A THESIS

FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

Larvicidal Activity of Constituents Identified in *Asarum heterotropoides* and *Millettia pinnata* against Four Mosquito Species and Their Potential Mode of Action

세신과 폰가미아 유래 화합물들의 감수성 및 저항성 모기 종들에 대한 살충활성과 잠재적인 작용기작

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UNDER THE DIRECTION ADVISER YOUNG JOON AHN
SUBMITTED TO THE FACULTY OF THE GRADUATE SCHOOL
OF SEOUL NATIONAL UNIVERSITY

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ABSTRACT

Mosquitoes are prevalent worldwide and common and serious disease vectoring insect pest. Mosquito larval abatement has been achieved principally by the use of organophosphorus insecticides (OPs), insect growth regulators, and bacterial larvicides. Continued and repeated use of these larvicides has disrupted natural biological control systems and led to resurgences of mosquitoes, often resulted in the widespread development of resistance, and has undesirable effects on aquatic nontarget organisms, and fostered environmental and human health concerns. Therefore, right now use of eco-friendly and cost-free plant based products for the control of mosquitoes is inevitable. Currently, numerous products of botanical origin, especially the secondary metabolites, have received considerable renewed attention as potentially bioactive agents used in insect vector management. In addition, studies on the mode of action of insecticides or plant-derived constituents are very important because it helps chemists to design additional chemicals with similar mode of action and because it could give scientists important clues as to the cause of resistance development in pests, particularly

that involving target insensitivity, and thereby helps in designing countermeasures to avoid resistance or reverse the development of resistance. In this study, an assessment was made of the toxicity of constituents identified in *Asarum heterotropoides* root steam distillate, methanolic extract of the plant root, and Indian native plant *Millettia pinnata* (formerly known as *Pongamia pinnata*) seeds against third instars from insecticide-susceptible and -resistant mosquito species. The possible mode of action and delivery of the constituents are also examined.

The toxicity of *A. heterotropoides* root steam distillate constituents to third instars of *Culex pipiens pallens*, *Aedes aegypti*, and *Ochlerotatus togoi* (formerly *Aedes togoi*) was examined using a direct-contact mortality bioassay. Results were compared with those following the treatment with OPs fenthion and temephos. *A. heterotropoides* root steam distillate exhibited good larvicidal activity (21.07–27.64 mg/L) against three mosquito species, based on LC_{50} values. Potent activity was produced by safrole (LC_{50}, 8.22–16.10 mg/L), terpinolene (11.85–15.32 mg/L), γ-terpinene (12.64–17.11 mg/L), (–)-β-pinene (12.87–18.03 mg/L), (+)-limonene (13.26–24.47 mg/L), 3-carene (13.83–19.19 mg/L), and α-phellandrene (13.84–23.08 mg/L), although the larvicidal activity of these compounds was less toxic than either fenthion (LC_{50}, 0.023–0.029 mg/L) or temephos (0.016–0.020 mg/L).

The toxicity of (–)-asarinin, α-asarone, methyleugenol, pellitorine, and pentadecane identified in *A. heterotropoides* root to third instars from insecticide-susceptible *Cx. p. pallens* (KS-CP strain), *Ae. aegypti*, and *Ochlerotatus togoi*, as well as field-collected *Cx. p. pallens* (DJ-CP colony), identified by polymerase chain reaction, were compared with

those of two mosquito larvicides: fenthion and temephos. Pellitorine (LC_{50}, 2.08, 2.33, and 2.38 mg/L) was 5.5, 10.8, and 25.6 times, 4.5, 11.6, and 24.7 times, and 6.9, 11.1, and 24.6 times more toxic than (–)-asarinin, α-asarone, and methyleugenol against susceptible *Cx. p. pallens*, *Ae. aegypti*, and *Oc. togoi* larvae, respectively. Pentadecane was least toxic. Overall, all the compounds were less toxic than either fenthion or temephos.

The toxic effect of pellitorine alone or in combination with (–)-asarinin, α-asarone, methyleugenol, and pentadecane (1:1, 1:2, 1:3, 2:1, and 3:1 ratios) to third instars from insecticide-susceptible KS-CP strain and -resistant DJ-CP colony of *Cx. p. pallens* was likewise evaluated. Binary mixture of pellitorine and asarinin (3:1 ratio) was significantly more toxic against KS-CP larvae (0.95 mg/L) and DJ-CP larvae (1.07 mg/L) than either pellitorine (2.08 mg/L for KS-CP and 2.33 mg/L for DJ-CP) or asarinin (11.45 and 12.61 mg/L) alone. The toxicity of the other binary mixtures (1:1, 1:2, 1:3, and 2:1 ratios) and pellitorine did not differ significantly from each other. Based on the co-toxicity coefficient (CC) and synergistic factor (SF), the three binary mixtures (1:3, 2:1, and 3:1) operated in a synergy pattern (CC, 250–390 and SF, 1.4–2.2 for KS-CP; CC, 257–279 and SF, 1.1–2.1 for DJ-CP).

The toxicity of constituents derived from the seeds of *Millettia pinnata* to third instars of insecticide-susceptible *Cx. p. pallens* and *Ae. aegypti* and wild *Aedes albopictus* was examined. Results were compared with those of fenthion and temephos. The active principles of *M. pinnata* were identified as the karanjin (**1**), karanjachromene (**2**), pongamol (**3**), pongarotene (**4**), oleic acid (**5**), and palmitic acid (**6**) by spectroscopic analysis. Based on 24 h LC_{50} values, karanjin (14.61 and 16.13 mg/L) was the most toxic

compound, followed by oleic acid (18.07 and 18.45 mg/L) and karanjachromene (18.74 and 20.57 mg/L). These constituents were less toxic than either fenthion ($LC_{50,}$ 0.0031 and 0.0048 mg/L) or temephos (0.021 and 0.050 mg/L) against *Ae. aegypti* and *Cx. p. pallens*. Low toxicity was produced by pongamol ($LC_{50,}$ 23.95 and 25.76 mg/L), pongarotene (25.52 and 37.61 mg/L), and palmitic acid (34.50 and 42.96 mg/L). Against third instars from *Ae. alpopictus*, oleic acid ($LC_{50,}$ 18.79 mg/L) was most toxic. Low toxicity was observed with the other five constituents (LC_{50}, 35.26–85.61 mg/L).

Enzyme kinetics analysis acetylcholinesterase (AChE) was analyzed to identify compounds and also observed gastrointestinal changes of treated compounds through histopathological section. In AChE inhibition assay, there is no potent inhibition was observed (IC_{50} $>10^{-7}$) in isolated and identified compounds when compare with commercially available larvicides dichlorvos (DDVP) (IC_{50} $>4 \times 10^{-7}$) against third instars of *Ae. aegypti.* The histopathological effects of pellitorine on larval segments of third instars of *Aedes aegypti* were examined by transmission electron microscopy. Results were compared with those of deltamethrin. At a concentration of 5 mg/L, pellitorine was targeted mainly on midgut epithelium and anal gills, indicating variably dramatic degenerative response of the midgut through a sequential epithelial disorganization. The anterior midgut was almost entirely necrosed, bearing only residues inside the plasma membranes on the basal lamina. The compound has shown complete damage of all glandular cells of anal gills, because cuticle damage was occurred by pellitorine. The histopathological effects 2.5 mg/L of deltamethrin on the different regions of the midgut clearly indicate the epithelial disorganization and delocalization of cell

organelles which have been correlated with their morphofunctional status. Based on these results, pellitorine merit further study as a potential larvicide with a specific target site and mode of delivery or a lead molecule for the control mosquito populations.

In conclusion, *A. heterotropoides* root-derived materials, particularly (–)-asarinin and pellitorine, and *M. pinnata* seed-derived materials, particularly karanjin, karanjachromene, and oleic acid, merit further study as potential mosquito larvicides for the control of insecticide-resistant mosquito populations in the light of global efforts to reduce the level of highly toxic synthetic insecticides in the aquatic environment.

Key words: mosquito, botanical insecticide, *Asarum heterotropoides*, *Millettia pinnata*, synergy, resistance, acetylcolinesterase inhibition, histopathology

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LIST OF ABBREVIATIONS

AChE	Acetylcholinesterase
COSY	Correlation spectroscopy
DEPT	Distortionless enhanced by polarization transfer
EI-MS	Electron impact-mass spectroscopy
EPA	Environmental protection agency
GC	Gas chromatography
GC-MS	Gas chromatography-mass spectroscopy
HPLC	High-pressure liquid chromatography
NMR	Nuclear magnetic resonance
OPs	Organophosphorus insecticides
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
RH	Relative humidity
ROK	Republic Of Korea
RR	Resistance ratio
TEM	Transmission electron microscope
TMS	Tetramethylsilane
TLC	Thin layer chromatography
WHO	World health organization

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INTRODUCTION

Mosquitoes alone transmit disease to more than 700 million persons annually (Taubes, 1997). The northern house mosquito, *Culex pipiens pallens* (Coquillett), and the yellow fever mosquitoes, *Aedes aegypti* (L.) and *Aedes albopictus* (Skuse), are widespread and transmit a number of diseases, such as dengue fever, dengue hemorrhagic fever, Japanese encephalitis, and filariasis, are increasing in prevalence, particularly in tropical and subtropical zones. Anopheline mosquito is one of the most important vectors of tropical diseases. Malaria ranks amongst the world's most prevalent tropical infectous diseases. Worldwide it causes over a million deaths annually, the majority among African children (WHO, 2008a). Lee *et al*., (2007) suggested that *Anopheles kleini*, *Anopheles pullus*, and *Anopheles sinensis* are vectors of malaria found in Korea.

Mosquito larval abatement has been achieved primarily by the use of insecticides, and many synthetic agents have been developed and employed in the field with considerable success. Their continued or repeated use has disrupted natural biological control systems and led to resurgences of mosquitoes (Croft and Brown, 1975), has often resulted in the widespread development of resistance (WHO, 1992; Anonymous. 2011), and has undesirable effects on aquatic nontarget organisms (Cooper, 1991; Rozendaal, 1997). Particularly, the use of conventional insecticides for the control of rice, horticultural, and veterinary pests has accelerated these adverse effects. Increasing levels of resistance to the commonly used insecticides have caused multiple treatments and excessive doses, raising serious human health and environmental concerns. Major

mechanisms of resistance to insecticides currently available to control mosquitoes are target site insensitivity that reduces sodium channel sensitivity to pyrethroid insecticides or acetylcholinesterase (AChE) sensitivity to organophosphorus (OP) and carbamate insecticides and enhanced metabolism of various groups of insecticides (Hemingway *et al*., 2004). The number of approved OP and carbamate insecticides will likely be reduced in the near future in the United States (U.S) by the U.S. Environmental Protection Agency (EPA) occurs as reregistration under the 1996 Food Quality and Protection Act (USEPA, 2010). These problems substantiate the need for the development of selective mosquito control alternatives to establish an efficient resistance management strategy and tactics based on all available information on the extent and nature of resistance.

The plant world comprises a rich untapped pool of phytochemicals that may be widely used in the place of synthetic insecticides. Plant-based products have been used to control domestic pests for a very long time. The search for and investigation of natural and environmentally friendly insecticidal substances are ongoing worldwide (Balandrin *et al*., 1985; Ghosh *et al.,* 2006; Kuo *et al*., 2007). Insecticidal effects of plant extracts vary not only according to plant species, mosquito species and plant parts, but also to extraction methodology (Swain, 1977). Much effort has been focused on them as potential sources of commercial mosquito larvicides, in part, because certain plants and their constituents meet the criteria of minimum-risk pesticides (USEPA, 2006). Various compounds, including phenolics, terpenoids, and alkaloids, exist in plants (Wink, 1993). They jointly or independently contribute to behavioral efficacy such as repellence and feeding deterrence and physiological efficacy such as acute

toxicity and developmental disruption against various arthropod species (Ahn *et al*., 2006; Isman, 2006). Studies on the mode of action of chemicals are very important from several points of view. The knowledge gained by such studies yields valuable basic information on the nature of the target systems (*i.e.* the weakness of sensitive insects) in terms of physiological, biochemical, and biophysical knowledge of vital biological system (Matsumura, 1986). Possessing a different mode of action would make these compounds more valuable for further commercial development as they would be particularly beneficial in areas with documented insecticide resistance.

In this study, an assessment is made of the toxicity of constituents from *Asarum heterotropoides* root steam distillate and from methanolic extract of the plant were against larvae of mosquito species. Also, synergistic effects of binary mixtures of the constituents were investigated against larvae of both susceptible and wild *Culex pipiens pallens*. Indian native plant *Millettia pinnata* (L.) Panigrahi (formerly known as *Pongamia pinnata*) seed-derived principles were also examined against mosquito species larvae. The possible mode of action and delivery of isolated active principles are also examined.

LITERATURE REVIEW

1. Mosquitoes

There are estimated to be over 3000 species of mosquito world wide, with hundreds of species in North America. The summing up-to-date records of Korean mosquitoes, a total of 51 species and 2 forms in 10 genera in 3 subfamilies in culicidae are listed. The major mosquito species are *Anopheles sinensis, Culex pipiens pallens, Culex pipiens molestus*, *Ochlerotatus togoi* and *Aedes albopictus* etc. in Korea (Ree, 2003). There is still some debate regarding the primary vector species for malaria in Korea. *An. sinensis* used to be considered the most important vivax malaria vector in Korea, but recently, *An. pullus* and *An. kleini* have been proposed to play important roles in malaria transmission. Lee *et al*., (2007) suggested that *An.kleini*, *An. pullus*, and *An. sinensis* are vectors of malaria in Korea based on the finding that higher proportions of *An. kleini* and *An. pullus*. The majority of mosquito species feed on plant nectar and on animal blood, but it is only the females that feed on blood to provide additional nutrition for their eggs. Mosquitoes act as vectors for many human diseases including Malaria, West Nile, yellow fever, encephalitis, and dengue.

2. Public Health Importance

Mosquitoes acts as vector and transmit many diseases. All mosquito-borne diseases are zoonoses; that is, they are diseases of animals that can be transmitted to people. Normally, these diseases can only be transmitted by the bite of an infected mosquito. An infected animal or person cannot pass the infection on to another animal

or person. Taking a blood meal from an infected animal infects the mosquito. The exception is La Crosse encephalitis, where infected female mosquitoes pass the infection to their offspring through the eggs. Once a mosquito becomes infected, it remains so for life, which is normally only a few weeks. In their lifetime, mosquitoes may take three or four blood meals.

Mosquito-borne diseases are an important public health problem in most tropical countries. In Seychelles, a tropical country, cases of imported malaria, dengue fever, and filariasis have been diagnosed over the last decades. Positive serology has been found in the population for dengue fever (Calisher *et al*., 1981; Metselaar, 1968), West Nile fever (Metselaar, 1968), filariasis (Frolich, 1968; Nuti, 1982), Sindbis and Chikungunya virus infections (Calisher *et al.,* 1981). Many of the diseases that currently occur in the tropics are mosquito borne (Cook, 1996). It is commonly assumed that their distribution is determined by climate and that warmer global temperatures will increase their incidence and geographic range (McMichael, 1996; Watson, 1998). Mosquito borne disease occurs it is the direct injection of this fluid into the capillaries that enables several life forms such as viruses, protozoa, and nematode worms to exploit mosquitoes as a means of transfer between vertebrate hosts. In nearly all cases, there is an obligatory phase within the insect. This includes a stage in which they multiply prodigiously in the salivary glands, from which they can be inoculated into a new host during a later blood meal. Although most such organisms do not appear to affect either the mosquitoes or their vertebrate hosts, some are pathogens of important human and animal diseases (Table 1).

Table 1. Influences on emergent/resurgent mosquito-borne diseases

Urbanization	Deforestation	Agricultural Practices
Dengue fever	Loaiasis	Malaria
Malaria	Onchocerciasis	Japanese encephalitis
Yellow fever	Malaria	St. Louis encephalitis
Chickungunya	Leishmaniasis	West Nile fever
Epidemic polyarthritis	Yellow fever	Oropouche
West Nile fever	Kyasanur Forest disease	Western equine encephalitis
St. Louis encephalitis	La Crosse encephalitis	Venezuelan equine encephalitis

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Malaria is one of the most widespread and prevalent of infectious human diseases by mosquitoes. It is the only protozoan disease transmitted by the bite of mosquitoes and it is inoculated by infected *Anopheles* species. Due to increasing tourism world-wide, an increasing number of sporadic cases are imported in countries outside of endemic areas. In addition, infected mosquitoes imported by planes can also cause sporadic cases in residents of these countries (Jenkin, 1997). The parasites multiply inside the red blood cells, which then break open within 48 to 72 h, infecting more red blood cells. The first symptoms usually occur 10 days to 4 weeks after infection, although they can appear as early as 8 days or as long as a year after infection. The World Malaria Report 2011 summarizes data received from 106 malaria-endemic countries for 2010. Ninety-nine of these countries had ongoing malaria transmission. There were 216 million cases of

malaria in 2010; 81% of these were in the World Health Organization (WHO) African Region. An estimated 3.3 billion people were at risk of malaria in 2010 and estimated 655,000 persons died of malaria in 2010. Eighty-six percent of the victims were children under 5 years of age, and 91% of malaria deaths occurred in the WHO African Region. The number of rapid diagnostic tests was delivered by manufacturers climbed from 45 million in 2008 to 88 million in 2010, and the testing rate in the public sector in the WHO African.

Dengue is a mosquito-borne infection found in tropical and subtropical regions around the world. In recent years, transmission has increased predominantly in urban and semiurban areas and has become a major international public health concern. Dengue viruses (Flaviviridae) are widely distributed in the tropical and subtropical countries and are transmitted by day-biting mosquitoes of the genus *Aedes*. Dengue fever is endemic on all continents and affects tens of millions of persons annually (Halstead, 1992). There are four types of dengue viruses that cause dengue fever worldwide (DEN-1, DEN-2, DEN-3, and DEN-4). The incidence of dengue has grown dramatically around the world in recent decades. Over 2.5 billion people-over 40% of the world's population-are now at risk from dengue. WHO currently estimates there may be 50–100 million dengue infections were recorded worldwide every year. Cases across the Americas, South-east Asia and Western Pacific have exceeded 1.2 million cases in 2008 and over 2.2 million in 2010 (based on official data submitted by Member States). Recently, the number of reported cases has continued to increase. In 2010, 1.6 million

cases of dengue were reported in the Americas alone, of which 49,000 cases were severe dengue (WHO, 2010).

West Nile virus (family Flaviviridae) is one of the most widely spread arboviruses in Africa and Asia. A major outbreak of West Nile fever in Europe occurred in the city of Bucharest and in the lower Danube valley (Romania) in 1996; later North America during the summer of 1999 (Anderson *et al*., 1999; Lanciotti *et al*., 1999). West Nile virus (Flaviviridae) is one of the most widely spread arboviruses in Africa and Asia. The vectors are mosquitoes of the genus *Culex* (*Culex pipiens*, *Culex restuans*, *Culex salinarius*, *Culex quinquefasciatus*, and *Culex tarsalis*) and rarely *Aedes* and *Anopheles* (Fontenille, 1989; Baqar, 1993). Affected people (mostly children, but epidemics can also affect persons of all ages) show fever, sore throat, lymphadenopathy and sometimes a morbilliform rash. WNV is maintained in an enzootic cycle between mosquitoes and birds, with humans, horses, and other domestic and wild animals as incidental hosts (Hayes, 1989). In the past 5 years, the geographic range of WNV has expanded from the Old World into the Americas, resulting in disease outbreaks in humans, domestic animals, and birds (Kathrn *et al*., 2003). As no human immunization is currently available, mosquito population control and use of repellents are the only methods to prevent outbreaks.

Yellow fever virus is an arbovirus of the genus *Flavivirus*, and the mosquito is the primary vector. The "yellow" in the name refers to the jaundice that affects some patients. It carries the virus from one host to another, primarily between monkeys, from monkeys to humans, and from person to person. Several different species of the *Aedes*

and *Haemogogus* mosquitoes transmit the virus. The mosquitoes either breed around houses (domestic), in the jungle (wild), or in both habitats (semidomestic). As *A. aegypti* is the classical vector and *A. albopictus* a serious potential vector for yellow fever (Miller, 1989; Mondet, 1996), an outbreak for this typical haemorrhagic fever could occur in if a traveler contracts the disease overseas. Up to 50% of severely affected persons without treatment will die from yellow fever. There are an estimated 200,000 cases of yellow fever, causing 30,000 deaths, worldwide each year (WHO, 2011). A total of 18,735 yellow fever cases and 4,522 deaths were reported in 44 countries from Africa and South America from 1987 to 1991, which represents the greatest amount of yellow fever activity reported to the WHO for any 5-year period since 1948 (Robertson *et al*., 1992). Case-fatality rate ranges from 19 to 50% (De Cock, 1986; Thonnon, 1998).

