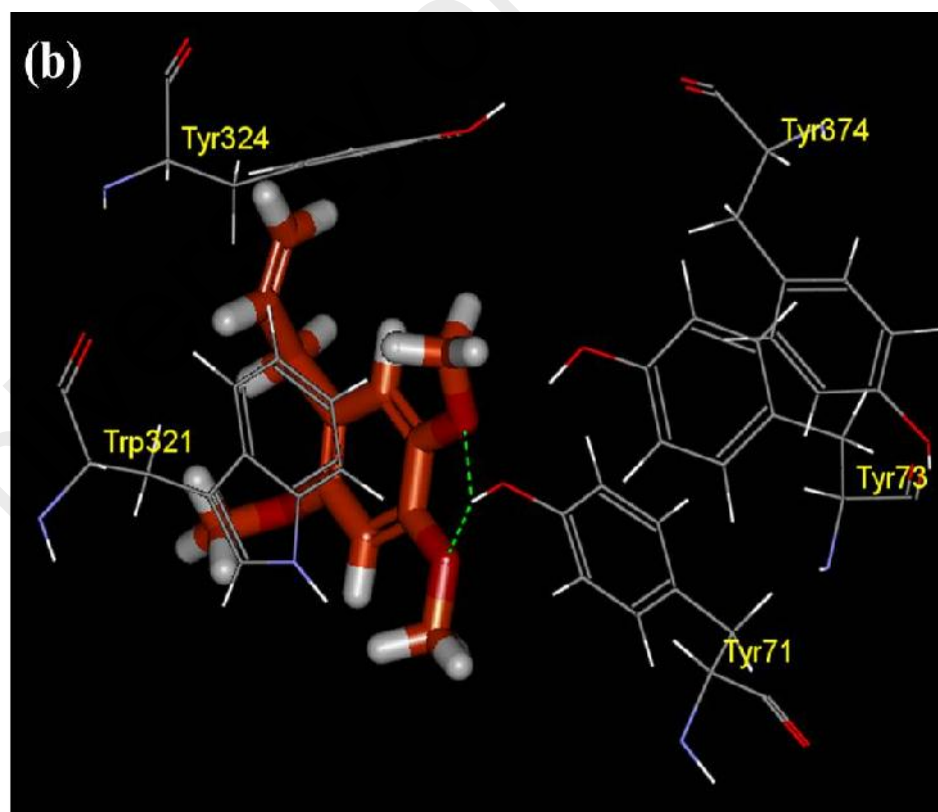
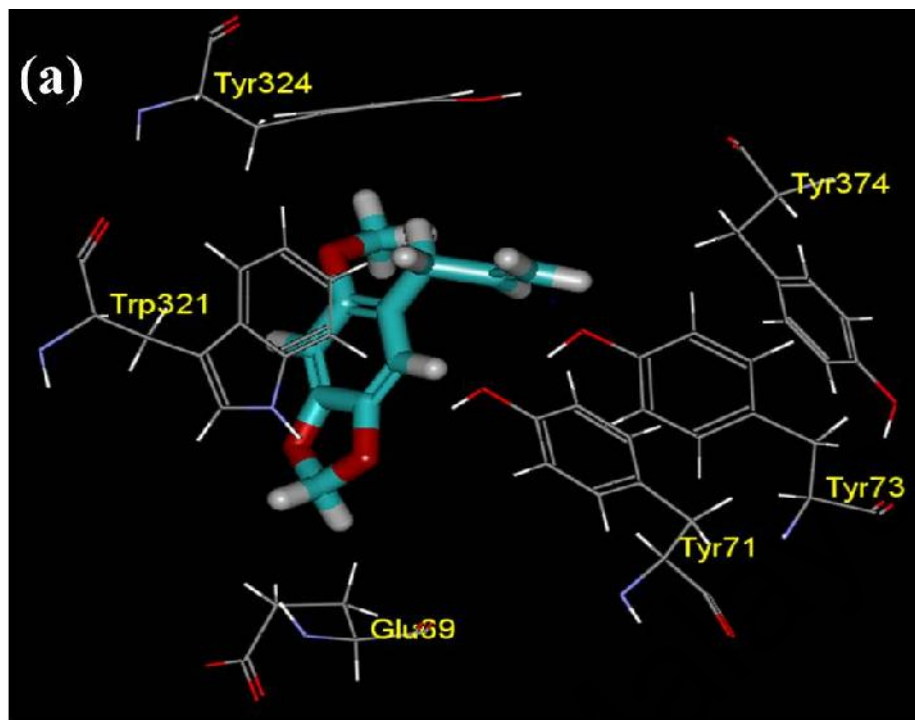
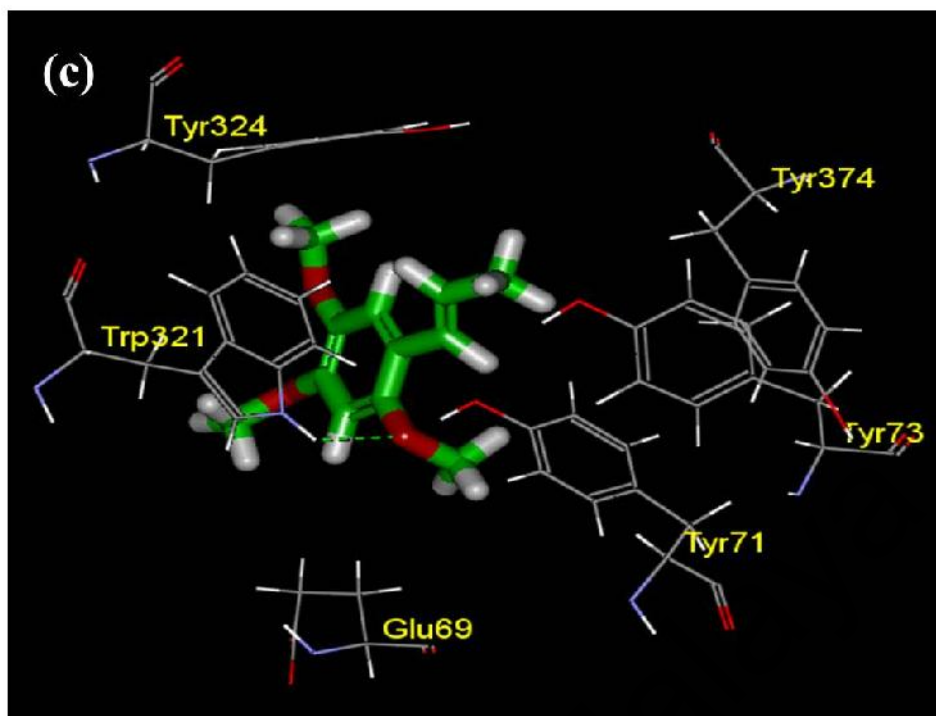
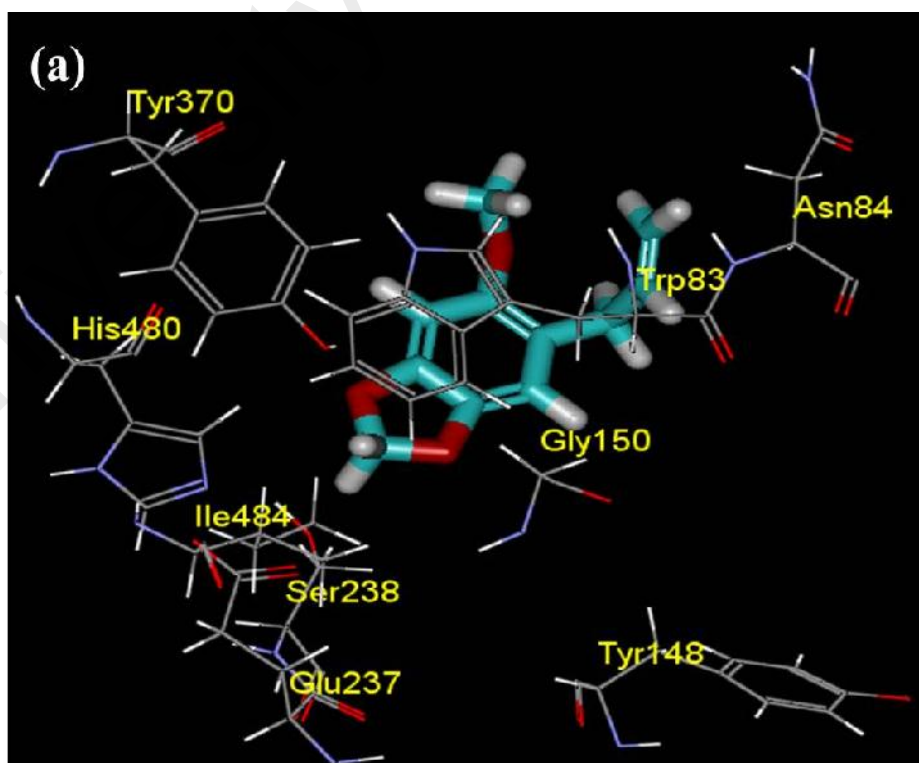