Japanese encephalitis (JE) is a viral infection of the membranes around the brain, is transmitted by some mosquitoes of the genus *Culex*. These mosquitoes prefer to breed in vast expanses of freshwater, and normally are associated with flooded rice fields in the early stages of the cropping cycle. Key species are *Cx. gelidus* and *Cx. tritaeniorrhynchus*. The culicine mosquitoes that transmit JE prefer to bite domestic animals rather than humans, and pigs are an important part of the transmission chain, as they serve as "amplifying" hosts for the virus. JE is the most important cause of viral encephalitis in Asia (mostly Far East but also India and Singapore). It causes 50,000 cases per year and it resulted, for example, 45,000 cases and 4,300 deaths in 1990 (WHO, 1995). Recently, JE cases occurred in areas where it had not been recognised previously (Australia, Nepal, and Papua New Guinea) (Spicer, 1997; Zimmerman, 1997).

Mosquitoes of the genus *Culex* (mainly *Cx. tritaeniorhyncus*) are the main vectors of JE. Avian vertebrates are natural hosts and infected pigs represent an additional amplification factor for the virus (Isselbacher, 1994). JE (incubation period, 4-15 days; case-fatality rate, 10-40%) does not always produce an encephalitis syndrome but can also cause lower motor neurone, cranial nerve, limb or urinary bladder paralysis alone and can therefore mimic acute poliomyelitis or Guillain-Barré syndrome (Mirsha, 1997).

Chikungunya ("the thing causing bending up") virus (Togaviridae) has primates as the reservoir. It is transmitted mostly by *Aedes* mosquitoes, *Ae. aegypti* and *Ae. albopictus* (Turell, 1992). But *Cx. tritaeniorhyncus* can be an additional vector in Southeast Asia. Similarly to yellow fever virus, the virus is maintained among non human primates living in Africa (sylvatic cycle) and is also readily transmitted among humans in urban areas (urban cycle). So far, 18 countries of Africa or Asia have reported the disease (Neogi, 1995). After an incubation period of two to three days, there is a brusque onset of fever and arthralgia with chills, headache, photophobia, conjunctival injection, and abdominal pain. Chikungunya is an acute febrile illness with sudden onset of fever and joint pains, particularly affecting the hands, wrists, ankles, and feet. Most patients recover after a few days but in some cases the joint pains may persist for weeks, months or even longer. There is no direct person-to-person transmission.

Filariasis is a disease group affecting humans and animals caused by nematode parasites of the order Filariidae, commonly called filariae. Filarial parasites may be classified according to the habitat of the adult worms in the vertebral host. The cutaneous group includes *Loa loa, Onchocerca volvulus,* and *Mansonella streptocerca.*

The lymphatic group includes *Wuchereria bancrofti, Brugia malayi,* and *Brugia timori.* The body-cavity group includes *Mansonella perstans* and *Mansonella ozzardi.* Natural vectors for *W. bancrofti* are *Cx. pipiens* in urban settings and anopheline, aedean mosquitoes in rural areas. Mosquitoes of the genus *Mansonia* are accessory vectors. The common clinical manifestations of lymphatic filariasis are asymptomatic microfilaraemia, chyluria, lymphatic inflammation, and obstruction culminating in hydrocele and elephantiasis. Infection is usually acquired in childhood, but the painful and profoundly disfiguring visible manifestations of the disease occur later in life. Filariasis is mostly a disease of the poor and can serve as an indicator of underdevelopment (WHO, 1992). Humans are the only definitive host for this parasite. Currently, more than 1.3 billion people in 72 countries are at risk. Approximately 65% of those infected live in the WHO South-East Asia Region, 30% in the African Region, and the remainder in other tropical areas.

3. Mosquito management

Mosquito management plans generally include monitoring of mosquito populations and mosquito-transmitted diseases, and they define action levels based on anticipated health threats, nuisance concerns, and other societal expectations. Mosquito control programs should follow an established mosquito management plan that incorporates the principles of integrated pest management (IPM).

Biological control is typically focused on the aquatic stages of the mosquito cycle. At present, small larvivorous fishes are the only biological control agents against mosquito

population for larval mosquitoes that is practical and economically viable (Coykendall 1980; Swanson *et al*., 1996). However, pond-breeding salamanders have been reported to regulate population levels of mosquito larvae and zooplankton (Brodman *et al*., 2003). The primary predator species used for biological control is the mosquitofish (*Gambusia* spp.) (Poeciliidae) (Gratz *et al*., 1996; Rupp, 1996; Pyke, 2008). One important issue is the risks to native ecosystems of introducing a nonnative species. Although there are drawbacks to using nonnative larvivorous fishes, there are settings where this approach can be ecologically-sound, practical and cost-effective, while avoiding or reducing the need for insecticide treatments (Sakolsky-Hoopes and Doane, 1998; Kent and Sakolsky-Hoopes, 1999). The creation or restoration of aquatic habitat for native larvivorous fishes such as killfish in salt marshes, or certain species of minnows in freshwater wetlands, can substantially assist with mosquito control (Harrington and Harrington, 1961; Nelson and Keenan, 1992; Kent and Sakolsky-Hoopes, 1999; Meredith and Lesser, 2007; Van Dam and Walton, 2007).

There has been an increased interest in recent years in the use of biological control agents for mosquito control. The discovery of bacteria like *Bacillus sphaericus* (Bs) and *Bacillus thuringiensis* subsp. *israelensis* (Bti) which are highly toxic to dipteran larvae opened up the possibility of the use of these biolarvicides in mosquito eradication programs (Goldberg and Margalit, 1977; deBarjac and Larget-Thiery, 1984). Bti synthesizes intracellular crystal inclusions by sporulation that contains multiple protein components of 134 kDa, 125 kDa, 67 kDa, and 27 kDa (Sekar, 1986; Hofte and whitely, 1989; Federici *et al*., 1990; Wirth *et al*., 1998). These proteins have been cloned

individually and are toxic to mosquito larvae (Sekar and Carlton, 1985; Delecluse *et al*., 1991, 1993). Although the high efficacy and specificity of Bs and Bti are useful in controlling mosquitoes, the cost to grow and produce Bs or Bti formulations through a highly refined laboratory bacterial culture medium is exorbitant. When passive and biological control methods are insufficient, current mosquito control programs prefer to use larvicides because control efforts are focused on the source of the problem and the area treated with larvicides is typically much smaller than with adulticides that are applied after adult mosquitoes have emerged and dispersed widely.

When mosquito problems necessitate the use of insecticides, it is generally best to employ larvicides and pupicides, as they are directed efficiently at the most concentrated developmental stages of the mosquito population and reduce the need for large-scale and expensive adulticiding. Only the public county and state commissions or agencies charged with the responsibility for mosquito control may perform mosquitocidal applications on any scale, large or small. If weather or environmental concerns prevent such efforts, adulticides can be used shortly after emergence when adults are still concentrated in their source area and before they have dispersed. But the chemical approach has demerits, such as the development of insecticide resistance, environmental pollution, bioamplification of contamination of food chain, and harmful effects to beneficial insects. List of insecticide recommended by WHO is given in Table 2.

Table 2. List of insecticides for mosquito control

Insecticde	Use	WHO[a]	US EPA[b]	KFDA[c]
Pyrethroid				
Bifenthrin	adult	○	○	-
S- Bioallethrin	adult	-	○	○
Cyfluthrin	adult	○	-	○
λ-Cyhalothrin	adult	○	○	○
Cypermethrin	adult	○	○	-
α-Cypermethrin	adult	○	-	○
Cyphenothrin	adult	○	-	-
Deltamethrin	adult	○	○	○
Etofenprox	adult	○	○	○
Permethrin	adult	○	○	○
d-Phenothrin	adult	○	○	○
Resmethrin	adult	○	○	-
Organophosphate				
Fenitrothion	adult	○	○	-
Malathion	adult	○	○	-
Pirimiphos-methyl	Adult (larvae)	○	○	○
Temephos	larva	○	○	○
Carbamate				
Bendiocarb	adult	○	-	○
Propoxur	adult	○	○	-
Chitin synthesis inhibitor				
Benzoylurea				
Diflubenzuron	larva	○	○	○
Novaluron	larva	○	○	○
Triflumuron	larva	-	-	○
Pyrazole				
Chlorfenapyr	adult	-	○	○
Neonicotinoid				
Imidacloprid	adult	-	○	○
Juvenile hormone mimics				
S-Methoprene	larva	○	○	○
Pyiproxyfen	larva	○	○	○
Microbial				
B. thuringiensis israelensis	larva	○	○	○

[a] World Health Organization (WHO, 2006)

[b] Pesticide Action Network (PAN0 Pesticide Database (Kegley *et al*., 2011)

[c] Korea Food and Drug Administration (KFDA, 2009)

4. Insecticide mode of action

Studies on the mode of action of insecticides or plant derived compounds are very important from several points of view. Firstly, such knowledge is needed to understand the health hazards of these chemicals to man and other nontarget organisms. Secondly, it helps chemists to design additional chemicals with similar mode of action. Thirdly, it could give scientists important clues as to the cause of resistance development in pests, particularly that involving target insensitivity, and thereby helps in designing countermeasures to avoid resistance or reverse the development of resistance. Finally, the knowledge gained by such studies yields valuable basic information on the nature of the target systems (*i.e.* the weakness of sensitive insects) in terms of physiological, biochemical, and biophysical knowledge of vital biological system (Matsumura, 1986). Most of the insecticides in current use act by interfering with the passage of impulse in the insect nervous system. Insects depend, like mammals, on an integrated nervous system which enables external stimuli to be translated into effective action.

A number of different chemicals have been implicated in transmission at various insect synapses including acetylcholine. Acetylcholine is the transmitter at central nervous system synapses in insects. In order for the nervous system to operate properly it is necessary that, once the appropriate message has been passed, excess acetylcholine should be removed from the synapse, both to prevent repetitive firing and to allow a succeeding message to be transmitted. This removal is effected AChE, which catalyses hydrolysis of the ester bond. OP and carbamate insecticides inhibit AChE. The result of this inhibition is that acetylcholine accumulates in the synapses so that nerve function is

impaired. This leads ultimately to the death of the insect. Enzyme kinetics analysis has identified insensitive forms of AChE in insecticide resistant strains, often apparently involving just one mutant form of the enzyme (Hemingway *et al*., 1986) or as in the housefly, a family of alleles each conferring a distinct pattern of sensitivity (Devonshire, 1987). Neurotoxicity of pyrethroids has been attributed to their activity on the nervous system. Ion channels are the primary target sites for several classes of natural and synthetic pyrethroids (Bloomquist, 1996). The pyrethroids appear to be acting at virtually every part of the insect nervous system: on sensory neurons (Roeder & Weiant, 1946), on interneurons (Narahashi, 1971a), on motor neurons (Yeager & Munson, 1945), and on neurosecretion (Singh & Orchard, 1983). Through *in vivo* and *in vitro* assays, several enzymes and cellular processes have been proposed as targets of pyrethroid modification: synaptic neurotransmitter release, voltage-dependent sodium channels, potassium channels, calcium channels, calmodium, peripheral benzodiazepine receptors, ATPase, nicotinic acetylcholine receptors, $Na+/Ca^{2}+$ exchangers, receptors for gamma-aminobutyric acid (GABA) (Rossignal, 1991; Bloomquist, 1996), acetylcholine-receptor complex (Kiss and Osipenko, 1991) and release of neurohormones (Singh and Orchard, 1983). Peripheral actions are action on the peripheral nervous system which in insects consists of sensory neurons and their axons, motor neurons, and their terminals, and all neurosecretory axons and neurohaemal organs that lie outside of the ventral nerve cord and paired ganglia. The central nervous system (CNS) is considered to be the ganglia, connectives, and commissures from the brain to the terminal abdominal ganglia.

The toxicological significance of ATPase inhibition by pyrethroids is wholly unknown. The Ca^{2}+, Mg^{2}+-ATPase are thought to be involved in sequestering calcium, so their inhibition should increase intracellular calcium. It was suggested by Clark and Matsumura (1982) this could account for the increase of spontaneous transmitter release by pyrethroids, but Salgado *et al*. (1983) showed that the increase of spontaneous transmitter release by pyrethroids was due to depolarization of the nerve terminals by pyrethroid-induced sodium influx. The GABA-gated channel is the proposed target in insects and mammals for several types of commercial and experimental insecticides. Gammon and Casida (1983) reported that insecticidal isomers of the type II pyrethroids, deltamethrin, cypermethrin, and fenvalerate blocked the GABA-activated conductance in crayfish muscles, while permethin, resmethrin, and S-bioalethrin had no effect. GABA receptors are much less sensitive to pyrethroids than the sodium channels suggesting that their contribution to pyrethroid poisoning is normally secondary importance (Chalmers *et al*., 1987). A work on *Ae. aegypti* and *Drosophila melanogaster* demonstrated that cyclodiene insecticide resistanec is associated with change in GABA receptor/chloride ion channel (Ffrench-Constant *et al*., 1994). Bloomquist (1996) stated that endosulfan like pyrethroids block the GABA-gated chloride channel.

The phototoxic effects on mosquito larvae and its potential application as a larvicide make its mechanism of action a topic of considerable importance (Aranason, 1981; Philogene, 1985; Aranason, 1987). It has been reported that α-terthienyl acts by Type II photodynamic action by sensitizing singlet oxygen (Bakker, 1979; Wat *et al*., 1980). It

has been shown that α-terthienyl generates a superoxide anion radical in aqueous media (Kagan *et al*., 1988). Mosquito larvae treated with cr-terthienyl in the presence of long wave ultraviolet light or sunlight show an accumulation of this compound in the anal gills and occurrence of gill membrane damage as a consequence of terthienyl treatment as can be seen by the halide leakage technique (Aranason, 1987).

5. Insecticide resistance and mechanisms of resistance

Vector control is facing a threat due to the development of resistance to insecticides. Several strategies have been adopted to control these dipteran pests and to reduce vector-borne diseases. Synthetic insecticides have been effectively used during the past several decades for mosquito control operations. Synthetic insecticides are at the forefront of mosquito control agents. However, the environmental threat that these chemicals pose, effects on nontarget organisms, and the resistance of mosquitoes to insecticides have all increased during the last five decades (Wattanachai and Tintanon, 1999). The development of resistance by mosquitoes to the compounds used against them as larvicides and adulticides was first observed in 1947, when the salt-marsh mosquitoes *Ae. taeniorhynchus* and *Aedes solliitans* began to show resistance to DDT in Florida (Brown, 1986). Since then, populations which have developed resistance to organochlorines (DDT and/or dieldrin) are known in 109 mosquito species throughout the world; 58 species have developed resistance to OP insecticides. Also among these species, l7 have now shown resistance of adults to the carbamates propoxur or bendiocarb, and 10 have shown either resistance or cross-resistance to certain

pyrethroids. Multiple resistances to all 4 of the above-mentioned chemical groups in the same population of a mosquito species has been developed in certain areas by *Ae. aegypti, Cx. pipiens, Cx. quinquefasciatus, Anopheles albimanus, Anopheles culcifacies, Anopheles pseudopunctipennis, Anopheles sacharoui*, and *Anopheles stephensi* (Brown, 1986).

The development of insecticide resistance is observed in the field as a progressive decrease in the control obtained by the dosage recommended on the basis of its effectiveness when the insecticide was first introduced. To obtain proof that the control failure observed is due to resistance in the target mosquitoes themselves, and not to such factors as deficiency of the formulation, inefficient application, or unfavorable meteorological conditions, it is necessary to submit a sample of the target population to a set test of its susceptibility to the insecticide. Methods for such susceptibility-resistance tests of international validity have been standardized by WHO for both adult and larval mosquitoes. The characterstics of insecticide resistance is inherited, and in most cases it has proved to be due to unitary genetic factors (gene alleles) for resistance. The resistance allele may be either recessive (as in certain DDT resistance), or dominant (as in OP resistance), or codominant, the resistant susceptible hybrids being intermediate (as in dieldrin resistance). In a mosquito population, resistance is induced by a process of selection which increases the proportion of resistant genotypes by killing off, generation after generation, the individuals with the normal susceptible alleles. Laboratory strains are known which are genetically pure for resistance, all the individuals being homozygous for the resistance allele, but resistant field populations

almost invariably contain some heterozygotes and the susceptible alleles are always infiltrating back from surrounding untreated areas (Georghiou, 1980a).

In *Cx. p. pipiens, Cx. quinquefasciatus* and other culicids, OP resistance is due to esterase isozymes which can break down OP compounds by phosphatase-type hydrolysis (Pasteur and Georghiou, 1980). In *An. albimanus* strains in El Salvador, malathion resistance is due to their AChE being an isozyme which is insensitive to inhibition by malathion and malaoxon; it is also insensitive to propoxur (Ayad and Georghiou, 1975). The widespread use of insecticides can, however, lead to the development of insecticide resistance, making insecticide use ineffective and limiting the available options for disease control (WHO, 1998). Insecticide resistance, including resistance to multiple types of insecticides, has arisen in all the insect species that are the major vectors of human diseases. Insecticide resistance in culicine mosquitoes is given in Table 3.

Insecticides are used to control vector populations, as they are relatively easy to apply compared to other control methods. Therefore, mosquitoes are exposed to repeated use of various types of insecticides, not only for vector control but also in agriculture, which has resulted in the development of insecticide resistance. Increased levels of resistance to the commonly used insecticides have led to excessively high application rates that raise serious human health and environmental concerns, increased frequency of application, and/or use of alternative insecticides, all of which are major obstacles to cost-effective integrated mosquito management in South Korea (Kim *et al*., 2007). An effective insecticide resistance management strategy, based on historical and

current information on the distribution and potential for insecticide resistance, is necessary.

Control failures in South Korea have occurred most likely as a result of the development of field resistance (Shim *et al*., 1995; Kim *et al*., 2007; Chang *et al*., 2009). Early detection of trends in the development of potential resistance can facilitate the use of synergists, rotation of insecticides and/or classes of insecticides, or alternative technologies that reduce the dependence and usage of chemical insecticides (Yilma *et al*., 1991; Lee *et al*., 1997). Strategies to decrease environmental pollution and human exposure to insecticides have accelerated to reduce the number of insecticide applications to an annual application, thus decreasing the usage of pyrethroid insecticides in South Korea since the early 2000s (Chang *et al*., 2009). Pyrethroid insecticides have been used frequently, due to their strong insecticidal activity and relatively lower human toxicity compared to organophosphorus insecticides.

Environmental issues associated with some synthetic insecticides (such as DDT) has indicated that additional approaches to control the proliferation of mosquito population would be an urgent priority research. However, high cost of synthetic pyrethroids, environment and food safety concerns, the unacceptability and toxicity of many organophosphates and organochlorines, and increasing insecticide resistance on a global scale argued for stimulated research towards potential botanicals (Severini, 1993) Modern synthetic chemicals could provide immediate results for the control of mosquitoes; on the contrary they bring irreversible environmental hazard, severe side effects and pernicious toxicity to human being and beneficial organisms.

Table 3. Insecticide resistance in culicine mosquitoes

Species	Cases	Insecticides
Culex pipiens	63	DDT, chloropyriphos, deltamethrin, dichlorvos, fenthion, malathion, parathion, permethirn, piperonyl butoxide, propoxur, temephos, tribufos
Culex pipiens pallens	54	DDT, chlorpyrifos, deltamethrin, diemthoate, dipterex, fenitrothion, fenthion, malathion, parathion, parathion-methyl, pirimifos-methyl, temephos, trichlorofon
Culex pipiens molestus	15	Chlorpyrifos, chlorpyrifos-methyl, diazinon, dichlorvos, etofenprox, fenitrothion, fenthion, malathion, permethrin, phenothrin, propetamphos, temephos
Culex tritaeniorhynchus	42	DDT, carbaryl, chloropyriphos, deltamethrin, dichlorvos, diazinon, dichlorvos, dieldrin, fenitrothion, fenthion, malathion, permethrin, proxpur, temephos
Aedes aegypti	260	DDT, bioresmethrin, chlorphoxim, chlorpyrifos, cyfluthrin, cyhalthrin-lambda, cybermethrin, dichlorvos, fenthion, malathion, permethrin, piperonyl butoxide, propoxur, temephos, tribufos, pirimiphos-methyl
Aedes albopictus	29	DDT, fenitrothion, fenthion, malathion, permethrin
Aedes dorsalis	3	Fenthion, malathion, parathion
Ochelerotatus togoi	3	DDT, chlorpyrifos, fenthion
Anopheles sinensis	39	DDT, fenitrothion, fenthion, malathion,
Anopheles gambiae	15	DDT, cyhalothrin-lambda

Kim, 2011.

6. Plant-derived active principles

Synthetic insecticides have continued to be commonly used for controlling mosquitoes in many parts of the world. Initially, their use was focused on the control of mosquitoes, either by killing or repelling them. However, the appearance of resistance to conventional insecticides in mosquitoes, together with public concern about the safety and availability of the insecticides have prompted the necessity to search for alternative insecticides that would be environmentally acceptable and less costly. Therefore, the use of environmentally friendly and easily biodegradable natural insecticides of plant origin has received renewed importance for malaria and other diseases control. Interest in this field is based on the fact that these substances do not leads to the accumulation of chemical residues in flora, fauna, soil and the entire environment in general. In concern to the quality and safety of life and the environment, the emphasis on controlling mosquito vectors has shifted steadily from the use of conventional chemicals toward alternative insecticides that are target-specific, biodegradable, and environmentally safe, and these are generally botanicals in origin. Therefore, right now use of eco-friendly and cost-free plant-based products for the control of mosquitoes is inevitable. Currently, numerous products of botanical origin, especially the secondary metabolites, have received considerable renewed attention as potentially bioactive agents used in insect vector management. However, there is a little other than anecdotal, traditional, or cultural evidence on this topic (Grodner, 1997). The use of herbal products is one of the best alternatives for mosquito control. The search for herbal preparations that do not produce any adverse effects in the nontarget organisms and are easily biodegradable

remains a top research issue for scientists associated with alternative vector control (Chowdhury, 2008). Many plant species are known to possess biological activity that is frequently assigned to the secondary metabolites. Among these, essential oils and their constituents have received considerable attention in the search for new biopesticides. Many of them have been found to possess an array of properties, including insecticidal activity, repellency, feeding deterrence, reproduction retardation, and insect growth regulation against various mosquito species (Rice, 1994; Isman, 2000; Cheng, 2004; Traboulsi, 2005; Yang, 2005).

Even though plants and their preparations were the only pest management agents available before the advent of synthetic organic chemicals, only a few insecticides of plant origin are now commercially available. Furthermore, most of them have lower and shorter-lived efficacy than the synthetic substances. Due to the high degree of biodegradation, however, plant-derived bioproducts are currently attractive as replacements for synthetic insecticides or for use in IPM programs to minimize human health hazards and reduce the accumulation of harmful residues in the environment. Furthermore, resistance to mosquitocidal botanical agents has not previously been documented (Shaalan *et al*., 2005). In recent years, the active insecticidal constituents isolated from plants have received much attention due to their pronounced larvicidal efficacy.

Plants synthesize secondary metabolites that may possess insecticidal, antimicrobial (Leeja and Thoppil, 2007), herbicidal and other biological activities (Tonk *et al*., 2006; Setia *et al*., 2007). Phytochemicals such as nicotine, pyrethrins, rotenoids,

brassinosteroids, and azadirachtin obtained from plants have been evaluated and a few of them are also exploited commercially. The similarity in chemical structure and/or mechanism of action between the pyrethroid insecticides and used plant products might be a key to the development of tolerance or resistance in natural populations of mosquitoes. In order to clarify this suspicion, isolation and identification of the active ingredients responsible for such larvicidal activity need to be performed. In recent years, interest in plant-based products has been revived because of the development of resistance, cross-resistance, and possible toxicity hazards associated with synthetic insecticides and their rising cost. Phytochemicals obtained from the huge diversity of plant species are important source for safe and biodegradable chemicals, which can be screened for mosquito repellent and insecticidal activities and tested for mammalian toxicity (Mittal, 2003).

Numerous products of botanical origin, especially essential oils, have received considerable renewed attention as potent bioactive compounds against various species of mosquitoes. Due to the fact that application of adulticides may only temporarily diminish the adult population (El Hag *et al*., 1999, 2001), a more efficient and attractive approach in mosquito control programs is to target the larval stage in their breeding sites with larvicides (Amer and Mehlhorn, 2006a; Knio *et al*., 2008). A large number of plant extracts have been reported to have mosquitocidal or repellent activity against mosquito vectors (Sukumar, 1991), but very few plant products have shown practical utility for mosquito control. Plant products can be obtained either from the whole plant or from a specific part by extraction with different types of solvents such as aqueous, methanol,

chloroform, hexane depending on the polarity of the phytochemicals. Several other plants have demonstrated toxic effects on mosquitoes mostly under laboratory conditions. *Tagetes* spp., commonly known as marigold, has shown both larvicidal as well as adulticidal activity against mosquitoes (Green, 1991; Perich, 1994; Macedo, 1997; Pathak, 2000).

Steam distillate from root of *Asarum heterotropoides* was shown to have potent insecticidal activity against third instars of *Cx. p. pallen*. *A. heterotropoides* essential oil contains various compounds such as asaricin, 1,8-cineole, croweacin, methyleugenol, myrcene, myristicin, α-pinene, β-pinene, safrole, terpinen-4-ol, and α-terpineol (Tang and Eisenbrand, 1992; Gong *et al*., 2006). Historically, *A. heterotropoides* has long been used as an analgesic and antitussive agent for the treatment of influenza, headache, rheumatic pain, and asthma (Tang and Eisenbrand, 1992). Very little work has been done to consider its potential to manage mosquitoes despite excellent pharmacological actions of *A. heterotropoides*. Sukumar *et al*. (1991) has pointed out that the most promising botanical mosquito control agents are plants in the families Asteraceae, Cladophoraceae, Lamiaceae (formerly: Labiatae), Meliaceae, Oocystaceae, and Rutaceae, although *A. heterotropoides* belongs to the family Aristolochiaceae.

7. Perspectives

The use of many conventional insecticides such as OP and carbamate insecticides will be restricted by recent pesticide regulation policies in the USA (USEPA, 2010), EU (Anonymous, 2005), and South Korea (KLRI, 2007). Untill 2003, the US government

banned or severly restricted 64 insecticides belonging to the categories of UN PIC (Prior Informed Consent), UN Severely Hazardous Pesticide Formulations (SHPF), and US PIC lists such as chlordimeform and ethyl parathion (USEPA, 2003). These problems have prompted renewed interest in the search and development of better vector control strategies that destroy vectors over a wide range, but cause no harm to nontarget organisms and the environment. The use of indigenous plant products in vector control might be one potentially alternative approach.

Modern synthetic chemicals could provide immediate results for the control of mosquitoes. They bring irreversible environmental hazard, severe side effects, and pernicious toxicity to human being and beneficial organisms. The search for herbal preparations that do not produce any adverse effects in the nontarget organisms and are easily biodegradable remains a top research issue for scientists associated with alternative vector control (Chowdhury, 2008). Research has proved the effectiveness of plant derived secondary compounds, such as saponine (Wiseman, 2005), steroinds (Chowdhury, 2008), isoflavonoids (Ghosh, 2008), essential oil (Joseph, 2004), alkaloids (Cavalcanti, 2004), tannins (Khanna, 2007) as mosquito larvicides. The plant-derived natural products as larvicides have the advantage of being harmless to beneficial nontarget organisms and environment when compared to synthetic ones (Pitasawat *et al*., 2007). The essential oils such as basil, cinnamon, citronella, and thymus are promising as mosquito larvicides (Mansour *et al*., 2000; Carvalho *et al*., 2003; Cheng *et al*., 2004; Cavalcanti *et al*., 2004) have received much attention as potentially useful bioactive compounds against insects (Kim *et al*., 2001).

Factors that induce resistance are numerous and the mechanism adopted by organism depends on the prevailing pressure and on the mode of action of the insecticide in use. Intoxication of arthropod by an insecticide encompasses different levels of pharmacokinetic interaction: penetration of barrier tissue, distribution, storage, metabolism in internal tissue, and molecular interaction with the ultimate target site (Soderlund *et al*., 1989). Insecticide resistance is an inherited characteristic that allows an insect to survive a dose of a pesticide that would normally prove fatal. According to WHO (1957) resistance has been defined as “the developed ability in a strain of insects to tolerate doses of toxicant which would prove lethal to the majority of individuals in a normal population of the same species". Management of resistance can help avoid resistance development in vector populations, slow the rate of resistance development, and cause resistant vector to "revert" to a more susceptible level. Tactics for management of resistance in vector populations should be followed to avoid resistance of inseceticdes.

In recent years, the top priority in finding new insecticides is that, they must be plant origin and does not have any ill effect on ecosystem. Research has proved the effectiveness of plant derived secondary compounds, such as saponine (Wiseman, 2005), steroinds (Chowdhury, 2008), isoflavonoids (Ghosh, 2008), essential oil (Joseph, 2004), alkaloids (Cavalcanti, 2004), tannins (Khanna, 2007) as mosquito larvicides. Plant preparations provide alternative source of mosquito repellent agents (Yang *et al*., 2004). The plant-derived natural products as larvicides have the advantage of being harmless to beneficial nontarget organisms and environment when compared to synthetic ones

(Pitasawat *et al*., 2007). The synthetic insecticides do not only affect non-target organisms but also constantly increase resistance to the insecticides by the vector (Wattal *et al*., 1981). The essential oils such as basil, cinnamon, citronella, and thymus are promising as mosquito larvicides (Mansour *et al*., 2000; Carvalho *et al*., 2003; Cheng *et al*., 2004; Cavalcanti *et al*., 2004) have received much attention as potentially useful bioactive compounds against insects (Kim *et al*., 2001).

Tactics for management of resistance in vector populations according to (WHO, 1992a; Georghiou, 1980; Croft, 1990; Leeper *et al*., 1986; Plapp, 1986; Taylor, 1986) should be followed to avoid resistance of inseceticdes. In recent years, the emphasis to control the mosquito populations has shifted steadily from the use conventional chemicals towards more specific and environmentally friendly materials, which are generally of botanical origin. Therefore, right now use of eco-friendly and cost-free plant based products for the control of insects/mosquitoes is inevitable. Efforts should be made to promote the use of easy accessible and affordable traditional insect/mosquito repellent plants.

CHAPTER I

Isolation and Identification of Larvicidal Constituents Identified in *Asarum heterotropoides* Root

1.1. Larvicidal Activity of *Asarum heterotropoides* Root Steam Distillate Constituents against *Culex pipiens pallens*, *Aedes aegypti*, and *Ochlerotatus togoi* (Diptera: Culicidae)

1.2. Larvicidal Activity of *Asarum heterotropoides* Root Constituents against Insecticide-Susceptible and -Resistant *Culex pipiens pallens* and *Aedes aegypti* and *Ochlerotatus togoi*

1.3. Enhanced Toxicity of Binary Mixtures of Larvicidal Constituents from *Asarum heterotropoides* Root to Insecticide-Susceptible and -Resistant *Culex pipiens pallens* (Diptera: Culicidae)

1.1. Larvicidal Activity of *Asarum heterotropoides* Root Steam Distillate Constituents against *Culex pipiens pallens*, *Aedes aegypti*, and *Ochlerotatus togoi* (Diptera: Culicidae)

INTRODUCTION

Plant essential oils and their constituents have been suggested as alternative sources for mosquito larval control products, in part, because they constitute a potential source of bioactive chemicals that often produce only minor adverse effects on nontarget organisms and the environment and often act at multiple and novel target sites, thereby reducing the potential for resistance (Sukumar *et al*., 1991; Wink, 1993; Isman, 2006). In addition, essential oils are widely available with some being relatively inexpensive compared with plant extracts (Isman, 2006). These potential new mosquito larvicides can be applied to mosquito breeding places in the same manner as the larvicides currently used (Rozendaal, 1997). Much effort has been focused on essential oils and their constituents as potential sources of commercial mosquito larval control products largely because certain essential oils and their constituents meet the criteria of minimum risk pesticides (USEPA, 1996; 2004). In a preliminary experiment, steam distillate from root of *Asarum heterotropoides* was shown to have potent insecticidal activity against third instars of *Culex pipiens pallens*. *A. heterotropoides* essential oil contains various compounds such as asaricin, 1,8-cineole, croweacin, methyleugenol, myrcene, myristicin, α-pinene, β-pinene, safrole, terpinen-4-ol, and α-terpineol (Tang and

Eisenbrand, 1992; Gong *et al*., 2006).

In this paper, an assessment is made of the mosquito larvicidal activity of the constituents that comprise *A*. *heterotropoides* root steam distillate against third instars of *Cx. p. pallens*, *Aedes aegypti*, and *Ochlerotatus togoi*. The mosquito larvicidal activity of the steam distillate constituents was then compared with that of two commonly used mosquito larvicides, fenthion and temephos.

MATERIALS AND METHODS

1.1.1. Chemicals

Twenty-eight compounds used in this study were as follows: β-asarone, borneol, camphene, 3-carene, 3,5-dimethoxy toluene, eucarvone, fenchene, (+)-limonene, linalool, pentadecane, α-phellandrene, (–)-β-pinene, methyleugenol, 3,4,5-trimethoxy toluene, and safrole purchased from Sigma-Aldrich (St. Louis, MO); estragole, myristicin, (+)-α-pinene, (+)-β-pinene, γ-terpinene, terpinen-4-ol, and verbenone purchased from Fluka (Buchs, Switzerland); 1,8-cineole, (–)-α-pinene, and α-terpineol purchased from Wako (Osaka, Japan); and β-caryophyllene, myrcene, and terpinolene purchased from Tokyo Chemical Industry (Tokyo, Japan). Fenthion (98.4% purity) and temephos (97.3% purity) were supplied by Supelco (West Chester, PA) and Riedel (Seelze, Germany), respectively. Triton X-100 was obtained from Shinyo Pure Chemicals (Osaka). All other chemicals were of reagent grade and available commercially.

1.1.2. Mosquitoes

The stock cultures of *Cx. p. pallens*, *Ae. aegypti*, and *Oc. togoi* (Yang *et al*., 2004) were maintained in the laboratory without exposure to any known insecticide. Adult mosquitoes were maintained on a 10% sucrose solution and blood fed on live mice. Larvae were reared in plastic trays (24 × 35 × 5 cm) containing 0.5 g of sterilized diet (40–mesh chick chow powder:yeast, 4:1 by weight). They were held at 27 ± 1°C and 65–75% relative humidity, and a photoperiod of 16:8 (L:D) h.

1.1.3. Steam Distillation

Air-dried root (600 g) of *A. heterotropoides* was pulverized and subjected to steam distillation at 100°C for 2 h using a Clevenger-type apparatus. The volatile oil was dried over anhydrous sodium sulfate and stored in a sealed vial at 4°C until use. The yield of the distillate was 2.98% based on dried weight of the plant.

1.1.4. Gas Chromatography (GC)

A Shimadzu GC 2010 gas chromatograph (Kyoto, Japan) equipped with splitless injector was used to separate and detect the constituents of *A. heterotropoides* steam distillate. Analytes were separated with a 30 m × 0.25 mm i.d. (d_f = 0.25 μm) DB−5 MS bonded-phase fused-silica capillary column (J&W Scientific, Ringoes, NJ). The flow velocity of the helium carrier gas was 1.1 ml/min. The oven temperature was kept at 50°C (5 min isothermal) and programmed to 280°C at a rate of 5°C/min, then isothermal at 280°C for 10 min. The injector temperature was 280°C. The steam distillate constituents were identified by co-elution of authenticated samples following co-injection.

1.1.5. Gas Chromatography-Mass Spectroscopy (GC-MS)

GC-MS analysis was performed using a Shimadzu GC 2010 gas chromatograph-Shimadzu QP 2010 mass spectrometer. The capillary column and temperature conditions for the GC-MS analysis were the same as described above for GC analysis. Helium carrier gas was used at a column head pressure of 8.89 psi (61.3 kPa). The ion source temperature was 200°C. The interface temperature was kept at 290°C, and mass spectra were obtained at 70 eV. The sector mass analyzer was set to scan from 50 to 650 amu

every 0.50 s. Constituents were identified by mass spectra library (Anonymous, 2000).

1.1.6. Bioassay

A direct contact mortality bioassay (Kim *et al*., 2008) was used to evaluate the toxicity of *A*. *heterotropoides* root steam distillate constituents to early third instar larvae of *Cx*. *p. pallens*, *Ae*. *aegypti*, and *Oc*. *togoi*. Briefly, each compound in methanol was suspended in distilled water with Triton X–100 (20 μL/L). Groups of 20 mosquito larvae were separately put into paper cups (270 mL) containing each test compound solution (250 mL). The toxicity of each test compound was determined with six concentrations ranging from 1 to 200 mg/L. Fenthion and temephos served as positive controls for comparison in larval mortality tests. Controls received methanol-Triton X–100 solution. Treated and control (methanol-Triton X–100 solution only) larvae were held at the same conditions used for colony maintenance. Larval mortalities were determined 24 h post-treatment. Larvae were considered to be dead if they did not move when they were prodded with fine wooden dowels. Because all bioassays could not be conducted at the same time, treatments were blocked over time with a separate control treatment included in each block. Freshly prepared solutions were used for each block of bioassays (Robertson and Preisler, 1992).

1.1.7. Data Analysis.

The LC_{50} values were calculated by probit analysis (SAS Institute, 2004). Larvicidal activity was considered to be significantly different when 95% confidence limits of the LC_{50} values failed to overlap. The toxicity was classified as follows: very highly active,

LC_{50} <10 mg/L; highly active, LC_{50} 10–20 mg/L; moderately active, LC_{50} 21–50 mg/L; poorly active, LC_{50} 51–100 mg/L; and very poorly active, LC_{50} >100 mg/L. Susceptibility ratio (SR) was determined as the ratio of LC_{50} of *Ae*. *aegypti* or *Oc*. *togoi* larvae/LC_{50} of *Cx*. *p. pallens* larvae as described previously (Kim *et al*., 2008).

RESULTS

1.1. Chemical Constituents of *A. heterotropoides* Root Steam Distillate

A. heterotropoides root steam distillate was composed of three major and 14 minor constituents by comparison of mass spectral data and by GC with authentic sample co-injection (Fig. 1). The three major constituents, methyleugenol, safrole, and 3,5-dimethoxytoluene, comprised 20.63, 20.31, and 10.32% of the steam distillate, respectively. They constituted about 51% of total steam distillate. *Asarum* root steam distillate GC-MS identified compounds were confirmed by the co-injection with steam distillate (Fig. 2). As a result of co-injection (Table 4), 15 compounds were identical which is present in the steam distillate of *A. heterotropoides*.

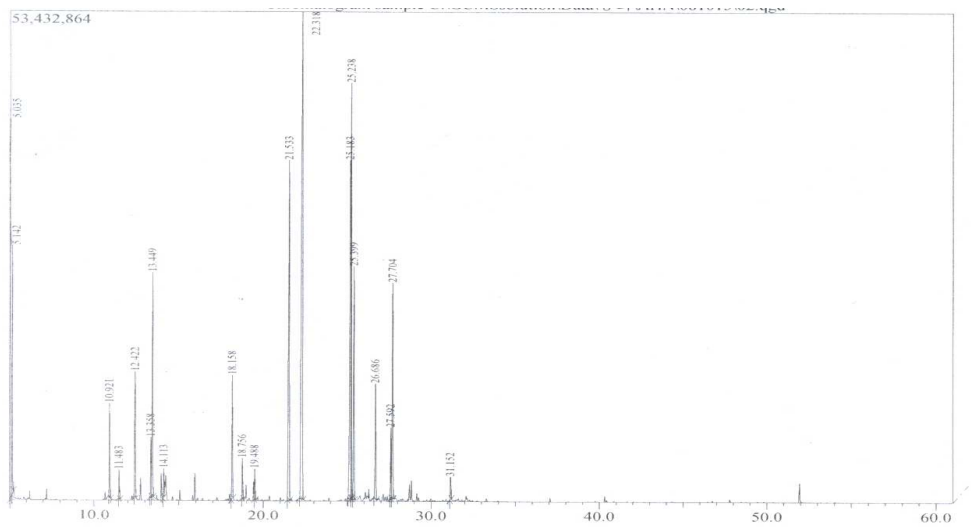

Fig. 1. GC-MS chromatogram of staeam distillate from *Asarum heterotropoides*.

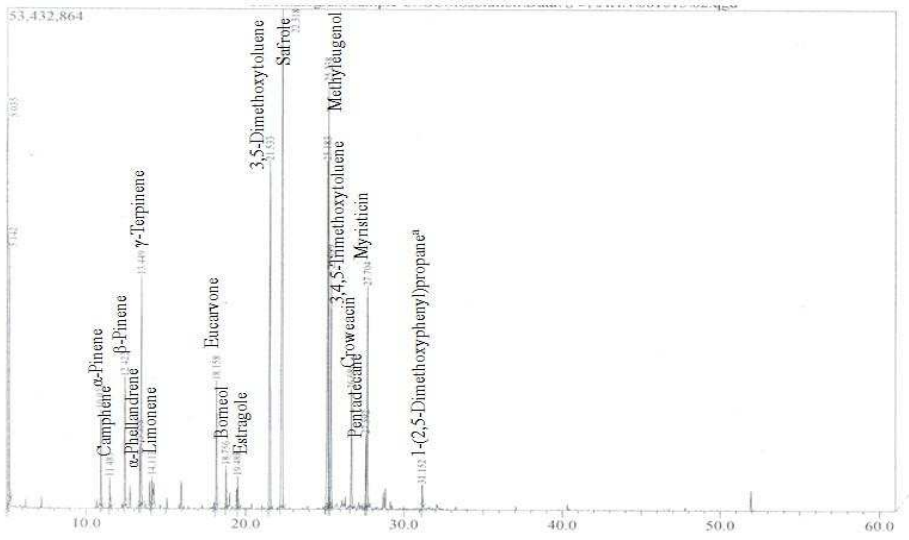

Fig. 2. Co-injection of identified compounds from staeam distillate from *Asarum heterotropoides*.