Figure 7.3, continued'

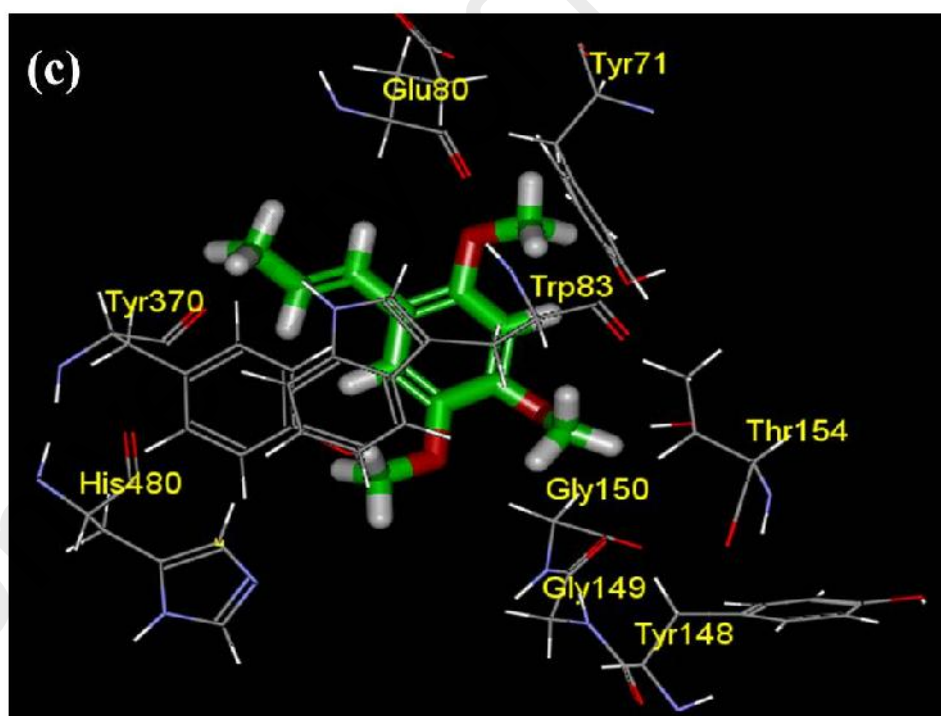
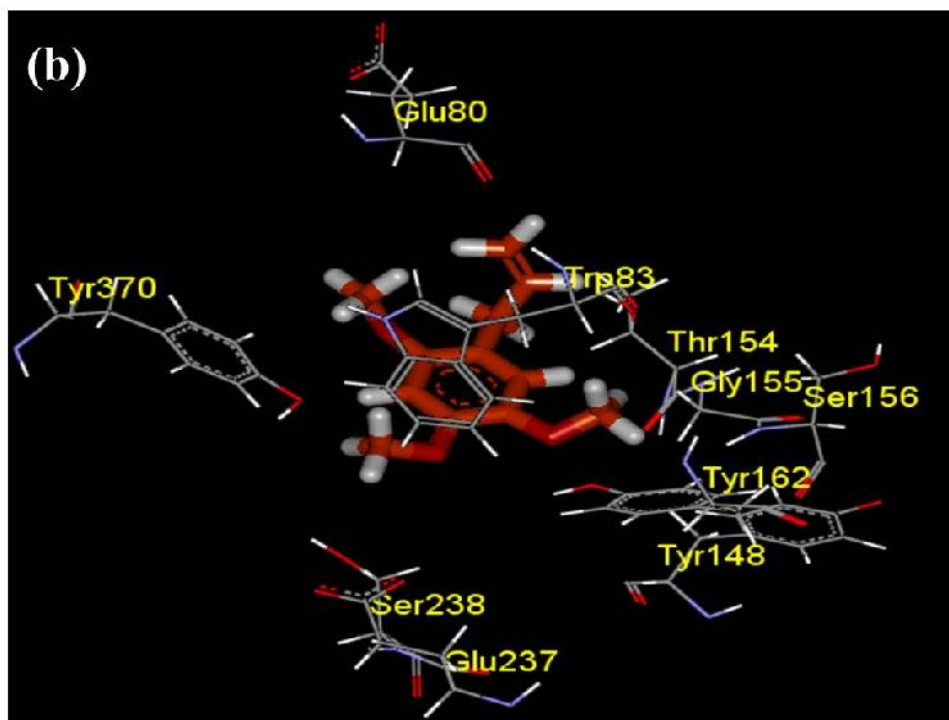