1.2. Larvicidal Activity of Test Compounds

The toxicity to third instars of *Cx. p. pallens* of *A. heterotropoides* root steam distillate, 28 test compounds, and two mosquito larvicides, fenthion and temephos, was evaluated by comparing the LC_{50} values estimated from the direct-contact application (Table 5). Based on 24 h LC_{50} values, safrole (8.22 mg/L) was the most toxic compound but less active than either fenthion (0.029 mg/L) or temephos (0.016 mg/L). Strong activity was also produced by terpinolene, γ-terpinene, (–)-β-pinene, (+)-limonene, 3-carene, and α-phellandrene (LC_{50}, 11.85–13.84 mg/L). Moderate toxicity was observed with *A. heterotropoides* root steam distillate, (+)-β-pinene, and β-asarone (LC_{50}, 21.07–22.38 mg/L). Poor to very poor toxicity was obtained from 17 and two compounds, respectively. There was no mortality for methanol-treated larvae.

Table 4. Chemical constituents of *Asarum heterotropoides* root steam distillate identified by gas chromatography and gas chromatography-mass spectrometry (GC-MS)

Compound	RT[b] (min)	% Area
α-Pinene	10.92	1.85
Camphene	11.48	0.52
β-Pinene	12.42	2.53
α-Phellandrene	13.36	1.32
γ-Terpinene	13.45	4.67
Limonene	14.11	0.56
Eucarvone	18.16	2.66
Borneol	18.76	0.82
Estragole	19.45	0.62
3,5-Dimethoxytoluene	21.53	10.32
Safrole	22.32	20.31
Methyleugenol	25.24	20.63
3,4,5-Trimethoxytoluene	25.40	5.34
Croweacin[a]	26.69	2.85
Pentadecane	27.59	1.39
Myristicin	27.70	5.57
1-(2,5-Dimethoxyphenyl)propane[a]	31.15	0.51

[a] Tentative identifications from mass spectral data. Other identifications were performed by comparison of GC-MS data and by GC with authentic sample co-injection.
[b] Retention time.

Table 5. Toxicity of *Asarum heterotropoides* root steam distillate and its constituents and two mosquito larvicides to third instars of *Culex pipiens pallens* during a 24 h exposure

Compound	Slope ± SE	LC_{50},[b] mg/L (95% CL[c])
Asarum steam distillate	2.2 ± 0.50	21.07 (17.19–27.49)
β-Asarone	2.7 ± 0.40	22.38 (19.22–26.00)
Borneol[a]	7.4 ± 1.09	91.56 (86.94–98.48)
Camphene[a]	8.6 ± 1.26	70.46 (67.23–74.70)
3-Carene	2.4 ± 0.31	13.83 (11.67–16.40)
β-Caryophyllene	5.2 ± 1.01	93.65 (87.05–106.16)
1,8-Cineole	10.3 ± 1.45	78.98 (75.97–82.64)
3,5-Dimethoxytoluene[a]	5.4 ± 1.61	55.12 (47.19–59.75)
Estragole[a]	6.1 ± 0.98	53.96 (50.47–57.67)
Eucarvone[a]	7.1 ± 1.47	117.93 (106.00–149.00)
Fenchene	8.2 ± 1.26	72.17 (68.65–77.17)
(+)-Limonene[a]	2.1 ± 0.30	13.26 (10.87–16.14)
Linalool	5.2 ± 1.01	94.80 (87.98–108.07)
Methyleugenol[a]	7.0 ± 1.00	53.30 (50.23–56.43)
Myrcene	8.1 ± 1.20	66.28 (63.10–69.88)
Myristicin[a]	9.6 ± 1.40	76.99 (73.89–80.58)
Pentadecane[a]	6.2 ± 1.08	97.58 (91.08–109.62)
Phellandrene[a]	2.3 ± 0.31	13.84 (11.57–16.58)
(+)-α-Pinene[a]	6.2 ± 0.98	53.96 (50.47–57.67)
(–)-α-Pinene[a]	9.8 ± 1.31	70.36 (67.48–73.98)
(+)-β-Pinene[a]	2.5 ± 0.39	21.12 (17.77–24.79)
(–)-β-Pinene[a]	2.7 ± 0.32	12.87 (10.99–15.00)
Safrole[a]	2.3 ± 0.41	8.22 (6.44–10.01)
Terpinen-4-ol	6.6 ± 1.01	58.31 (54.96–62.54)

γ-Terpinene[a]	1.9 ± 0.29	12.64 (10.11–15.65)
α-Terpineol	5.2 ± 1.07	104.07 (94.91–125.40)
Terpinolene	1.7 ± 0.29	11.85 (9.26–14.81)
3,4,5-Trimethoxytoluene[a]	9.1 ± 1.37	74.76 (71.24–79.99)
Verbenone	12.5 ± 1.87	96.02 (92.56–101.20)
Fenthion	1.3 ± 0.17	0.029 (0.022–0.041)
Temephos	1.1 ± 0.16	0.016 (0.011–0.022)

[a] Compounds identified in this study. The other compounds were reported by Tang and Eisenbrand (1992) and Gong et al. (2006).

[b] Median lethal concentration.

[c] CL denotes confidence limit.

The larvicidal activity of *A*. *heterotropoides* root steam distillate, 28 test compounds, and two mosquito larvicides against third instars of *Ae*. *aegypti* was likewise examined (Table 6). As judged by 24 h LC_{50} values, safrole (9.88 mg/L) was the most toxic compound, followed by terpinolene, (–)-β-pinene, γ-terpinene, and 3-carene exhibited strong activity (LC_{50}, 15.32–19.19 mg/L). Moderate toxicity was observed with (+)-β-pinene, α-phellandrene, *A*. *heterotropoides* root steam distillate, (+)-limonene, β-asarone, and estragole (LC_{50}, 22.39–46.39 mg/L). Poor to very poor toxicity was obtained from 16 and two compounds, respectively. Overall, all the compounds were less toxic than either fenthion (0.023 mg/L) or temephos (0.017 mg/L).

Table 6. Toxicity of *Asarum heterotropoides* root steam distillate and its constituents, and two mosquito larvicides to third instars of *Aedes aegypti* during a 24 h exposure

Compound	Slope ± SE	LC_{50},[b] mg/L (95% CL[c])	SR[d]
Asarum steam distillate	3.1 ± 0.42	23.82 (20.87–27.62)	1.1
β-Asarone	2.8 ± 0.42	26.99 (23.42–31.88)	1.2
Borneol[a]	8.3 ± 1.19	94.89 (90.23–102.09)	1.0
Camphene[a]	7.8 ± 1.19	67.02 (63.75–70.87)	0.9
3-Carene	2.1 ± 0.32	19.19 (15.94–24.24)	1.4
β-Caryophyllene	6.4 ± 1.02	88.30 (83.52–95.30)	0.9
1,8-Cineole	11.0 ± 1.44	74.91 (72.16–77.80)	0.9
3,5-Dimethoxytoluene[a]	5.4 ± 1.11	64.05 (59.31–69.15)	1.2
Estragole[a]	6.8 ± 1.00	46.39 (46.03–52.33)	0.9
Eucarvone[a]	4.5 ± 1.24	130.35 (110.40–208.10)	1.1
Fenchene	7.7 ± 1.20	69.28 (65.83–73.79)	1.0
(+)-Limonene[a]	3.1 ± 0.42	24.47 (21.52–28.00)	1.8
Linalool	5.9 ± 1.06	96.60 (90.05–108.90)	1.0
Methyleugenol[a]	6.4 ± 1.00	57.65 (54.25–61.87)	1.1
Myrcene	7.0 ± 1.16	66.42 (62.82–70.66)	1.0
Myristicin[a]	10.6 ± 1.42	72.98 (70.12–75.81)	0.9
Pentadecane[a]	7.9 ± 1.18	96.71 (91.46–105.35)	1.0
α-Phellandrene[a]	2.7 ± 0.40	23.08 (19.90–26.80)	1.7
(+)-α-Pinene[a]	7.6 ± 1.03	50.92 (48.00–53.66)	0.9
(–)-α-Pinene[a]	9.5 ± 1.31	64.80 (62.04–67.70)	1.0
(+)-β-pinene[a]	2.5 ± 0.39	22.39 (18.94–26.38)	1.1
(–)-β-pinene[a]	2.7 ± 0.33	15.40 (13.22–18.09)	1.2
Safrole[a]	2.6 ± 0.42	9.88 (8.20–11.85)	1.2
Terpinen-4-ol	8.3 ± 1.20	64.76 (61.69–68.05)	1.1
γ-Terpinene[a]	2.0 ± 0.30	17.11 (14.01–21.50)	1.3
α-Terpineol	6.2 ± 1.22	111.78 (101.30–136.90)	0.9

Terpinolene	2.3 ± 0.31	15.32 (12.87–18.46)	1.3
3,4,5-Trimethoxytoluene[a]	7.4 ± 1.18	67.13 (63.69–71.28)	0.9
Verbenone	10.5 ± 1.65	93.16 (89.55–98.43)	1.0
Fenthion	1.3 ± 0.17	0.023 (0.018–0.032)	0.8
Temephos	1.2 ± 0.17	0.017 (0.013–0.023)	1.1

[a] For explanation, see Table 2.

[b] Median lethal concentration.

[c] CL denotes confidence limit.

[d] Susceptibility ratio = LC_{50} of *Ae*. *aegypti* larvae/LC_{50} of *Cx*. *pipiens pallens* larvae.

Toxic effects on third instars of *Oc*. *togoi* in direct contact application of *A*. *heterotropoides* root steam distillate and 28 test compounds were compared with those of fenthion and temephos (Table 7). Based on 24 h LC_{50} values, strong larvicidal activity was observed from terpinolene, γ-terpinene, safrole, α-phellandrene, 3-carene, (–)-β-pinene, and (+)-limonene (LC_{50}, 14.20–19.20 mg/L). The toxicity of these compounds was less toxic than either fenthion (LC_{50}, 0.023 mg/L) or temephos (0.017 mg/L). Moderate toxicity was observed with (+)-α-pinene, (+)-β-pinene, *A*. *heterotropoides* root steam distillate, and β-asarone (LC_{50}, 23.08–46.39 mg/L). Poor to very poor toxicity was obtained from 16 and two compounds, respectively.

Table 7. Toxicity of *Asarum heterotropoides* root steam distillate and its constituents, and two mosquito larvicides to third instars of *Ochlerotatus togoi* during a 24 h exposure

Compound	Slope ± SE	LC_{50},[b] mg/L (95% CL[c])	RT[d]
Asarum steam distillate	3.1 ± 0.44	27.64 (24.27–32.15)	1.3
β-Asarone	2.8 ± 0.40	26.38 (22.70–31.39)	1.2
Borneol[a]	7.8 ± 1.17	97.30 (91.92–106.20)	1.1
Camphene[a]	8.4 ± 1.92	68.69 (64.62–72.59)	1.0
3-Carene	2.0 ± 0.25	16.21 (13.48–19.35)	1.2
β-Caryophyllene	7.7 ± 1.19	97.90 (92.40–107.20)	1.0
1,8-Cineole	7.9 ± 1.07	83.21 (79.04–89.30)	1.1
3,5-Dimethoxytoluene[a]	5.2 ± 1.12	67.00 (62.27–73.27)	1.2
Estragole[a]	7.4 ± 1.18	58.52 (54.64–61.71)	1.1
Eucarvone[a]	5.2 ± 1.33	122.95 (107.90–168.50)	1.0
Fenchene	6.2 ± 0.97	95.22 (89.26–104.80)	1.3
(+)-Limonene[a]	2.2 ± 0.26	19.20 (16.29–22.80)	1.4
Linalool	6.4 ± 1.11	98.97 (92.35–111.30)	1.0
Methyleugenol[a]	7.4 ± 1.18	58.52 (54.64–61.71)	1.1
Myrcene	7.4 ± 1.67	64.76 (61.69–68.05)	1.0
Myristicin[a]	15.9 ± 1.94	90.68 (88.26–93.57)	1.2
Pentadecane[a]	8.4 ± 1.26	99.19 (93.81–108.10)	1.0
α-Phellandrene[a]	1.9 ± 0.24	16.10 (13.27–19.41)	1.2
(+)-α-pinene[a]	5.7 ± 0.97	47.25 (42.84–50.67)	0.9
(–)-α-Pinene[a]	8.7 ± 1.22	57.93 (54.57–60.72)	0.9
(+)-β-pinene[a]	2.5 ± 0.40	25.60 (21.94–30.44)	1.2
(–)-β-pinene[a]	2.4 ± 0.27	18.03 (15.44–21.06)	1.4
Safrole[a]	1.9 ± 0.24	16.10 (13.27–19.41)	2.0
Terpinen-4-ol	7.4 ± 1.18	58.52 (54.64–61.71)	1.0
γ-Terpinene[a]	2.3 ± 0.31	14.36 (12.08–17.16)	1.1

α-Terpineol	6.9 ± 1.45	117.89 (105.80–149.40)	1.1
Terpinolene	1.6 ± 0.48	14.20 (8.56–18.50)	1.2
3,4,5-Trimethoxytoluene[a]	7.9 ± 1.52	91.41 (87.04–98.22)	1.2
Verbenone	16.9 ± 2.01	90.56 (88.25–93.25)	0.9
Fenthion	1.5 ± 0.18	0.024 (0.019–0.032)	0.8
Temephos	1.7 ± 0.31	0.020 (0.019–0.040)	1.3

[a] For explanation, see Table 2.

[b] Median lethal concentration.

[c] CL denotes confidence limit.

[d] Susceptibility ratio = LC_{50} of *Oc. togoi* larvae/LC_{50} of *Cx. pipiens pallens* larvae.

DISCUSSION

In the Chinese Pharmacopoeia, *A. heterotropoides* has long been used as an analgesic and antitussive agent for the treatment of influenza, headache, rheumatic pain, and asthma (Tang and Eisenbrand, 1992). Very little work has been done to consider its potential to manage mosquitoes despite excellent pharmacological actions of *A. heterotropoides*. Sukumar *et al*., (1991) has pointed out that the most promising botanical mosquito control agents are species in the families Asteraceae, Cladophoraceae, Lamiaceae (formerly Labiatae), Meliaceae, Oocystaceae, and Rutaceae, although *A. heterotropoides* belongs to the family Aristolochiaceae. In this study, *A. heterotropoides* root steam distillate exhibited good larvicidal activity against third instars of *Cx. p. pallens*, *Ae. aegypti*, and *Oc. togoi*. This original finding supports the contention that the steam distillate may be a good candidate for a naturally occurring mosquito larval control product.

Essential oils consist of highly complex mixtures of hydrocarbons, such as terpenes, monoterpenes, sesquiterpenes, and diterpenes, and oxygenated compounds, such as esters, aldehydes, ketones, alcohols, phenols, and oxides (Sellar, 2001; Lawless, 2002). They jointly or independently contribute to behavioral efficacy, such as repellency and feeding deterrence, and physiological efficacy, such as acute toxicity and developmental disruption, against various arthropod species (Isman, 2006). Many plant preparations and their constituents manifest larvicidal activity against different mosquito species and have been proposed as alternatives to the most widely used larvicides (Sukumar *et al*., 1991; Kim *et al*., 2004; Kim *et al*., 2008; Park *et al*., 2002). For example, it has been

reported that the isobutylamide alkaloids pellitorine, guineensine, pipercide, and retrofractamide A possess potent larvicidal activity against larvae of *Cx. p. pallens*, *Ae. aegypti*, and *Oc. togoi* and the *N*-isobutyiamine moiety appears to play a crucial role in the larvicidal activity (Park *et al*., 2002). Additionally, certain plant preparations or their constituents also provide an alternative for future resistance management because they can be highly effective against insecticide resistant insect pests (Ahn *et al*., 1997; Kim *et al*., 2008; Yang *et al*., 2008). Monoterpenoids such as linalool, (–)-terpinen-4-ol, and α-terpineol, possess remarkable insecticidal activity against a dual malathion- and permethrin-resistant strain of *Pediculus humanus capitis* (De Geer) (Yang *et al*., 2008). In the present study, the larvicidal principles of *A. heterotropoides* root steam distillate were identified as the phenylpropanoid safrole and monoterpenoids 3-carene, (+)-limonene, α-phellandrene, (–)-β-pinene, γ-terpinene, and terpinolene. These compounds may hold promise as novel and effective larvicidal products, although their larvicidal activity was less effective than either fenthion or temephos. Although not yet proven, the octopaminergic and γ-aminobutyric acid (GABA) receptors have been suggested as novel target sites for some essential oil constituents by Kostyukovsky *et al*., (2002) and Priestley *et al*., (2003), respectively.

Variation in mosquito responses to chemicals has been well noted (Sukumar *et al*., 1991; Park *et al*., 2002; Kim *et al*., 2008). For example, *Ae. aegypti* and *Oc. togoi* larvae are found to be more tolerant than *Cx. p. pallens* larvae to pellitorine, guineensine, pipercide, and retrofractamide A (Park *et al*., 2002). In this study, responses were dependent on mosquito species and compound tested. *Ae. aegypti* and *Oc. togoi* larvae

were slightly more tolerant than *Cx. p. pallens* larvae to some compounds such as (+)-limonene and safrole. Different susceptibility of the compounds to three mosquito species larvae might be attributed to differences in one or more of physiological or biochemical characteristics: penetration, detoxifying enzyme activity, and the relative sensitivity to the toxic lesion at the target site (Terriere, 1984; Graham-Bryce, 1987).

In conclusion, *A. heterotropoides* root steam distillate and its constituents, particularly 3-carene, (+)-limonene, α-phellandrene, (–)-β-pinene, safrole, γ-terpinene, and terpinolene, could be useful as larvicides in the control of mosquito populations. For the practical use of *A. heterotropoides* essential oil and its constituents as novel larvicides to proceed, further research is needed to establish their safety issues on human health, nontarget aquatic organisms and the environment. Because of carcinogen action of β-asarone, a typical constituent of *Acorus calamus* L. and *Asarum europaeum* L., the European Council limits the maximum content of the compound: 0.1 mg/kg in nonalcoholic drinks and 1 mg/kg in alcoholic drinks and food stuffs containing in these medicinal herbs (Anonymous, 1968). In addition, their larvicide modes of action need to be established and formulations for improving larvicidal potency and stability, thereby reducing costs, need to be developed.

1.2. Larvicidal Activity of *Asarum heterotropoides* Root Constituents against Insecticide-Susceptible and -Resistant *Culex pipiens pallens* and *Aedes aegypti* and *Ochlerotatus togoi*

INTRODUCTION

Numerous plant products have been reported either as insecticides for killing larvae or adult mosquitoes and are one of the best alternatives for mosquito control (Brown, 1986, Sukmuar *et al*., 1991). *Asarum heterotropoides* (Aristolochiaceae) root steam distillate and its several constituents had larvicidal activity against third instars of *Cx. p. pallens*, *Ae. aegypti*, and *Oc. togoi* as stated in Chapter 1.1. Very little information has been done to consider potential of *A. heterotropoides* root-derived materials to manage insecticide-resistant mosquitoes.

In this study, an assessment is made of the mosquito larvicidal activity of the constituents that comprise *A. heterotropoides* root against third instars from insecticide-susceptible *Cx. p. pallens*, *Ae. aegypti*, and *Oc. togoi*, as well as a field-collected colony of *Cx. p. pallens*, identified by polymerase chain reaction (PCR), resistant to various insecticides. The toxicities of the constituents were compared with those of fenthion and temephos.

MATERIALS AND METHODS

1.2.1. General Instrumental Methods

^{1}H and ^{13}C NMR spectra were recorded in $CDCl_3$ on a Bruker AVANCE 600 spectrometer (Karlsruhe, Germany) using tetramethylsilane (TMS) as an internal standard, and chemical shifts are given in δ (ppm). UV spectra were obtained in ethanol on a UVICON 933/934 spectrophotometer (Kontron Instrument, Milan, Italy) and mass spectra on a Jeol JMS-DX 303 spectrometer (Tokyo, Japan). Optical rotation was measured with an Autopol III polarimeter (Rudolph Research Analytical, Flanders, NJ). Silica gel (0.063–0.2 mm) (Merck, Darmstadt, Germany) was used for column chromatography. Pre-coated silica gel plates (Kieselgel 60 F_{254}) (Merck) were used for analytical thin-layer chromatography (TLC). An Agilent 1200 series high-performance liquid chromatograph (HPLC) (Santa Clara, CA) was used for isolation of active principles.

1.2.2. Chemicals

Eleven insecticides examined in this study were as follows: bifenthrin (97.0% purity) β-cyfluthrin (98.0% purity), α-cypermethrin (97.5% purity), etofenprox (96.5% purity), fenitrothion (98.5% purity), and chlorfenapyr (99.0% purity) purchased from Sigma-Aldrich (St. Louis, MO, USA); deltamethrin (99.0% purity), chlorpyrifos (98.9% purity), fenthion (98.4% purity), and permethrin (98.0% purity) purchased from Supelco (West Chester, PA); and temephos (97.3% purity) purchased from Riedel (Seelze, Germany). Triton X-100 was obtained from Shinyo Pure Chemicals (Osaka, Japan). All other

chemicals of were of reagent-grade quality and available commercially.

1.2.3. Mosquitoes

The stock cultures of *Cx. p. pallens* (susceptible KS-CP strain), *Ae. aegypti*, and *Oc. togoi* were maintained as stated in Chapter 2.1. Larvae of *Cx. p. pallens* colony were collected near rice paddy fields and cowsheds in Daejeon (South Korea) in early August 2009. The collected larvae were immediately transferred to an insect rearing room. Larvae were reared in plastic trays (27 × 15 × 4 cm) containing 0.5 g of sterilized diet (Vivid-S: Super Terramin, 4:1 by weight). Vivid-S and Super Terramin were purchased from Sewhapet (Inchon, South Korea). Adult mosquitoes were maintained on a 10% sucrose solution and blood fed on live mice. They were held at 27 ± 1°C and 65–75% relative humidity under a 12:12 h light:dark cycle. Species identification based on PCR (Kasai *et.al*., 2008) revealed that larvae from the field-collected colony (designated DJ-CP) belonged to *Cx. p. pallens*.

1.2.4. Extraction and Isolation

Air-dried root (600 g) of *A. heterotropoides* was purchased from Boeun medicinal herb shop, Kyoungdong market (Seoul, South Korea). It was pulverized and extracted with methanol (2 × 3 L) at room temperature for 1 day and filtered. The combined filtrate was concentrated under vacuum at 40°C to yield ~74.63 g of a dark brownish tar. The extract (20 g) was sequentially partitioned into hexane- (2.84 g), chloroform- (4.63 g), ethyl acetate- (1.01 g), butanol- (2.80 g) and water-soluble (8.72 g) portions for subsequent bioassay. The organic solvent-soluble portions were concentrated to dryness

by rotary evaporation at 40°C and the water-soluble portion was freeze-dried. For isolation of active principles, 50 mg/L of each *A. heterotropoides* root-derived material was tested in a direct-contact mortality bioassay as described previously (Kim *et. al*., 2008).

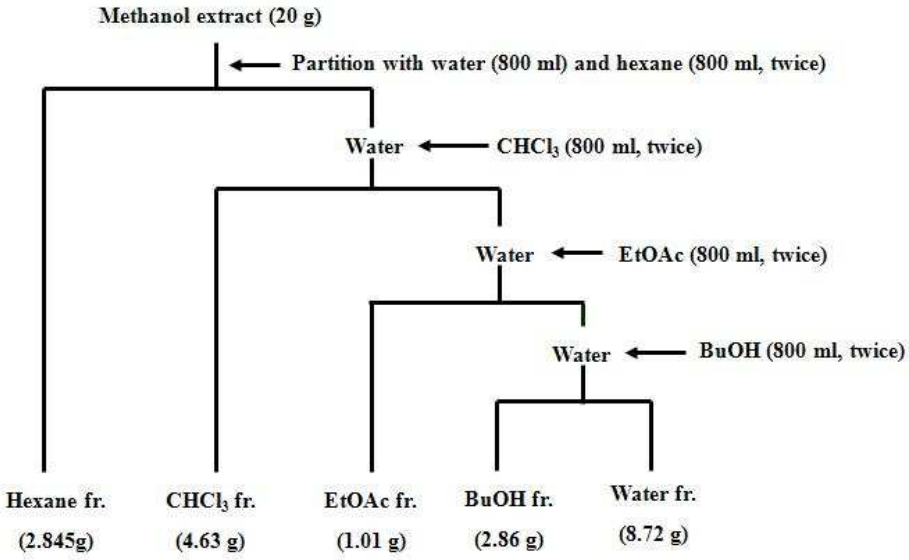

Fig. 3. Solvent fractionation of methanol extract of *A. heterotropoides.*

The most active hexane-soluble fraction (10 g) was chromatographed on a 70 × 5.5 cm silica gel column (600 g) and eluted with a gradient of hexane and ethyl acetate [(10:1 (2.2 L), 9:1 (2 L), 7:3 (2 L), 5:5 (1 L), and 3:7 (1 L) by volume] and finally with methanol (1 L) to provide 48 fractions (each about 250 mL). Column fractions were monitored by TLC on silica gel plates with hexane and ethyl acetate (7:3 by volume). Fractions with similar R_f values on the TLC plates were pooled. Spots were detected by

spraying with 2% H_2SO_4 and then heating on a hot plate. Fractions 1 to 4 (2.92 g) were pooled and separated by preparative TLC [hexane: ethyl acetate (7:3) by volume] to yield compound **1** (720 mg, R_f = 0.94) and compound **2** (40 mg, R_f = 0.78). Both compounds **1** (Fig. 4) and **2** (Fig. 5) were purified by HPLC (21.2 mm i.d. × 250 mm Prodigy ODS (Phenomenex, Torrance, CA) using a mobile phase of acetonitrile and water (8:2 by volume), at 254 nm in flowrate 1 mL/min. Fractions 5 to 8 (450 mg) were purified by preparative TLC [hexane: ethyl acetate (7:3)] to provide compound **3** (725 mg, R_f = 0.61). The active fractions 15 to 27 (760 mg) were pooled and recrystallized in methanol at –4 °C to afford compound **4** (2.11 mg). Likewise compound **3** (Fig. 6) and compound **4** (Fig. 7) were purified by HPLC using UV detector at 254 nm and 241 nm, respectively

A preparative HPLC was used for separation of the constituents from the active fractions 28 to 32 (739 mg). The column was a 21.2 mm i.d. × 250 mm Prodigy ODS using a mobile phase of acetonitrile and water (8:2 by volume) at a flow rate of 1.5 mL/min. Chromatographic separation was monitored using a UV detector at 254 nm. Finally, an active principle **5** (12 mg) was isolated at a retention time of 10.89 min (Fig. 8).

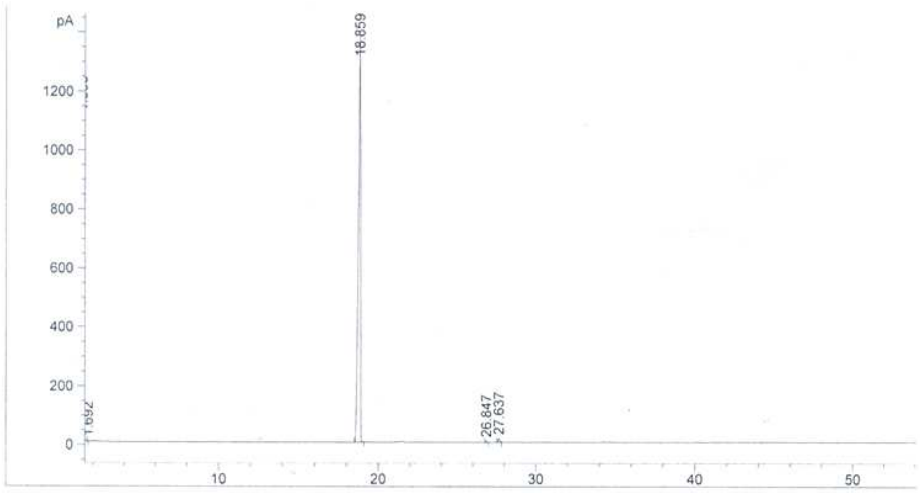

Fig. 4. HPLC chromatogram of compound **1**.

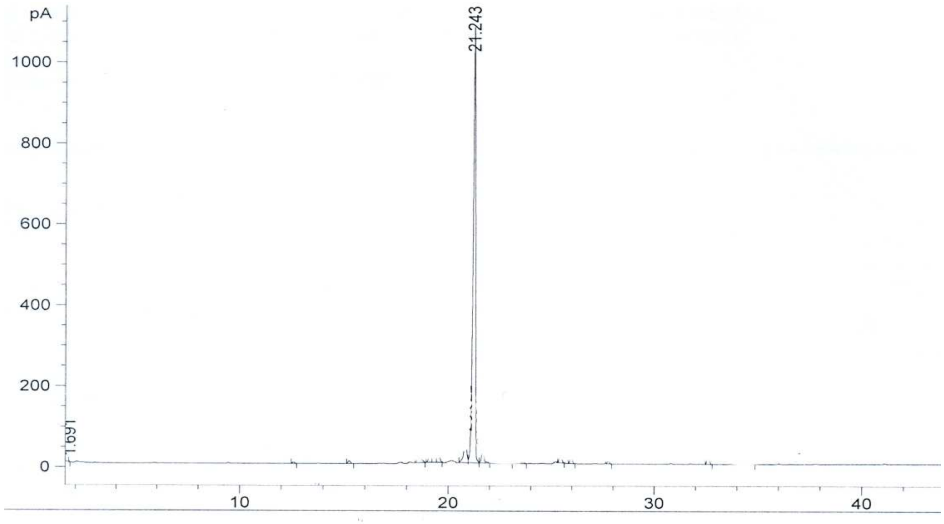

Fig. 5. HPLC chromatogram of compound **2**.

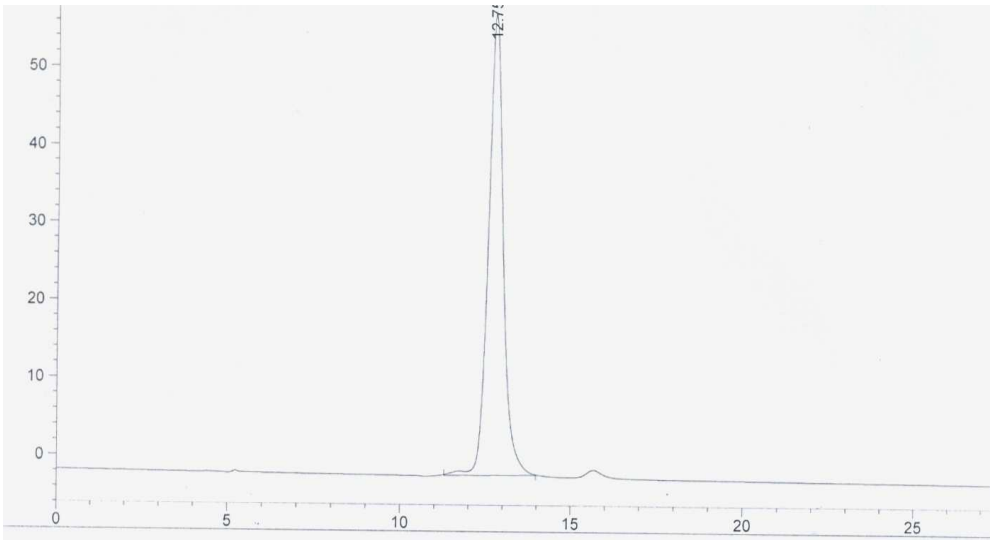

Fig. 6. HPLC chromatogram of compound **3**.

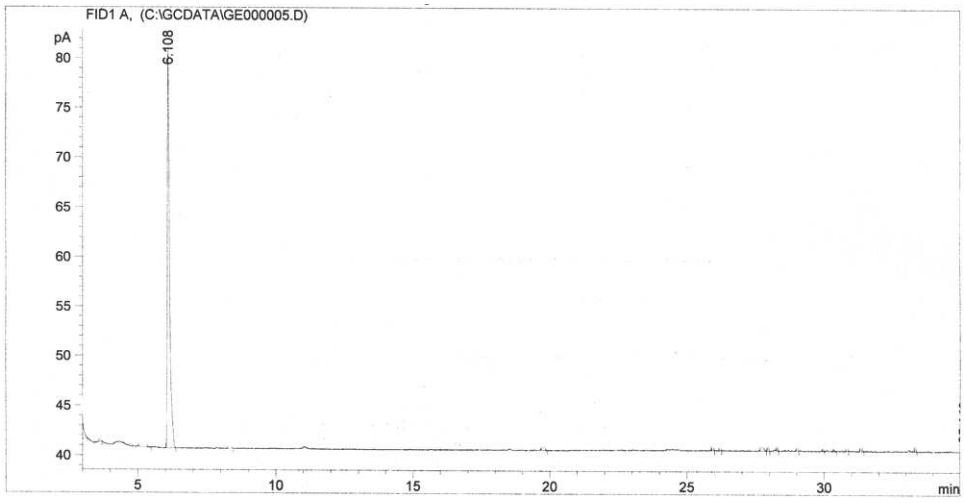

Fig. 7. GC chromatogram of compound **4**.

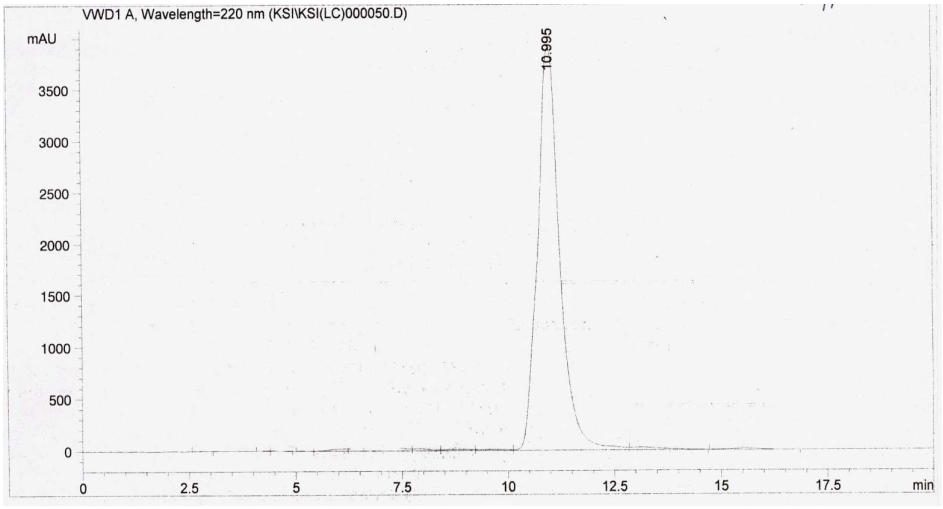

Fig. 8. HPLC chromatogram of compound **5**.

1.2.5. Bioassay

A direct-contact mortality bioassay (Kim *et al*., 2008) was used to evaluate the toxicity of test materials to third instar larvae from the susceptible and field-collected mosquitoes. Each compound in acetone (for (–)-asarinin) or methanol (for the other compounds) was suspended in distilled water with Triton X–100 (20 μL/L). Groups of 20 mosquito larvae were separately put into paper cups (270 mL) containing each test compound solution (250 mL). The toxicity of each test compound was determined with four to six concentrations ranging from 0.1 to 200 mg/L. Fenthion and temephos served as standard references and were similarly formulated as the test materials. Controls (*i.e.* no material or insecticide) consisted of the methanol- or acetone-Triton X–100 carrier solution in distilled water. All treatments were replicated three times using 20 larvae per replicate. Treated and control larvae were held at the same conditions as those used for

colony maintenance. Larvae were considered to be dead if they did not move when prodded with fine wooden dowels 24 h posttreatment.

1.2.6. Data Analysis

Concentration-mortality data were subjected to probit analysis (SAS Institute, 2004). The LC_{50} values for each species and their treatments were considered to be significantly different from one another when their 95% confidence limits failed to overlap. A resistance ratio (RR) was calculated according to the formula, RR = LC_{50} of larvae from the DJ-CP colony of *Cx. p. pallens*/LC_{50} of larvae of the susceptible KS-CP strain. RR values of <10, 10–40, 40–160, and >160 were classified as low, moderate, high, and extremely high resistance, respectively (Kim *et al*., 2004). Susceptibility ratio (SR) was determined as the ratio of LC_{50} of *Ae. aegypti* or *Oc. togoi* larvae/LC_{50} of susceptible *Cx. p. pallens* larvae (Kim *et al*., 2008).

RESULTS

1.2.1. Bioassay-Guided Fractionation and Isolation

Fractions obtained from the methanol extract of *A. heterotropoides* root were bioassayed against third instars from *Cx. p. pallens*, *Ae. aegypti*, and *Oc. togoi* by the direct-contact application (Table 9). Significant differences in larvicidal activity in fractions of the extract were observed, and they were used to identify peak activity fractions for the next step in the purification. Based on 24 h LC_{50} values, hexane-soluble fraction was the most toxic material, followed by chloroform- and ethyl acetate-soluble fractions against three mosquito species. Low or no toxicity was obtained from the butanol- and water-soluble fractions. There was no mortality in methanol-Triton X–100 solution-treated controls for any of the species in this study.

Most active hexane soluble fraction was re-chromatographed using silica-gel column and was received six column fractions (H1-H6). All hexane fractions were assayed against third instars of *Cx. p. pallens* (Table 10) and *Ae. aegypti* (Table 11). Of six fractions obtained, fraction H5 showed the strongest activity againste both mosquito species.

Table 9. Toxicity of each solvent fraction-derived from methanol extract of *Asarum heterotropoides* root to third instars from three mosquito species using direct-contact mortality bioassay during a 24 h exposure

Material	LC_{50}, mg/L (95% CL[a])		
	Culex pipiens pallens	*Aedes aegypti*	*Ochlerotatus togoi*
Methanol extract	15.87 (12.91–20.06)	17.68 (14.28 21.72)	17.54 (14.73–21.49)
Hexane-soluble fr.	7.12 (5.57–10.55)	8.56 (6.19–10.79)	9.06 (7.32–11.55)
Chloroform-soluble fr.	14.47 (11.67–19.97)	13.68 (11.48–16.30)	16.49 (13.51–20.79)
Ethyl acetate-soluble fr.	16.87 (13.40–22.40)	17.54 (14.73–21.49)	19.15(15.43–25.50)
Butanol-soluble fr.	33.51 (29.48–38.84)	52.42 (40.93–77.50)	45.19 (40.54–53.23)
Water-soluble fr.	>200	>200	>200

[a] CL denotes confidence limit.

Table 10. Lethality of hexane-soluble subfractions derived from silica gel column chromatography against third instars of *Culex pipiens pallens*

Fraction	Mortality (%) (± SE)		
	10 (mg/L)	25 (mg/L)	50 (mg/L)
H1	10 ± 0.0cd	17 ± 1.7c	33 ± 1.7c
H2	17 ± 1.7c	15 ± 2.9c	28 ± 1.7cd
H3	3 ± 1.7d	17 ± 1.7c	18 ± 1.7d
H4	55 ± 2.9b	65 ± 2.9b	87 ± 1.7b
H5	87 ± 1.7a	97 ± 1.7a	100a
H6	0e	0e	0e

Table 11. Lethality of hexane-soluble subfractions derived from silica gel column chromatography against third instars of *Aedes aegypti*

Fraction	Mortality (%) (± SE)		
	10 (mg/L)	25 (mg/L)	50 (mg/L)
H1	8 ± 1.7c	17 ± 1.7c	25 ± 2.8c
H2	8 ± 1.7c	15 ± 2.9c	27 ± 1.7c
H3	7 ± 1.7c	17 ± 1.7c	18 ± 1.7c
H4	42 ± 4.4b	63 ± 3.3b	88 ± 1.7b
H5	75 ± 2.9a	93 ± 1.7a	100a
H6	0d	0d	0d

Direct-contact mortality bioassay-guided fractionation of *A. heterotropoides* root extract afforded five active principles identified by spectroscopic analyses, including MS and NMR. The five active principles were methyleugenol (**1**), pentadecane (**2**), α-asarone (**3**), (–)-asarinin (**4**), and pellitorine (**5**).

The compound **1** was obtained as pale yellow viscous oil and identified as methyleugenol (**1**) by spectroscopic analysis, including EI-MS (Fig. 9), ^{1}H NMR (Fig. 10), ^{13}C NMR (Fig. 11), and DEPT (Fig. 12). The ^{13}C NMR spectra showed 11 carbons in the molecule and including two oxynated methyl groups suggesting the molecular formula $C_{11}H_{14}O_2$. The interpretation of proton and carbon signals was largely consistent with those of Fontenelle *et al*. (2011).

Methyleugenol (**1**): viscous oil; UV (EtOH): λ_{max} = 254. EI-MS (70 eV), *m*/*z* (rel. int.): 178 $[M]^+$ (100), 163 (26.3), 147 (21.4), 135 (5.6), 115 (4.8), 91 (10.9), 77(5.8). ^{1}H NMR (MeOD, 600 MHz) and ^{13}C NMR (MeOD, 150 MHz) are given in Table 12.