**Figure 7.3:** Docking structures of compound (a) compound 1 (b) compound 2 (c) and compound 3 towards peripheral site of AChE and their closed contact residue interaction with the hydrogen bond interactions at TRP77:HH: ACO14 (2.17Å) and TRP77:HH: ACO12 (2.27Å) in (b) and TRP321:HE1: C1O22 (2.35Å) in (c). Only residue interacted with compound at  $< -2$  kcal/mol are shown.



**Figure 7.4, continued'**



**Figure 7.4:** Docking structures of compound (a) compound 1 (b) compound 2 (c) and compound 3 toward Anionic site of AChE and their closed contact residue interaction. Only residue interacted with compound at  $< -2$  kcal/mol are shown.

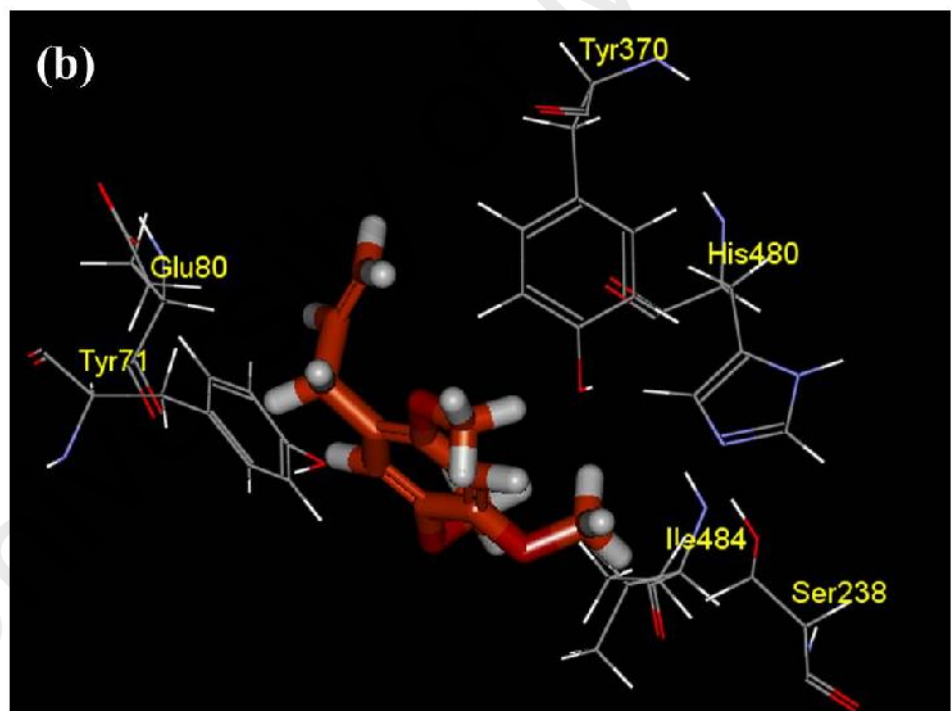
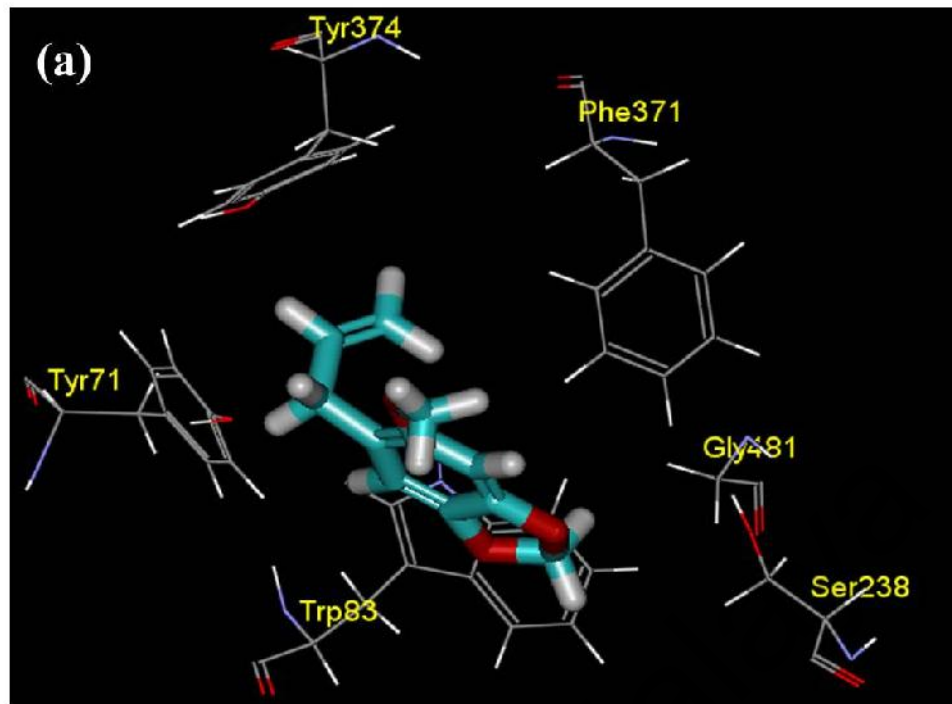
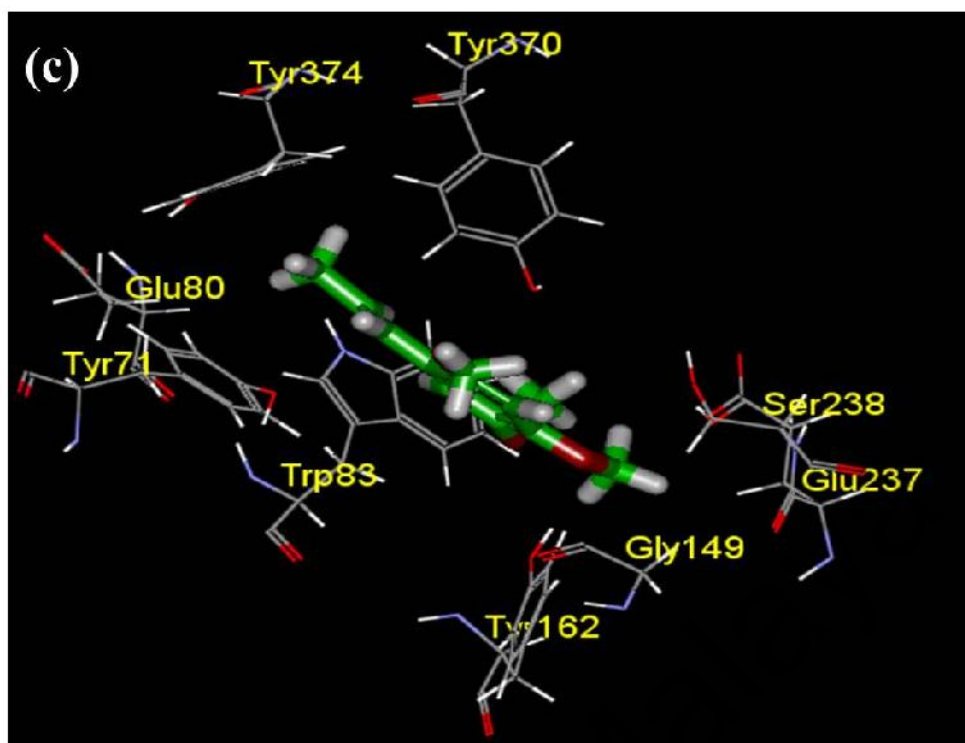
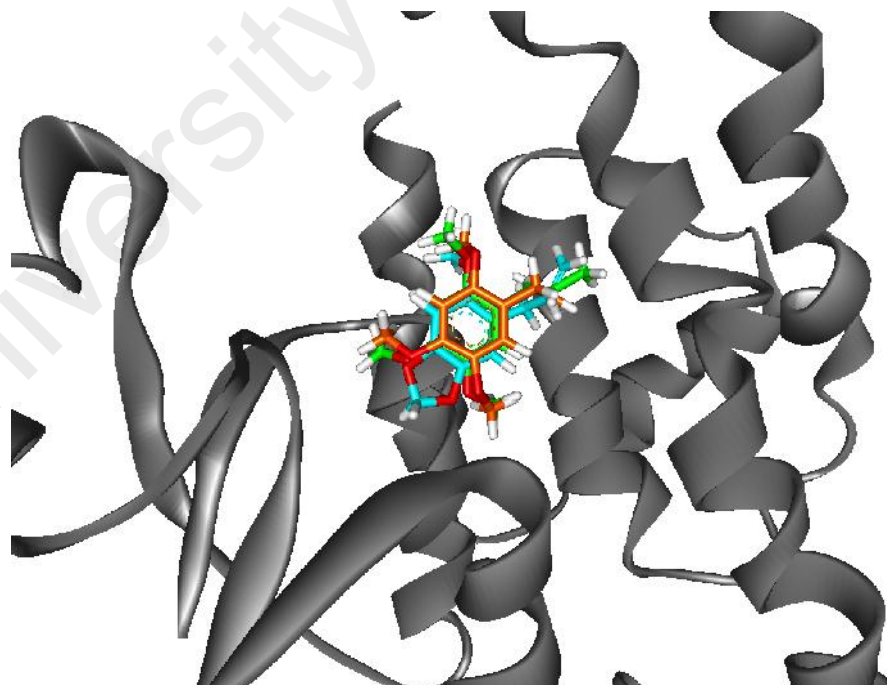