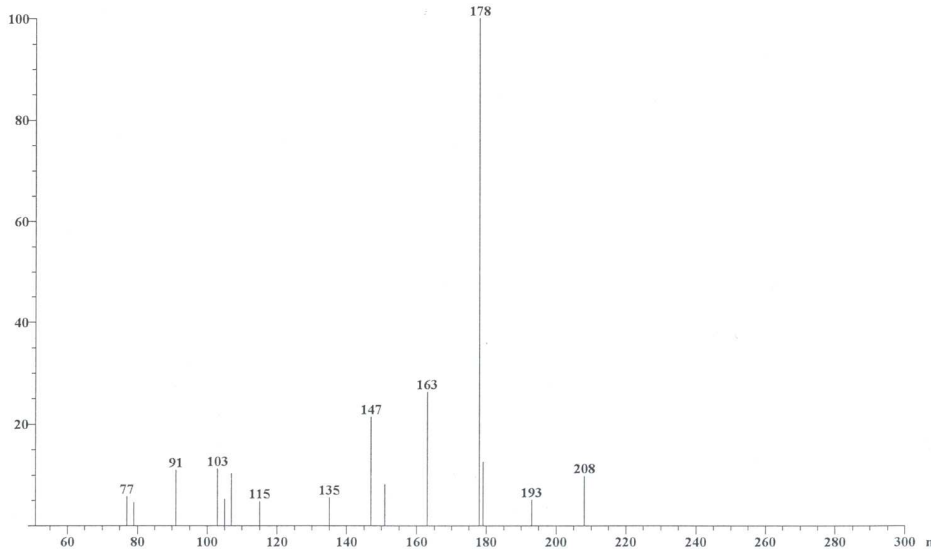

Fig. 9. EI-MS spectrum of compound **1**.

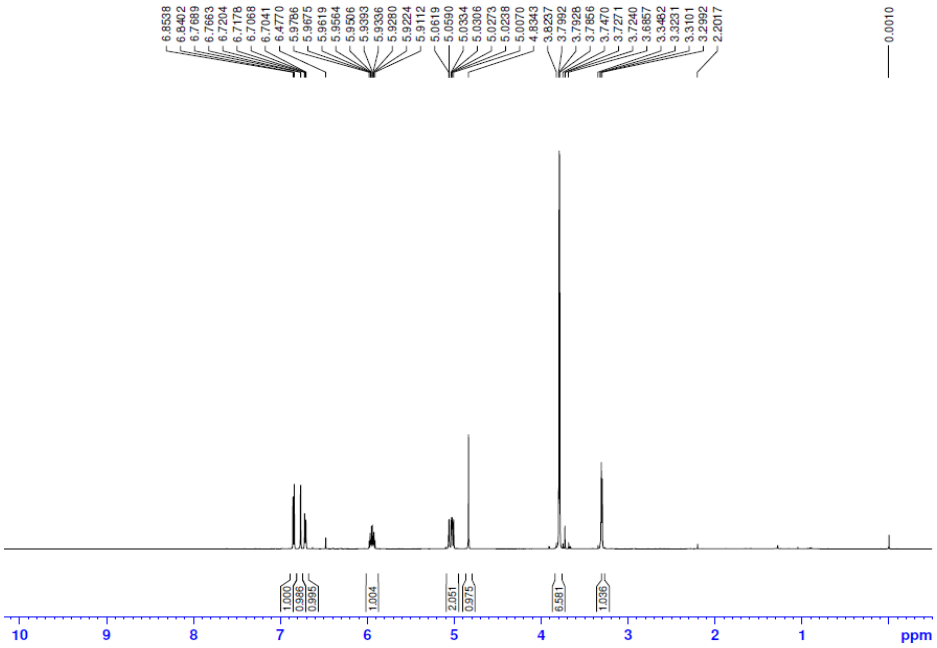

Fig. 10. ^{1}H NMR spectrum of compound **1**.

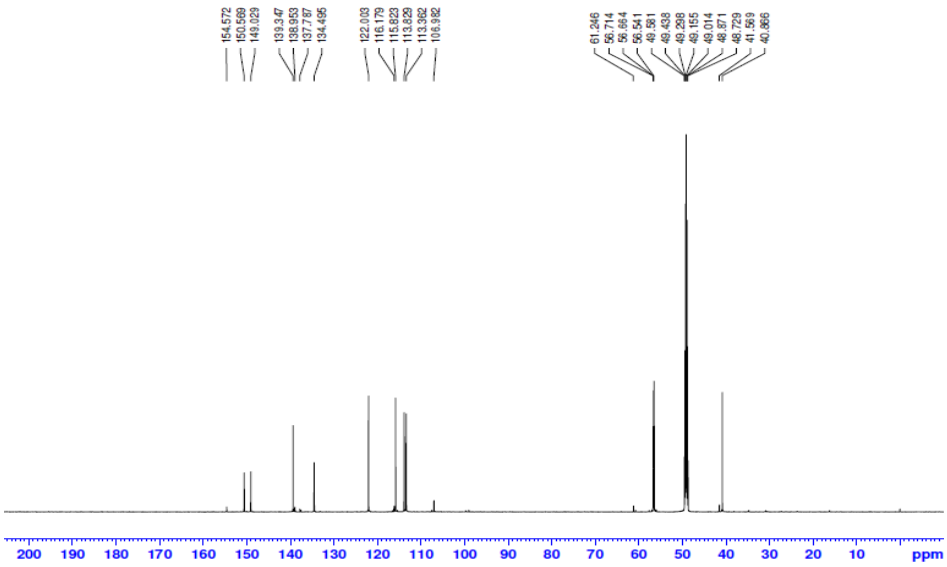

Fig. 11. ^{13}C NMR spectrum of compound **1**.

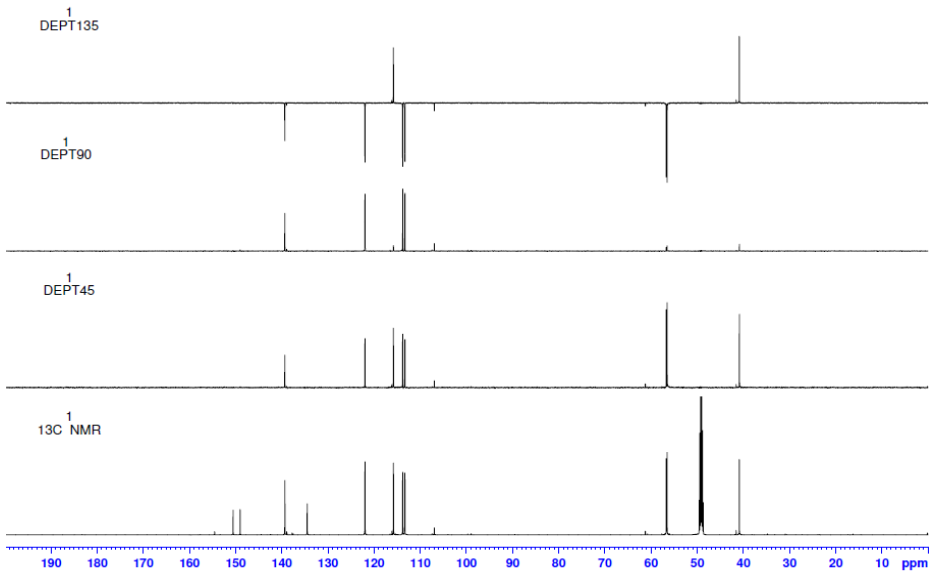

Fig. 12. DEPT spectrum of compound **1**

Table 12. ^{1}H NMR (600 MHz) and ^{13}C NMR (150 MHz) spectral data of compound **1**

Position	Partial structure	δ_C (ppm)	δ_H (ppm)	δ_C (ppm)	δ_H (ppm) Fontenelle *et al*., 2011
1	C	149.7		148.8	
2	C	146.8		147.3	
3	C	133.2		132.6	
4	CH	114.1	6.85, d (*J* = 8.16 Hz)	111.8	6.83, m
5	CH	112.3	6.76, s	111.2	6.83, m
6	CH	122.3	6.70, d (*J* = 1.56 Hz)	120.4	6.76, d (*J* = 4.70 Hz)
7	CH3	56.1	3.79, s	95.7	3.90, s
8	CH3	56.1	3.78, s	95.9	3.88, s
9	CH2	39.8	3.31, s	39.7	3.36, d d (*J* = 1.56 Hz)
10	CH	136.5	5.92, m	137.8	5.98, m
11	CH2	115.9	4.83, s	115.6	5.09, dd, (*J* = 9.3 and 2.9 Hz)

The compound **2** was obtained as viscous oil and identified as pentadecane (**2**) by spectroscopic analysis, including EI-MS (Fig. 13), ^{1}H NMR (Fig. 14), ^{13}C NMR (Fig. 15), and DEPT (Fig. 16). The ^{13}C NMR spectra showed 15 carbons in the molecule suggesting the molecular formula CH_3 $(CH_2)_{13}CH_3$. The interpretation of proton and carbon signals was mostly reliable with those of Siddiqui *et al.* (2004) and Piasecki *al.* (2009).

Pentadecane (**2**): UV (EtOH): λ_{max} = 254. EI-MS (70 eV), *m*/*z* (rel. int.): 212 $[M]^+$ (16.4), 183 (2.4), 155 (6.3), 141 (8.0), 127 (8.8), 113 (10.3), 99 (14.8), 85 (51.2), 71 (70.7), 57 (100) ^{1}H NMR (MeOD, 600 MHz) and ^{13}C NMR (MeOD, 150 MHz) are given in Table 13.

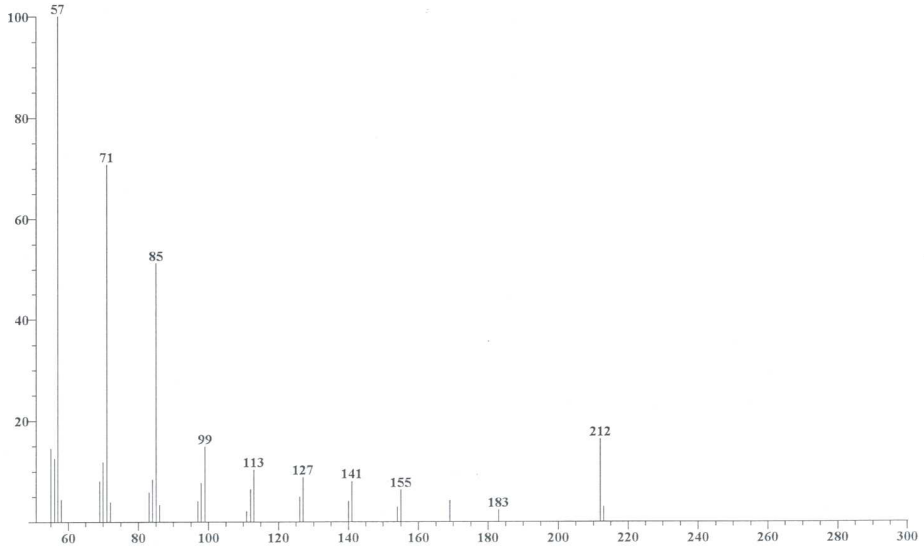

Fig. 13. EI-MS spectrum of compound **2**.

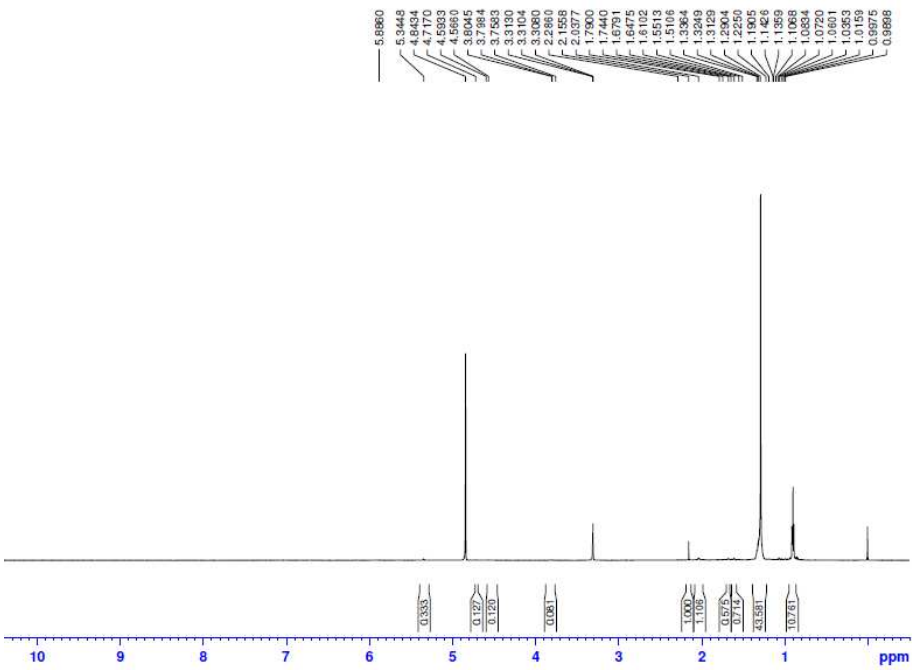

Fig. 14. [1]H NMR spectrum of compound **2**.

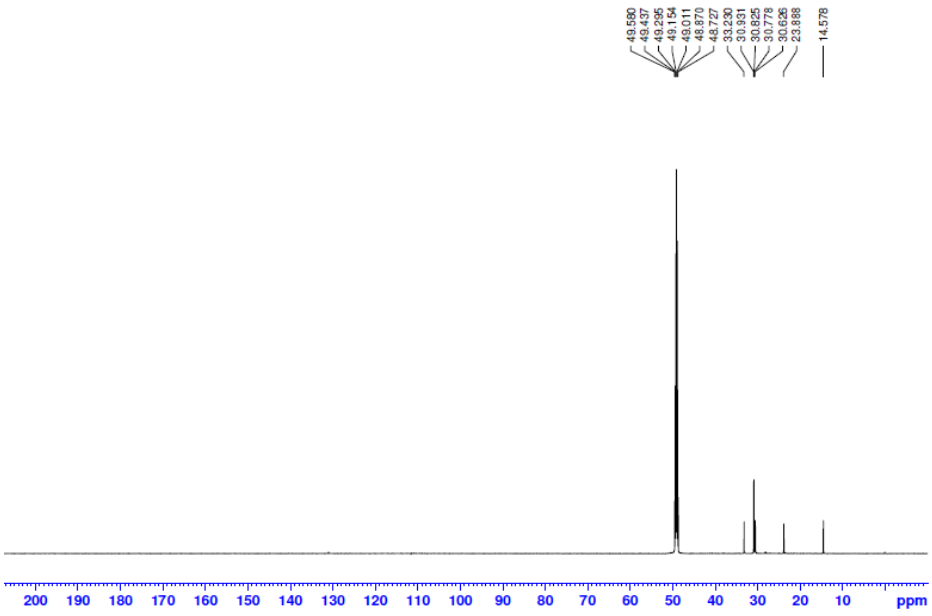

Fig. 15. [13]C NMR spectrum of compound **2**.

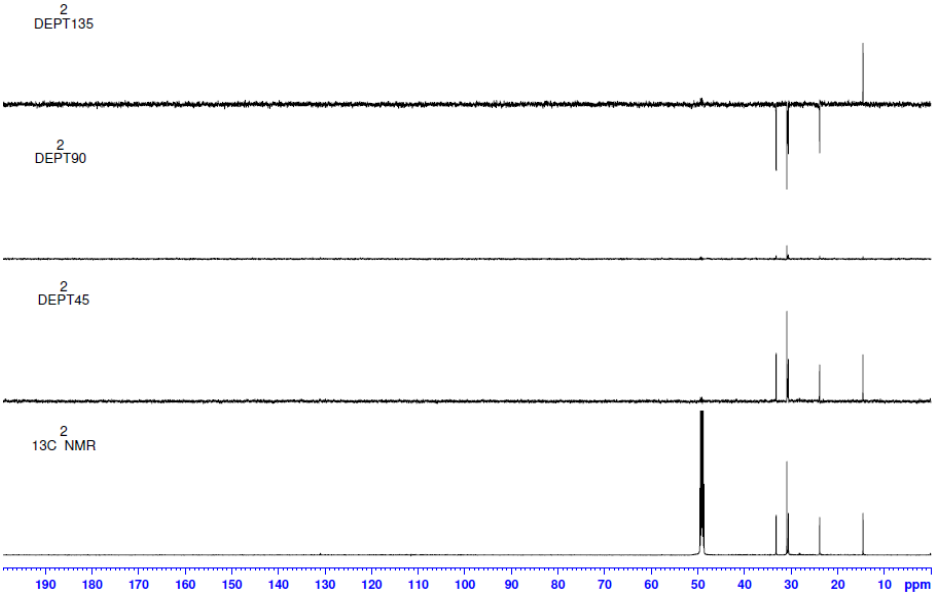

Fig. 16. DEPT spectrum of compound **2**.

Table 13. ^{1}H NMR (600 MHz) and ^{13}C NMR (150 MHz) spectral data of compound **2**

Position	Partial structure	δ_C (ppm)	δ_H (ppm)	δ_C (ppm) Siddiqui *et al.* 2004	δ_H (ppm) Piasecki *et al*., 2009
1	CH2	30.9	1.26, s	29.8	1.23, s
2	CH2	30.9	1.26, s	29.8	1.23, s
3	CH2	30.7	1.26, s	29.7	1.23, s
4	CH2	30.7	1.26, s	29.7	1.23, s
5	CH2	30.8	1.26, s	29.8	1.23, s
6	CH2	30.8	1.26, s	29.8	1.23, s
7	CH2	30.8	1.26, s	29.8	1.23, s
8	CH2	30.6	1.29, s	29.4	1.29, s
9	CH2	30.6	1.29, s	29.4	1.29, s
10	CH2	33.2	1.29, m	31.9	1.29, m
11	CH2	33.2	1.29, m	31.9	1.29, m
12	CH2	23.8	1.31, s	22.7	1.38, s
13	CH2	23.8	1.31, s	22.7	1.38, s
14	CH3	14.1	0.88, s	14.1	0.89, s
15	CH3	14.1	0.88, s	14.1	0.89, s

The compound **3** was obtained as viscous oil and identified as α-asarone (**3**) by spectroscopic analysis, including EI-MS (Fig. 17), ^{1}H NMR (Fig. 18), ^{13}C NMR (Fig. 19), and DEPT (Fig. 20). The EI-MS revealed a molecular ion at *m*/*z* 208 $[M]^{+}$ and its ^{13}C NMR spectra showed 12 carbons including three oxynated methyl groups in the molecule suggesting the molecular formula $C_{12}H_{16}O_3$.

α-Asarone (**3**): viscous oil; UV (EtOH): λ_{max} = 254. EI-MS (70 eV), *m*/*z* (rel. int.): 208 $[M]^{+}$ (100), 193 (51.8), 177 (10), 165 (7.5), 134 (3.3), 77 (4.9), 69 (1.6). ^{1}H NMR (MeOD, 600 MHz) and ^{13}C NMR (MeOD, 150 MHz) are given in Table 14. The interpretations of proton and carbon signals of compound **3** were largely consistent with those of Zuo *et al*. (2012).

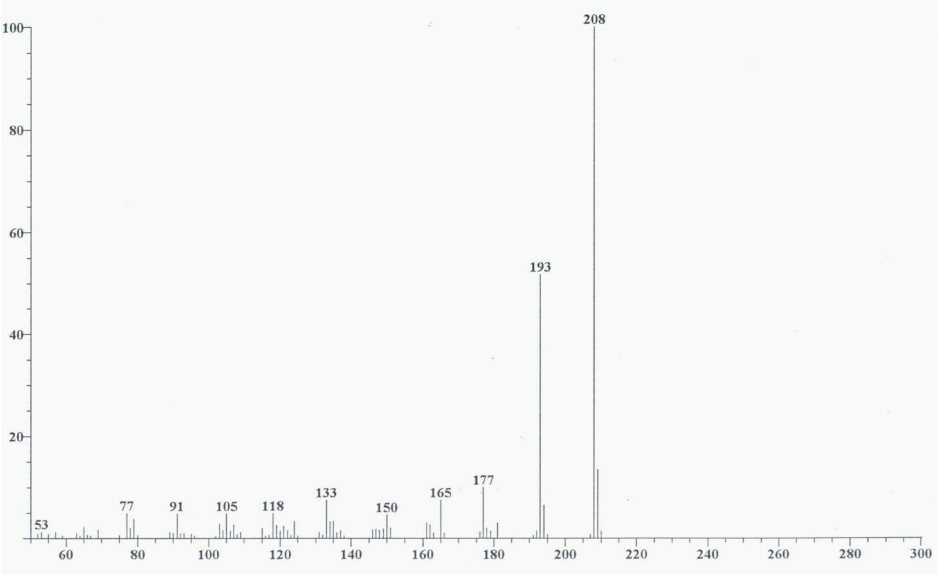

Fig. 17. EI-MS spectrum of compound **3**.

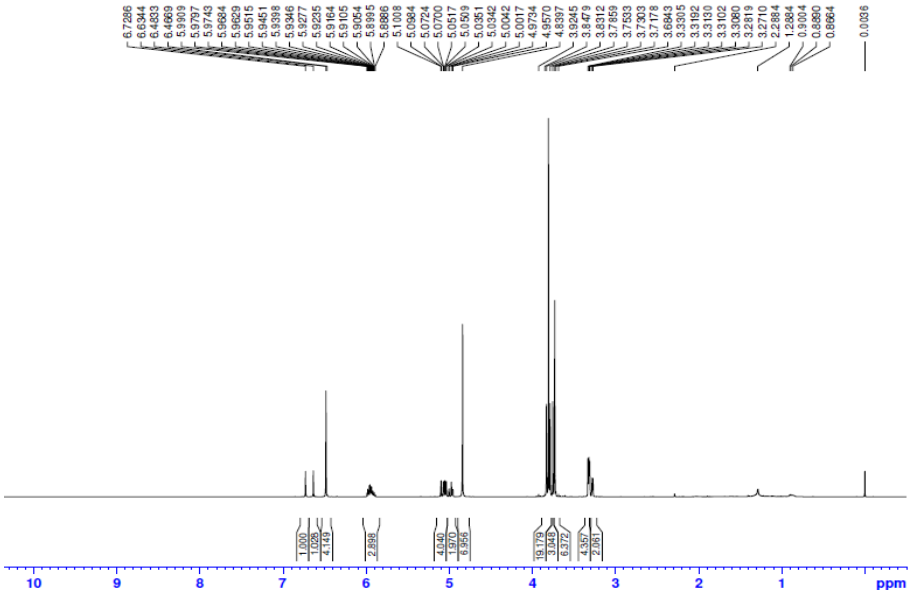

Fig. 18. ^{1}H NMR spectrum of compound **3**.

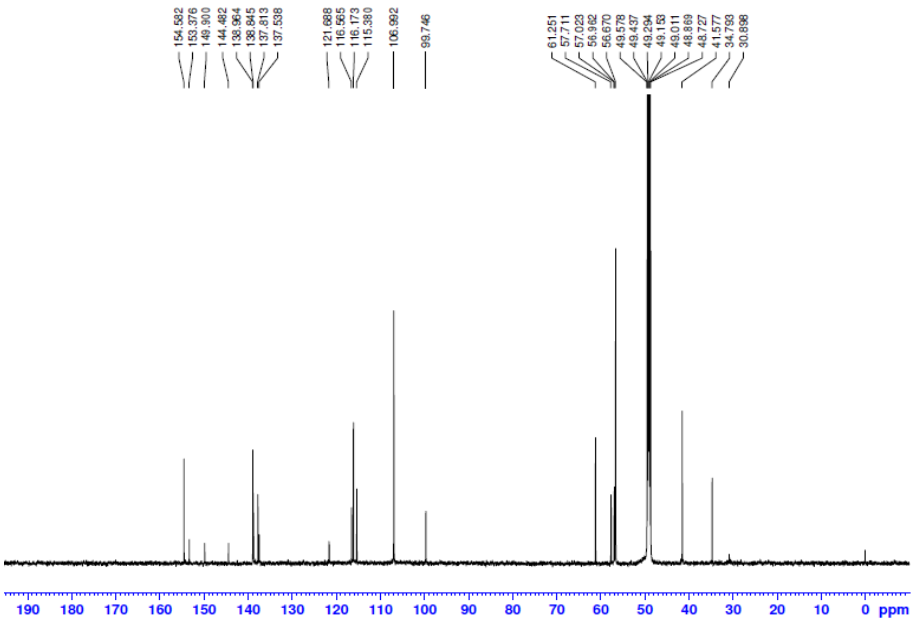

Fig. 19. ^{13}C NMR spectrum of compound **3**.

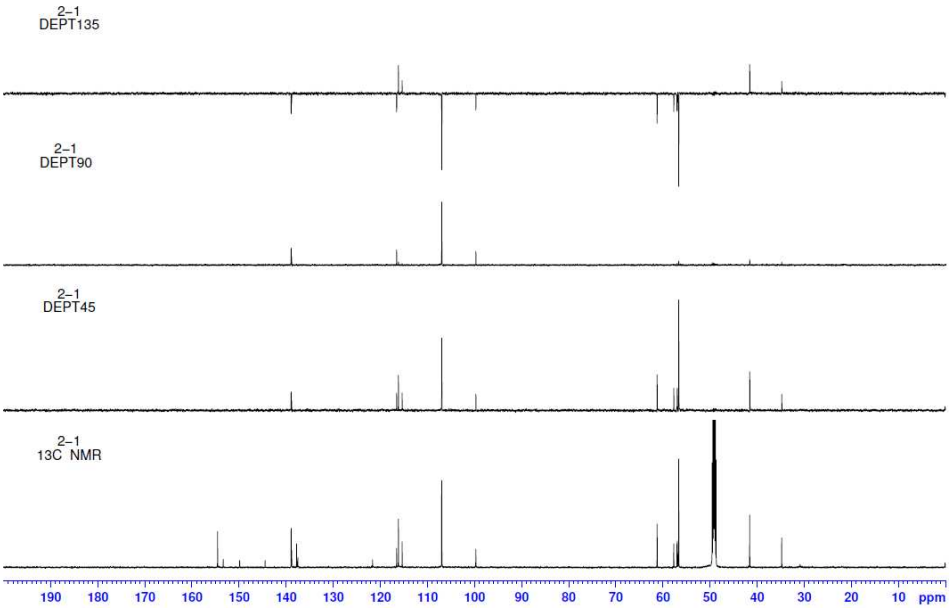

Fig. 20. DEPT spectrum of compound **3**.

Table 14. ^{1}H NMR (600 MHz) and ^{13}C NMR (150 MHz) spectral data of compound **3**

Position	Partial structure	δ_C (ppm)	δ_H (ppm)	δ_C (ppm)	δ_H (ppm) Zuo *et al*., 2012
1	C	154.5		151.6	
2	C	149.9		148.6	
3	C	144.4		142.5	
4	C	116.1		118.1	
5	CH	99.7	5.93, s	97.6	6.49, d (J = 1.5 Hz)
6	CH	115.3	6.72, s	114.2	6.85, s
7	CH3	57.0	3.73, s	56.5	3.81, s
8	CH3	56.7	3.75, s	56.7	3.84, s
9	CH3	56.9	3.78, s	56.2	3.90, s
10	CH	121.6	6.48, s	124.9	6.54, s
11	CH	137.8	5.03, s	130.0	5.77 (J = 7.0 Hz)
12	CH3	34.8	3.32, d (J = 5.4 Hz)	14.8	1.84, s

The compound **4** was obtained as powder or needle and identified as (–)-asarinin (**4**) by spectroscopic analysis, including EI-MS (Fig. 21), ^{1}H NMR (Fig. 22), ^{13}C NMR (Fig. 23), and DEPT (Fig. 24). The EI-MS revealed a molecular ion at *m*/*z* 354 $[M]^{+}$ and its ^{13}C NMR spectra showed 20 carbons in the big molecule suggesting the molecular formula $C_{20}H_{18}O_6$.

(–)-Asarinin (**4**): white powder or needle; $[\alpha]^{15.2}_{D}$: –152° (*c* +003; chloroform); UV (EtOH): λ_{max} = 241; EI-MS (70 eV), *m*/*z* (rel. int.): 354 $[M]^{+}$ (100, base peak), 323, 203, 178, 161, 149, 135, 122. ^{1}H NMR ($CDCl_3$, 600 MHz) and ^{13}C NMR ($CDCl_3$, 150 MHz) are given in Table 15. The interpretations of proton and carbon signals of compound **4** were largely consistent with those of Wimalasena *et al*. (1994) and Ju *et al*. (2001).

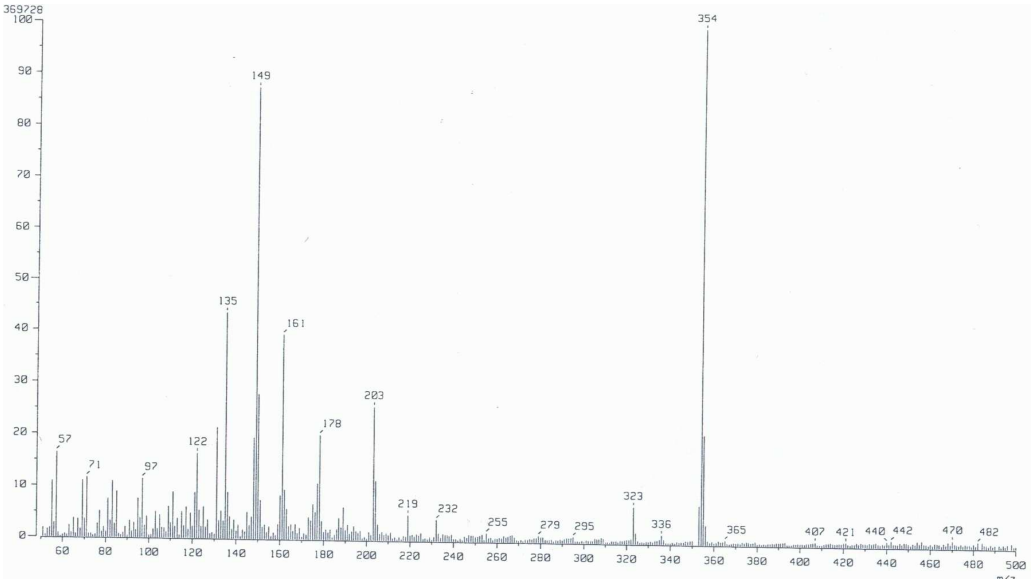

Fig. 21. EI-MS spectrum of compound **4**.

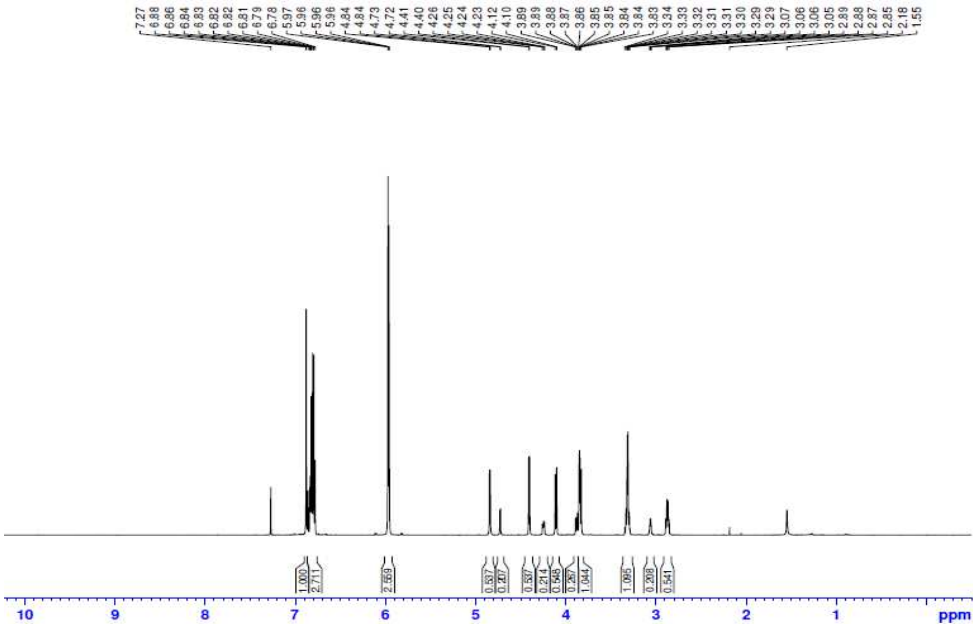

Fig. 22. ^{1}H NMR spectrum of compound **4**.

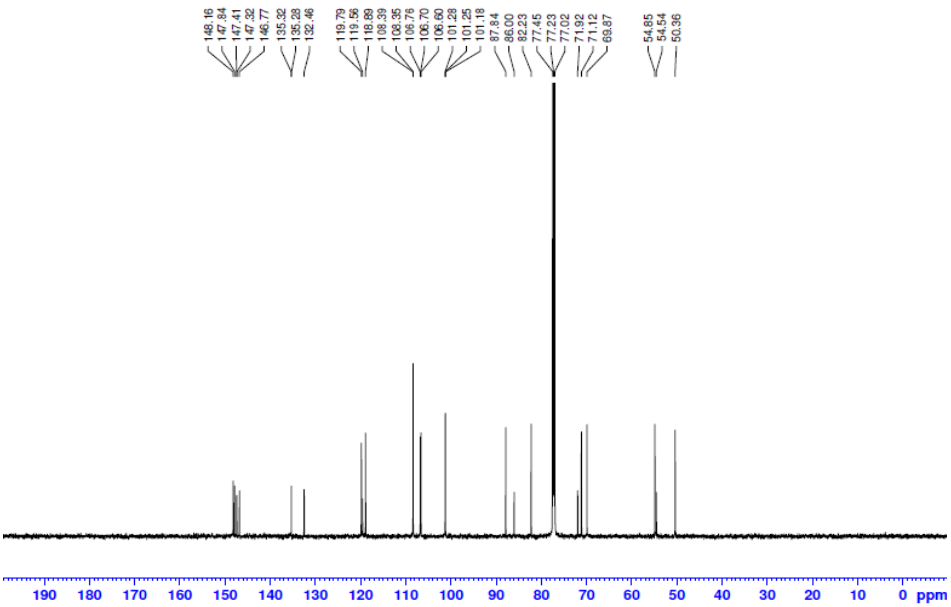

Fig. 23. ^{13}C NMR spectrum of compound **4**.

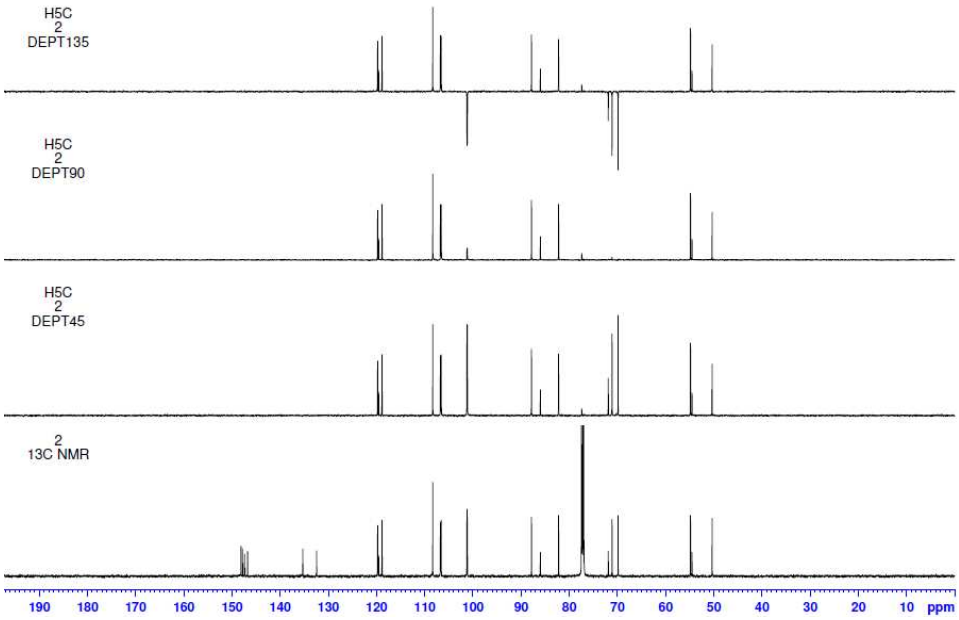

Fig. 24. DEPT spectrum of compound **4**.

Table 15. ^{1}H NMR (600 MHz) and ^{13}C NMR (150 MHz) spectral data of compound **4**

Position	Partial structure	δ_C (ppm)	δ_H (ppm)	δ_C (ppm)	δ_H (ppm) Wimalasena *et al.,* 1994
1	CH2	101.1	5.97, d (*J* = 7.2 Hz)	101.1	5.95, s
2	CH2	101.3	5.95, d (*J* =5.4 Hz)	101.0	5.95, s
3	C	148.1		147.9	
4	C	146.7		146.5	
5	C	147.8		147.6	
6	C	147.7		147.2	
7	CH	82.2	4.10, d (*J* = 11.4 Hz)	82.0	4. 38, d (*J* = 7.0 Hz)
8	CH	87.8	4.40, d (*J* = 6.6 Hz)	87.62	4.06, d (*J* = 9.0 Hz)
9	CH2	69.8	3.30, m	69.66	3.31, m
10	CH2	71.1	3.83, m	70.88	3.83, m
11	CH	50.3	2.85, d (*J* = 4.2 Hz)	50.12	2.85, m
12	CH	54.8	2.85, d (*J* = 13.2 Hz)	54.63	2.85, m
13	CH	106.6	6.88, s	106.3	6.99, m
14	CH	108.4	6.79, m	108.3	6.77, m
15	C	132.4		132.2	
16	C	135.5		135.0	
17	CH	106.8	6.86, m	106.3	6.86, m
18	CH	108.3	6.79, m	108.2	6.79, m
19	CH	118.8	6.79, m	116.6	6.77, m
20	CH	119.7	6.79, m	119.5	6.77,m

The compound **5** was obtained as viscous oil and identified as pellitorine (**5**) by spectroscopic analysis, including EI-MS (Fig. 25), ^{1}H NMR (Fig. 26), ^{13}C NMR (Fig. 27), and DEPT (Fig. 28). The EI-MS revealed a molecular ion at *m*/*z* 223 $[M]^{+}$ and its ^{13}C NMR spectra showed 14 carbons including methylpropyl and amide group in the molecule suggesting the molecular formula $C_{14}H_{25}NO$.

Pellitorine (**5**): viscous oil; UV (EtOH): λ_{max} = 220. EI-MS (70 eV), *m*/*z* (rel. int.): 223 $[M]^{+}$ (100, base peak), 208, 180, 167, 152, 113, 96, 72. ^{1}H NMR ($CDCl_3$, 600 MHz) and ^{13}C NMR ($CDCl_3$, 150 MHz) are given in Table 16. The interpretations of proton and carbon signals of compound **5** were largely consistent with those of Steyn *et al*. (1998) and Park *et al*. (2002).

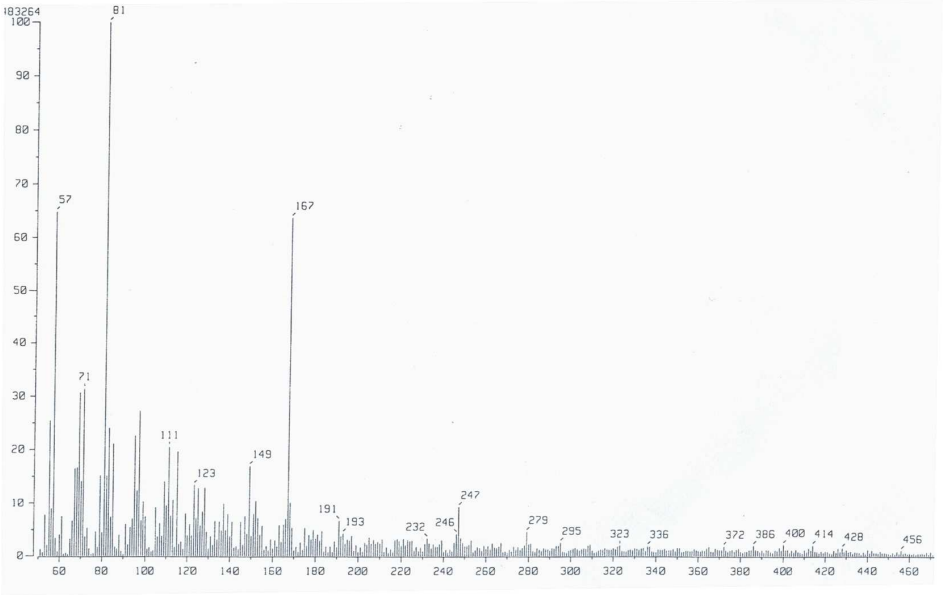

Fig. 25. EI-MS spectrum of compound **5**.