Figure 7.5, continued'



**Figure 7.5:** Docking structures of compound ( a) compound 1 (b) compound 2 (c) and compound 3 towards Catalytic site of AChE and their closed contact residue interaction. Only residue interacted with compound at  $< -2$  kcal/mol are shown.



**Figure 7.6:** Docking complexes of compounds 1 (blue), 2 (orange), and 3 (green) with GST.

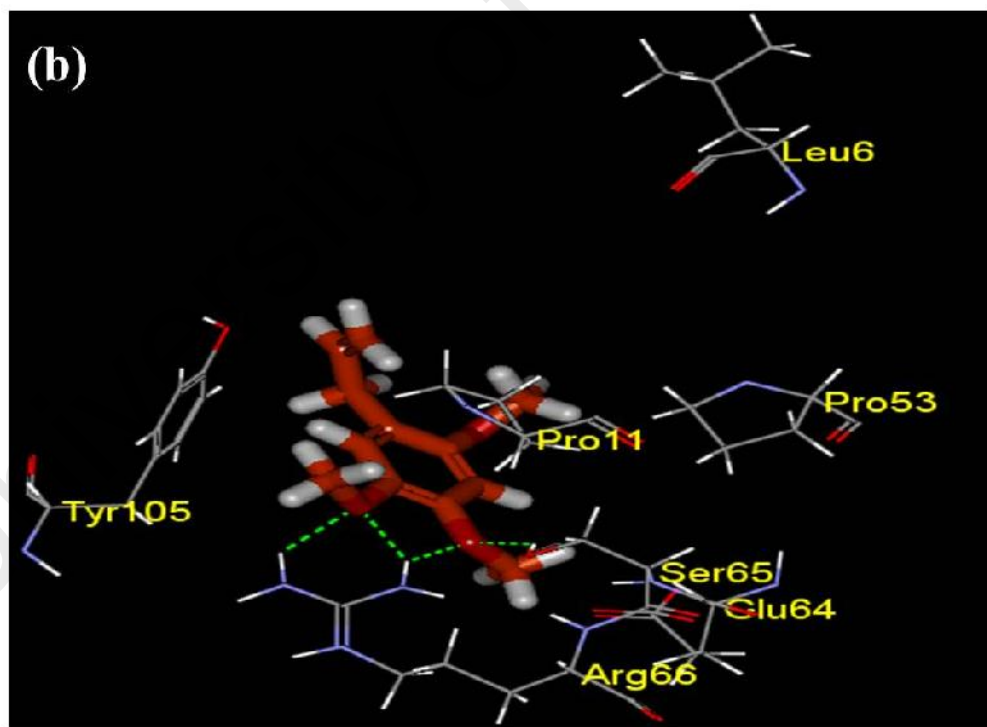
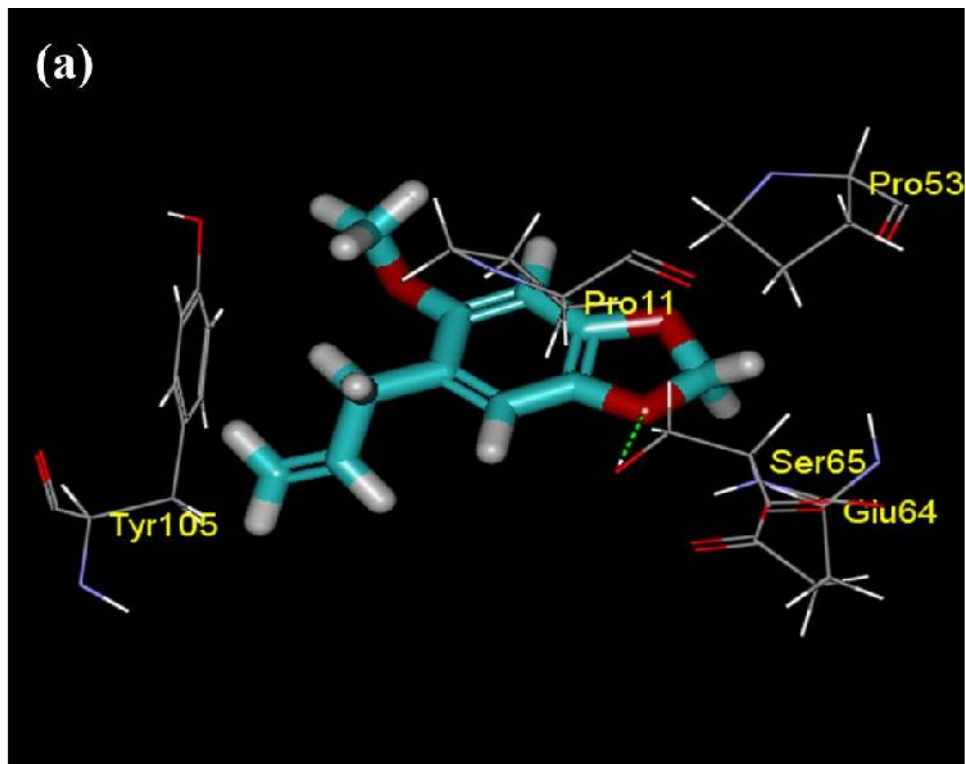
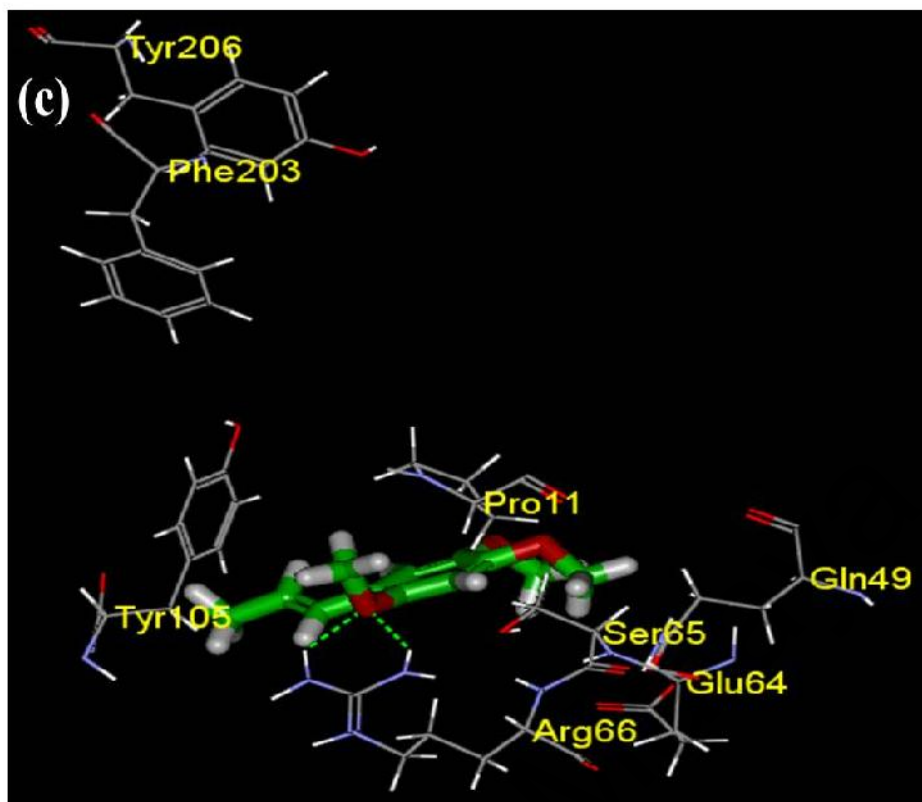


Figure 7.7, continued'



**Figure 7.7:** Docking structures of compound ( a) compound 1 (b) compound 2 (c) and compound 3 towards GST and their closed contact residue interaction with the hydrogen bond interactions at SER65HG:O9 in (a) SER65HG:O12, ARG66HH12:O14, ARG66HH12:O12 and ARG66H22:O14 in (b) ARG66HH12:O22 and ARG66HH22:O22 in (c) and TYR113OH:H30 in (d). Only residue interacted with compound at  $< -2$  kcal/mol are shown.

## 7.5 DISCUSSION

### 7.5.1 Binding affinity of compounds 1, 2 and 3 towards AChE and GST

Binding energy evaluation provided a correlation to the activity testing performed at the experimental stage. The more negative the binding energy, the better the binding activity. In another word, the binding between the compound and the target will be more favorable (Atterwill & Neal, 1978). Binding energy and interaction energy computed from Autodock/Vina and Discovery Studio program, respectively would reveal the binding affinity and interaction mediated by the compounds within the binding sites of enzyme. The detailed interaction formed between the binding residues

of enzyme towards the compounds could be dissected by calculating 3 Å interaction energy. Calculated interaction energy of both asaricin **1**, isoasarone **2** and trans-*asarone* **3** exhibited a negative value and also implying the binding affinity of the compounds to anionic site is higher compared to CAS site and PAS site, as summarized in Tables 7.2 to 7.4. The calculated binding energies were well correlated with experimental data that both compounds are active against AChE. Therefore, further analysis on the intermolecular interactions would explain the differences in binding activity in different binding site as showed in Figures 7.3 to 7.5. There were similar study has been conducted on similar compounds such as Huperzine A which is found in an extract from a club moss that has been used for centuries in Chinese folk medicine. In previous study done by Badia et al., (1998) Huperzine A (HupA) showed high AChE inhibition and docking ability with AChE enzyme. Its action has been attributed to its ability to strongly inhibit acetylcholinesterase (AChE).

### **7.5.2 Molecular interaction of compounds 1, 2 and 3 towards AChE at the PAS binding site**

Binding affinity of asaricin **1**, isoasarone **2** and trans-*asarone* **3** in catalytic binding site obtained from from Autodock/Vina is generally low, -4.7 kcal/mol, -3.9 kcal/mol and -3.9 kcal/mol, respectively (Table 7.2). It is also consistent with the high 3 Å interaction energy (IE) of asaricin **1**, isoasarone **2** and trans-*asarone* **3** at -14.26, -16.51 and -25.3 kcal/mol as tabulated in Table 7.2. The intermolecular interactions mostly contributed by aromatic-aromatic ring interactions (Renuga Parameswari et al., 2015) as the compounds containing aromatic rings that favoured the ring-ring interaction. Asaricin **1**, isoasarone **2** and trans-*asarone* **3** were found interacting with TYR 71, TRO 83 and TYR 370 in similar manner. Stacking effect between the ring of

the compounds and the ring of TYR 71 gave rise to very low interaction energy. It is however, distance between the rings gave energy contribution at different magnitude.

### 7.5.3 Molecular interaction of 1, 2 and 3 toward AChE at the PAS binding site.

The binding affinity (Table 7.2) and 3 Å IE of the asaricin **1**, isoasarone **2** and trans-*asarone* **3** in peripheral binding sites (PAS) is slightly higher than the catalytic site (Table 7.2). Although asaricin **1** did not form hydrogen bond with any of the binding residues, however due to its elongated structure, it penetrates deeper into the binding site and making contact with GLU 69 with a relatively low energy. Whereas isoasarone **2** formed two hydrogen bonds with residue TYR 71 through atom O12 and O14 at distance of 2.17Å and 2.27Å, respectively and therefore, very low interaction energy was observed, about -9.35 kcal/mol. trans-*asarone* **3** comparatively had higher energy bond -0.95 kcal/mol which means it is not as stable as **1** and **2**. Apart from that, the -OH group of residue TYR 73 directed itself towards the aromatic ring of the asaricin **1** and isoasarone **2** as presented in Figure 7.4. The presence of electron-donating group -OCH<sub>3</sub> attached to the ring resulted in the electron density in the ring to be high and hence, created a negatively charged site that possibly come in contact with the hydrogen of the OH group of TYR 73 which was partially electron deficient. Distance between the ring of asaricin **1**, isoasarone **2** and trans-*asarone* **3** hydrogen of TYR 73 gave rise to the difference in energy contribution. A great difference in opposition charge and short distance between the two interface groups would produce a tight binding. Another important binding residue was observed to be TRP 321. The ring structure of the two compounds interfaced with the rings of TRP 321 and therefore, initiated aromatic-aromatic interactions. Besides, isoasarone **2** was observed making a hydrogen contact with TRP 321 and it strengthens the interaction to a greater extend.



#### 7.5.4 Molecular interaction of **1**, **2** and **3** toward AChE at the anionic site.

Examining the binding affinities from anionic binding sites was selected for discussion due to the fact that other inhibitors in previous reports were exhibited the strongest binding affinity at this binding site which actually corresponding to the binding activity (Liew et al., 2015). The calculated binding affinity and 3 Å IE for asaricin **1**, isoasarone **2** and *trans*-asarone **3** when they are accommodated in anionic site are very much different from other binding sites. All three compounds are having the highest binding affinity, lower than -6 kcal/mol (Table 7.1) in this binding pocket despite the absence of hydrogen bond formation. TRP 83 is highlighted at the first place due to its distinctive low energy as tabulated in Table 7.4. The ring of compounds and the ring of TRP 83 have just come into right position that encouraged the stacking as displayed in Figure 7.4. Ring-ring interactions also occurred between the compounds and TYR 370. The side chain of isoasarone **2** that containing a double bond, together with its adjacent side chain were observed to form a very large interactive surface to interact with the residues of the enzyme, THR 154, GLY 155, SER 156, TYR 162 within the binding site. It is therefore contributed to a lower 3 Å IE as compared to asaricin **1** and *trans*-asarone **3**. On the other hand, the elongated structure of asaricin **1** and *trans*-asarone **3** enabled it to be embedded deeper into the binding pocket to interact with GLU 237, SER 238 and ILE 484 with a much lower energy. Within the 3 Å binding vicinity, van der Waals interactions are more vital than electrostatics forces to accommodate the ligand in the binding site. The **1**, **2** and **3** are found to be most active in this binding site most probably due to the ability in mediating interactions with more residues within 3 Å using a lower energy.

### 7.5.5 Molecular interaction of phenylpropanoids compounds toward GST

A compound may be less effective as an insecticide if it binds strongly and readily with GST. That particular insect may be able to get rid of the insecticidal compound before the compound could affect the targets. Total interaction energy and interaction energy (IE) within 3 Å residues (as highlighted in Table 7.6) indicated that highest interaction energy obtained from compound **1** (-25.07 kcal/mol) followed by compound **3** (-32.81 kcal/mol) and compound **2** (**-46.52 kcal/mol**). Figure 7.6 and 7.7 has illustrated the detailed interactions, in particular, hydrogen bonds of compounds formed in the binding cavity and denoted by green dotted line. All the compounds **1**, **2** and **3** were found to form hydrogen bond(s) with the binding residues within the active site. Compound **2** formed four hydrogen bonds through SER65HG:O12, ARG66HH12:O14, ARG66HH12:O12 and ARG66H22:O14, (Figure 7.7b), compound **3** formed two hydrogen bonds via ARG66HH12:O22 and ARG66HH22:O22 (Figure 7.7c) whereas compound **1** formed one hydrogen bond with the binding residues through SER65HG:O9 (Figure 7.7a). Hydrogen bond formed would accommodate a ligand tightly in the binding region and the interactions would be strengthened when number of hydrogen bonds increased. That explained a distinctive low IE recorded at residue ARG 66 when it interacted with compound 2 due to formation of 3 hydrogen bonds. Important residues that are commonly interacting with all the 3 compounds within 3 Å had been located, PRO11, GLU64, SER65, ARG66, and TYR105 with IE < -2 kcal/mol. Electrostatic contribution is dominating van der Waals contribution as a summation of IE in compounds **2** and **3** but not in compound **1**.

### 7.6 CONCLUSION

The binding affinity of the **1**, **2** and **3** towards the three binding pockets of AChE were explored and compared. Binding affinity from Auto-dock/Vina suggested that

peripheral binding site might be the most possible target site that **1**, **2** and **3** interacted with. This was further supported by calculated surface interaction energy and 3Å interaction energy. The low surface interaction energy implied that compounds **1** and **2** were actually bounded in the peripheral binding pocket readily and were with higher affinity compared to **2**. The interaction energy of the **1**, **2** and **3** was very comparable to the one contributed by previous researches, indicating that all three compounds were active. In addition, binding affinity of the **1**, **2** and **3** towards the detoxifying enzyme, GST was evaluated. All the compounds were observed to have a relatively low affinity towards GST. Compound **1** interacted with GST with the highest energy which suggested that it might not be easily neutralized by GST which affects its inhibitory activity. It might suggest that **1** could be the most potent toxic compound. Hence, the **1** and **2** were highly potent inhibitors as they could interact with target site AChE at a considerably low binding energy and it was consistent with the assay results from laboratory. On other hand and **3** was had low binding affinity and this was not correlate with it low activity and this may due to other detoxifying

## CHAPTER 8: GENERAL DISCUSSION

Many medicinal plants from Piper family such as *Piper auritum*, *Piper aduncum*, *Aegle marmelos* and *Piper retrofractum* are known to have insecticidal activities against insects pests (Yadav et al., 2009). *Piper sarmentosum* is medicinal local plant in Malaysia with insecticidal activities. The use of local medicinal plant extracts for storage insects pest and mosquito control will reduce dependence on expensive imported products and maximize the local efforts to enhance public health (Burkill, 1966; Nair & Burke, 1990). The insecticidal activity assays against tested insects indicated the high potency of asaricin **1**, isoasarone **2** and mild activity of *trans*-asarone **3**. Based on the LC<sub>50</sub> and LC<sub>95</sub> values and their respective 95% confidence intervals, **1** and **2** was found to be significantly effective towards *S. oryzae* and *R. dominica* adults and *P. interpunctella* larvae. Although the LC<sub>50</sub> and LC<sub>95</sub> values for all three species were variable but they were highly potent with LC<sub>50</sub> ≤ 5.6 µg/mL to LC<sub>50</sub> ≤ 17.3 µg/mL and LC<sub>95</sub> ≤ 14.3 µg/mL to LC<sub>95</sub> ≤ 37.7 µg/mL. In addition showing potent repellency and residual toxicity make the compounds **1** and **2** activity even more significant (Koul., 2004; Huangetal., 1997).

Similarly *in vivo* assays against *Ae. aegypti*, *Ae. albopictus* and *Cx. quinquefasciatus* larvae proved that the dosage that can cause 50% mortality was ≤ 7 µg/mL and 100% mortality was observed at concentration of ≤ 15 µg/mL which were relatively low for asaricin **1** and isoasarone **2**. Although *Cx. quinquefasciatus* larvae showed slightly highest resistance asaricin **1** and isoasarone **2** could cause 100% mortality to its larvae at low dosage µg/mL. In previous studies on Piper family including *P. sarmentosum*, ovicidal activity of extracts and essential oils has been reported (Silvia et al., 2008; Anees, 2008). Asaricin **1** and isoasarone **2** showed reliable ovicidal activity against three mosquito species; *Ae. aegypti*, *Ae. albopictus* and *Cx.*

*quinquefasciatus*. It is possible that this activity be significantly improved by future studies on formulation of the above compounds (Stroh et al., 1998). Although the repellent activity was not as significant but due to the structure of these compounds (hydrophilic head and hydrophobic tail) they may be useful in combining with other known natural repellent.

Toxic action of these compounds could be the result of enzyme inhibition related to hydrophobicity and the generation of organic radicals and reactive oxygen species (Kamaraj et al. 2010). To clarify the mechanism of action of asaricin **1**, isoasarone **2** and *trans*-asarone **3**, acetylcholinesterase (AChE), glutathione S-transferases (GST), oxidases and non-specific esterases activities were evaluated for tested insects. Asaricin **1**, and isoasarone **2** showed the high AChE inhibition while *trans*-asarone **3** AChE inhibition were moderate. The statistic analysis of these result indicated that there is meaningful and negative correlation between LC<sub>95</sub> value and AChE inhibition value of tested storage insects pests and mosquito vectors larvae (Hemingway, 2000). The significant negative correlation of asaricin **1**, isoasarone **2** and *trans*-asarone **3** toxicity with AChE inhibition suggest that asaricin **1** and isoasarone **2** can considered as neuron toxic compounds (Barry & Polavarapu, 2004; Bass et al., 2014). *Trans*-asarone **3** had lower AChE inhibition activity but still its result had meaning full correlation means that its mechanism of activity is similar to asaricin **1**, isoasarone **2** but in lower range. To the author's knowledge, this is the first report on the larvicidal and AChE inhibition activity of asaricin **1**, isoasarone **2** and *trans*-asarone **3** against tested storage pest insects and mosquito vectors.

As the GST participates in detoxification and the regulation in the cellular level it was essential to examine the relation between GST and **1**, **2** and **3** (Kotze et al., 2014).

All tested insects GST activity was significant in enzyme test. The GST activity was significantly higher in the tested insects with higher resistance to **1**, **2** and **3**; *P. interpunctella* and *Cx. quinquefasciatus* latvea. The GST activity in tested mosquito was positively correlated with **1**, **2** and **3** relative LC<sub>95</sub> value means. These results showed the GST role in the detoxification as one of the important factor in insects resistance (Almli et al., 2002). Oxidases and non-specific esterases did not show any meaningful activities in respond to **1**, **2** and **3**.

To study the binding affinity and interaction mediated by the compounds within the binding sites of enzymes Autodock/vina and Discovery Studio programme, were assisted. Calculated interaction energy of **1**, **2** and **3** exhibited a negative value and also implying the binding affinity of the compounds to anionic site was higher compared to CAS site and PAS site, as summarized in (Vitorović-Todorović et al., 2014). The calculated binding energies were well correlated with experimental data that both compounds are active against AChE. Asaricin **1**, isoasarone **2** and *trans*-asarone **3** were found interacting well at anionic site with TYR 71, TRO 83 and TYR 370 residue in similar manner. The **1** and **2** compounds are found to be most active in this binding site most probably due to the ability in mediating interactions with more residues within 3 Å using a lower energy.

On the other hand the GST computational analysis did show the interaction of **1**, **2** and **3** with GST and the importance of PRO11. The analysis suggested that **1**, **2** and **3** had stronger binding with GST enzyme PRO11, GLU64 and TYR105 residue compare SER65 and ARG66 residues (Zhang, Modén, Tars, & Mannervik, 2012). Although all three compounds could bond with GST enzyme but they still could have strong insecticidal activity. This may be due to their binding pocket as compare to previous

researchs mostly toxicant bind to SER65 and ARG66 residues while in this case they prefer PRO11, GLU64 and TYR105 (Hemingway et al, 2004 and Nkya et al, 2012).

In near future biopesticides will play larger and more important role in controlling agriculture and health related pests control (Peshin, Jayaratne & Sharma 2014). Since the compounds **1**, **2** and **3** are natural occurring compounds and have not been used as bioinsecticide therefore insects may not develop resistance as quickly as synthetic insecticide (Senghor, Liang & Ho, 2007). On other hand their isolation process can be done at low cost compare to common biopesticide such as azadirachtin and rotenone (Wright, 2014). Therefore **1**, **2** and **3** have high potential to be used as biopesticide in near future. The current search for new bioinsecticides from *P. sarmentosum* is based on this philosophy.

## CHAPTER 9: GENERAL CONCLUSION

Out of three extracts screening from *Piper sarmentosum* for efficacy as eco-friendly bioinsecticide, hexane and methanol were the most effective. Further bioassay guided separation study lead to identify the active fraction and isolation of four phenylpropanoids; Asaricin **1**, isoasarone **2**, *trans*-asarone **3** and asaraldehyde **4**. Compounds **1**, **2** and **3** were relatively isolated form hexane and methanol extracts of roots and aerial part while asaraldehyde **4** was only isolated from aerial part. Bioassay on tested storage insect pests showed that **1** and **2** were highly potent against *Sitophilus oryzae*, *Rhyzopertha dominica* adults and *Plodia interpunctella* larvae whereas **3** had mild activity. In addition having strong repellency activity followed by favorable residual toxicity brings high value to compounds **1** and **2** as natural storage grain protection. Compound **3** had very high LC value but more consistent activity within 60 days of residual test which can be consider in future investigation. In addition **1** and **2** were the main derivatives that showed strong larvicidal and ovicidal activity against *Aedes aegypti*, *Aedes albopictus* and *Culex quinquefasciatus* larvae. Compound **3** larvicidal activities were moderate and did not show strong ovicidal activity and compound **4** was not active. The low LC values of **1** and **2** against *Ae. aegypti*, *Ae. albopictus* and *Cx. quinquefasciatus* late 3<sup>rd</sup> early 4<sup>th</sup> instar larvae was remarkable. **1**, **2** and **3** were not highly active as adulticide and repellent. Since the compounds were used in their pure form it's possible that there adulticide activities improve by formulation in future work. The biochemical assay revealed the strong acetylcholinesterase (AChE) inhibition by **1** and **2**. Although compound **3** was able to inhibit the AChE as well its level of inhibition was significantly lower. The significant negative correlation between the AChE inhibition and LC<sub>95</sub> can imply that **1**, **2** and **3** toxicity are by disrupting the insects nervous system. Further biochemical assay on GST enzyme reveal high level of



activity in all tested insects (*S. oryzae*, *R. dominica*, *P. interpunctella*, *Ae. aegypti*, *Ae. albopictus* and *Cx. quinquefasciatus*). The GST activity in all tested insects was positively correlated with compounds **1**, **2** and **3** relative LC<sub>95</sub>. These results showed the GST role in the detoxification as one of the important factor in insects resistance. The computation work on docking the compounds **1**, **2** and **3** with AChE and GST enzyme showed the interaction between toxicants and the enzymes. The compounds **1**, **2** and **3** binding with active catalytic and anionic site of AChE showed their strong inhibition ability. In the GST docking study, **1**, **2** and **3** bind with PRO11, GLU64 and TYR105 which were different from known active residue of SER65 and ARG66. This can explain that although **1** and **2** bind to GST enzyme but they do not bind to the essential residue so they won't detoxify easily. Understanding the mechanism of action of **1**, **2** and **3** can help for controlling the resistance of insects pests in future work.

*P. sarmentosum* has been used in local cuisine for decades and did not show any mammalian toxicity. With global insects resistance and pollution problem finding a sustainable and environmental friendly bioinsecticide with natural origin is essential in IPM. With this context, the current study introduced potential candidates for future biopesticide investigation and formulation.

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