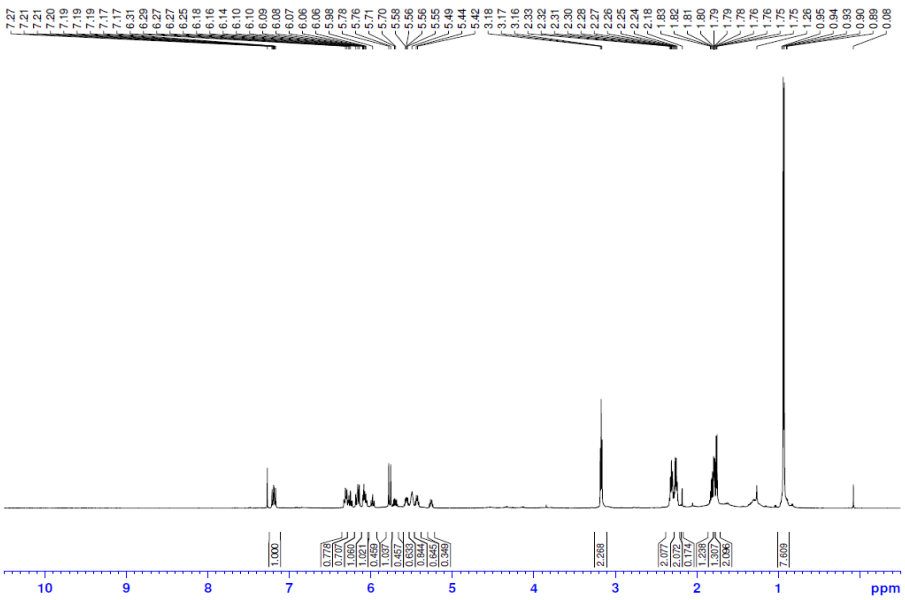

Fig. 26. ^{1}H NMR spectrum of compound **5**.

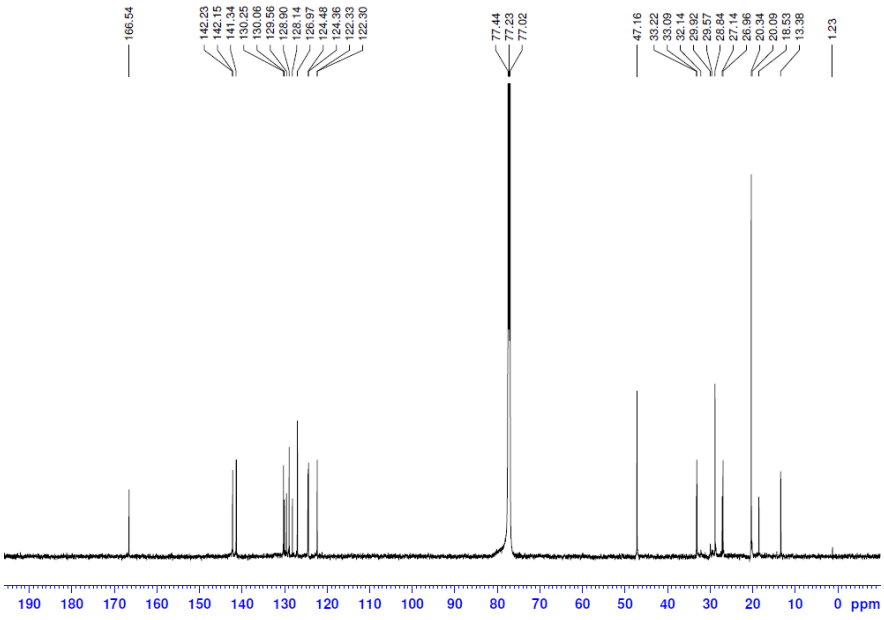

Fig. 27. ^{13}C NMR spectrum of compound **5**.

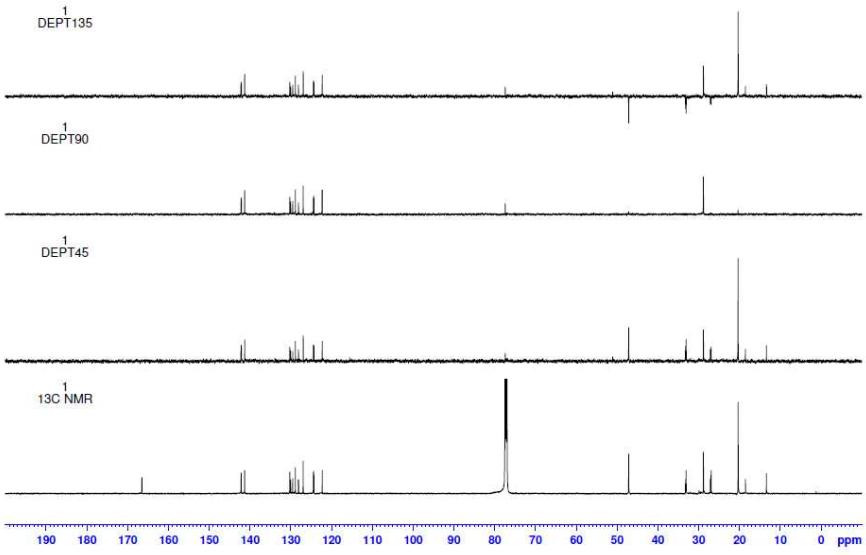

Fig. 28. DEPT spectrum of compound **5**.

Table 16. ^{1}H NMR (600 MHz) and ^{13}C NMR (150 MHz) spectral data of compound **5**

Position	Partial structure	δ_C (ppm)	δ_H (ppm)	δ_C (ppm)	δ_H (ppm) Steyn *et al*., 1998
1	C	166.4		166.6	
2	CH2	46.9	3.16, t (*J* =6.4 and 12.9 Hz)	46.8	3.10, t (*J* = 6.5 Hz)
3	CH	121.7	5.60, m	121.8	5.35, m
4	CH	142.2	7.19, m	143.2	7.15, m
5	CH	128.2	6.09, m	128.7	6.01, m
6	CH	141.2	5.76, m	141.3	5.74, d (*J* = 11.1 Hz)
7	CH	28.6	2.23, d (*J* = 6.6 Hz)	28.5	2.08, m
8	CH2	31.4	1.37, d (*J* = 12.0 Hz)	31.3	1.34, m
9	CH2	28.5	1.28, m	28.3	1.24, m
10	CH2	32.9	1.75, m	32.9	1.74, m
11	CH2	22.5	1.28, m	22.3	1.21, m
12	CH3	20.1	0.93, s	19.9	0.85, s
13	CH3	20.1	0.91, s	19.9	0.85, s
14	CH3	14.0	0.88, s	13.8	0.81, s
	NH		8.03, s		8.05, s

Structures of isolates from *A. heterotropoides* root, methyleugenol (**1**), pentadecane (**2**), α-asarone (**3**), (–)-asarinin (**4**), and pellitorine (**5**) are given in Fig. 29.

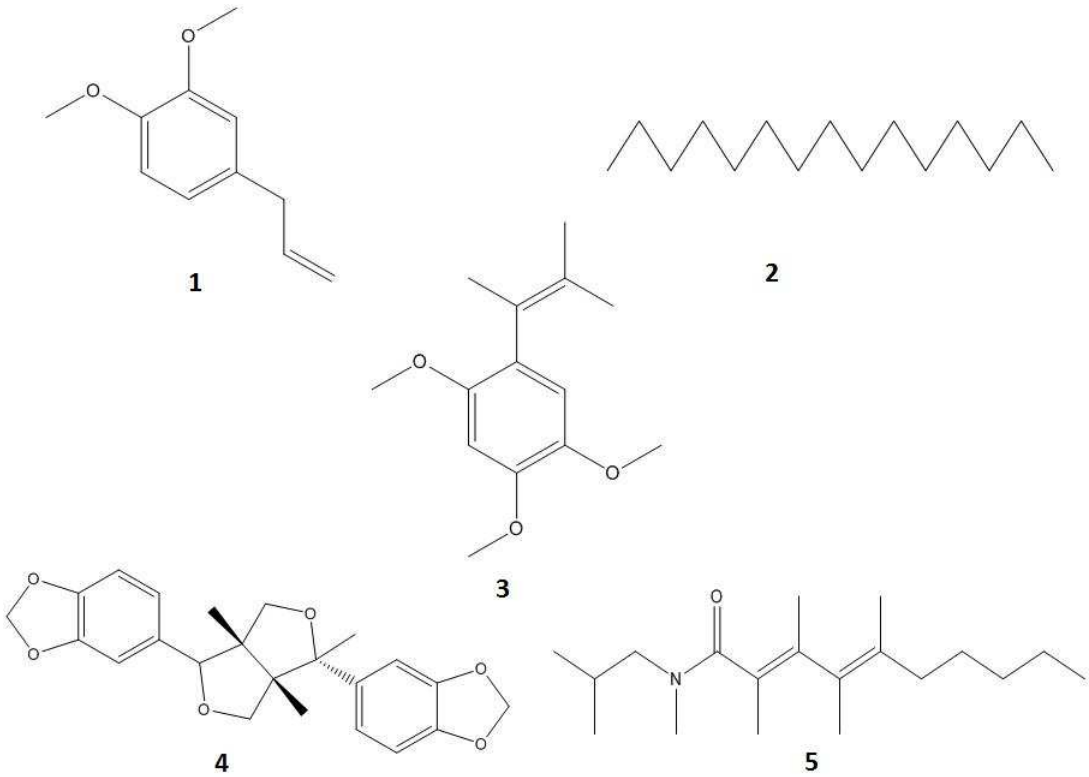

Fig. 29. Structures of isolated compounds from *A. heterotropoides* root.

1.2.2. Larvicidal Activity of Insecticides

The toxicity of 11 insecticides examined against third instars from the susceptible KS-CP strain of *Cx. p. pallens* was evaluated using the direct-contact mortality bioassay (Table 17). As judged by 24 h LC_{50} values, β-cyfluthrin and deltamethrin were the most toxic insecticides, followed by α-cypermethrin. Moderate toxicity was produced by bifenthrin, fenthion, and chlorpyrifos. The other five insecticides exhibited low toxicity.

Table 17. Toxicity of 11 insecticides to third instars from insecticide-susceptible KS-CP strain of *Culex pipiens pallens* using direct-contact mortality bioassay during a 24 h exposure

Insecticide	slope ± SE	LC_{50}, mg/L (95%CL[a])
β-Cyfluthrin	1.9 ± 0.17	0.00020 (0.00016–0.00024)
Deltamethrin	1.4 ± 0.13	0.00029 (0.00023–0.00038)
α-Cypermethrin	3.5 ± 0.43	0.00047 (0.00041–0.00052)
Bifenthrin	2.2 ± 0.23	0.00125 (0.00104–0.00149)
Fenthion	1.9 ± 0.16	0.0031 (0.0025–0.0037)
Chlorpyrifos	2.5 ± 0.26	0.0034 (0.0028–0.0040)
Fenitrothion	1.9 ± 0.19	0.0091 (0.0074–0.0113)
Permethrin	1.5 ± 0.13	0.0079 (0.0062–0.0102)
Chlorfenapyr	1.6 ± 0.14	0.0108 (0.0086–0.0136)
Etofenprox	2.0 ± 0.18	0.0186 (0.0153–0.0226)
Temephos	1.1 ± 0.16	0.016 (0.011–0.022)

[a] CL denotes confidence limit.

The comparative toxicity of the 11 test insecticides to third instars from the field-collected DJ-CP colony of *Cx. p. pallens* was likewise compared (Table 18). DJ-CP larvae exhibited extremely high levels of resistance to fenthion (RR, 1179), chlorpyrifos (RR, 1174), fenitrothion (RR, 428), deltamethrin (RR, 316) and chlorfenapyr (RR, 225), high level of resistance to α-cypermethrin (RR, 94), and low levels of resistance to bifenthrin, permethrin, etofenprox, β-cyfluthrin and temephos (RR, <9) compared to KS-CP larvae.

Table 18. Toxicity of 11 insecticides to third instars from in field-collected DJ-CP colony of *Culex pipiens pallens* using direct-contact mortality bioassay during a 24 h exposure

Insecticide	Slope ± SE	LC_{50}, mg/L (95% CL[a])	RR[b]
β-Cyfluthrin	1.6 ± 0.17	0.00118 (0.00091–0.00149)	5.9
Deltamethrin	0.7 ± 0.09	0.09163 (0.05768–0.14535)	316.0
α-Cypermethrin	0.9 ± 0.08	0.04435 (0.02895–0.06735)	94.4
Bifenthrin	0.8 ± 0.08	0.01120 (0.00658–0.01819)	9.0
Fenthion	1.9 ± 0.19	3.6549 (3.0720–4.4051)	1179.0
Chlorpyrifos	1.5 ± 0.12	3.9898 (3.1956–5.0173)	1173.5
Fenitrothion	1.3 ± 0.18	3.8930 (2.8366–5.0950)	427.8
Permethrin	1.1 ± 0.09	0.0541 (0.0398–0.0740)	6.8
Chlorfenapyr	0.7 ± 0.09	2.4312 (1.5035–3.9500)	225.1
Etofenprox	1.0 ± 0.09	0.1183 (0.0833–0.1676)	6.4
Temephos	1.9 ± 0.29	0.068 (0.054–0.096)	4.3

[a] CL denotes confidence limit.

[b] Resistance ratio.

1.2.3. Larvicidal Activity of Test Compounds

The toxicity of five isolated compounds and two mosquito larvicides to third instars from CP-KS strain of *Cx. p. pallens* was examined as stated above (Table 19). Based on 24 h LC_{50} values, pellitorine ($LC_{50,}$ 2.08 mg/L) was the most toxic constituent, followed by (–)-asarinin ($LC_{50,}$ 11.45 mg/L) and α-asarone ($LC_{50,}$ 22.38 mg/L). Moderate activity was observed in methyleugenol ($LC_{50,}$ 53.30 mg/L), whereas weak activity was observed in pentadecane ($LC_{50,}$ 97.58 mg/L). Overall, all the compounds were less toxic than either fenthion or temephos (Table 18).

Table 19. Toxicity of *Asarum heterotropoides* root-derived materials to third instars from insecticide-susceptible KS-CP strain of *Culex pipiens pallens* using direct-contact mortality bioassay during a 24 h exposure

Material	Slope ± SE	LC_{50}, mg/L (95% CL[a])
MeOH ext.	2.3 ± 0.32	14.25 (11.95–17.05)
Pellitorine	2.3 ± 0.44	2.08 (1.68 –2.92)
(–)-Asarinin	1.5 ± 0.39	11.45 (8.48–16.88)
α-Asarone	2.7 ± 0.40	22.38 (19.22–26.00)
Methyleugenol	7.0 ± 1.00	53.30 (50.23–56.43)
Pentadecane	6.2 ± 1.08	97.58 (91.08–109.62)

[a] CL denotes confidence limit.

The toxicity of the isolated constituents to third instars of field collected DJ-CP colony of *Cx. p. pallens* was likewise compared (Table 20). Interestingly, all five constituents and *A. heterotropoides* root extract were of equal toxicity against both the KS-CP and DJ-CP larvae, indicating a lack of cross-resistance in the DJ-CP.

Table 20. Toxicity of *Asarum heterotropoides* root-derived materials to third instars from a field-collected DJ-CP colony of *Culex pipiens pallens* using direct-contact mortality bioassay during a 24 h exposure

Material	Slope ± SE	LC_{50}, mg/L (95% CL[a])
MeOH ext.	2.2 ± 0.31	16.26 (13.64–19.75)
Pellitorine	2.2 ± 0.28	2.19 (1.81–2.68)
(–)-Asarinin	1.7 ± 0.40	12.69 (9.89–18.05)
α-Asarone	3.1 ± 0.42	23.82 (20.87–27.62)
Methyleugenol	7.8 ± 1.19	67.02 (63.75–70.87)
Pentadecane	7.1 ± 1.47	117.93 (106.00–149.00)

[a] CL denotes confidence limit.

Toxic effects on third instars from *Ae. aegypti* of five test compounds and two larvicides were likewise compared (Table 21). Pellitorine ($LC_{50,}$ 2.33 mg/L) was 4.5-, 11.6-, 24.7-, and 41.5-fold more toxic than (–)-asarinin, α-asarone, methyleugenol, and pentadecane, respectively. However, these compounds were less toxic than either fenthion or temephos.

Against third instars from *Oc. togoi*, pellitorine ($LC_{50,}$ 2.38 ppm) was 6.9, 11.1, 24.6, and 41.7 times more toxic than (–)-asarinin, α-asarone, methyleugenol, and pentadecane, respectively (Table 22). These compounds were less toxic than either fenthion or temephos.

Table 21. Toxicity of test compounds and two larvicides to third instars from *Aedes aegypti* using direct-contact mortality bioassay during a 24 h exposure

Compound	Slope ± SE	LC_{50}, mg/L (95% CL[a])	SR [b]
Pellitorine	2.0 ± 0.26	2.33 (1.89 – 2.93)	1.1
(–)-Asarinin	2.6 ± 0.21	10.49 (9.03–12.09)	0.9
α-Asarone	2.8 ± 0.42	26.99 (23.42–31.88)	1.2
Methyleugenol	6.4 ± 1.00	57.65 (54.25–61.87)	1.1
Pentadecane	7.9±1.18	96.71(91.46–105.35)	0.9
Fenthion	1.9 ± 0.28	0.0043 (0.0035–0.0054)	1.4
Temephos	1.2±0.17	0.017(0.013–0.023)	1.0

[a] CL denotes confidence limit.

[b] Susceptibility ratio = LC_{50} of *Ae. aegypti* larvae/LC_{50} of *Cx. p. pallens* larvae.

1.2.4. Species Susceptibility

Based on SR (Tables 21 and 22), there were no significant differences in the toxicity of each test compound among *Cx. pipiens pallens*, *Ae. aegypti*, and *Oc. togoi* larvae.

Table 22. Toxicity of test compounds and two larvicides to third instars from *Ochlerotatus togoi* using direct-contact mortality bioassay during a 24 h exposure

Compound	Slope ± SE	LC_{50}, mg/L (95% CL[a])	SR [b]
Pellitorine	1.8 ± 0.27	2.38 (1.91–3.08)	1.1
(–)-Asarinin	1.9 ± 0.30	16.49 (13.51–20.79)	1.4
α-Asarone	2.8 ± 0.40	26.38 (22.70–31.39)	1.2
Methyleugenol	7.4 ± 1.18	58.52 (54.64–61.71)	1.1
Pentadecane	8.4 ± 1.26	99.19 (93.81–108.10)	1.0
Fenthion	1.5 ± 0.18	0.0047 (0.0039–0.0059)	1.5
Temephos	1.7 ± 0.31	0.020 (0.019–0.040)	1.2

[a] CL denotes confidence limit.

[b] Susceptibility ratio = LC_{50} of *Oc. togoi* larvae/LC_{50} of *Cx. p. pallens* larvae.

DISCUSSION

Various compounds, including phenolics, terpenoids, and alkaloids, exist in plants (Wink, 1993). They jointly or independently contribute to behavioral efficacy such as repellence and feeding deterrence and physiological efficacy such as acute toxicity and developmental disruption against various arthropod species (Ahn *et al*., 2006; Isman, 2006). Many plant preparations and their constituents manifest larvicidal activity against different mosquito species (Sukumar *et al*., 1991; Park *et al*., 2002; Kim *et al*., 2008) and have been proposed as alternatives to the widely available larvicides. For example, it has been reported that the isobutylamide alkaloids pellitorine, guineensine, pipercide, and retrofractamide A possess potent larvicidal activity against larvae of *Cx. p. pallens*, *Ae. aegypti*, and *Oc. togoi* and *Ae. aegypti* and *Oc. togoi* larvae were more tolerant than *Cx. p. pallens* larvae to these compounds (Park *et al*., 2002). In this study, the larvicidal principles of *A. heterotropoides* root were identified as the phenylpropanoids methyleugenol (**1**) and α-asarone (**3**), the saturated hydrocarbon pentadecane (**2**), the lignan (–)-asarinin (**4**), and the isobutylamide alkaloid pellitorine (**5**). No significant difference in toxicity among three mosquito species larvae was observed. Pellitorine and (–)-asarinin were highly effective against three mosquito species larvae. In addition, these compounds were also effective against *Cx. p. pallens* larvae resistant to various insecticides. This original finding indicates that the *A. heterotropoides* root-derived materials may hold promise for the development of novel and effective mosquito larvicides even against currently insecticide-resistant mosquito populations.

Investigations on the modes of action and the resistance mechanisms of natural

insecticidal products are of practical importance for mosquito control because it may give useful information on the most appropriate formulations and delivery means to be adapted for their future commercialization and for future resistance management. Major mechanisms of resistance to insecticides currently available to control mosquitoes are target site insensitivity that reduces sodium channel sensitivity to pyrethroid insecticides or acetylcholinesterase sensitivity to OP and carbamate insecticides and enhanced metabolism of various groups of insecticides (Hemingway *et al*., 2004). Alternative control agents with novel modes of action, low mammalian toxicity, and little environmental impact are urgently needed. In addition, certain plant-derived compounds were found to be highly effective against insecticide-resistant insect species (Ahn *et al*., 1997; Yang *et al*., 2009) and they are likely to be useful in resistance management strategies. Although available information is limited in mosquitoes, the phenylpropanoids such as ethyl cinnamate and ethyl *p*-methoxycinnamate are effective against larvae from field-collected *Cx. p. pallens* with low levels of resistance to fenthion and temephos (Kim *et al*., 2008). The current finding that pellitorine, (–)-asarinin, α-asarone, and methyleugenol are virtually equal in toxicity to both insecticide-susceptible and -resistant larvae of *Cx. p. pallens* suggests that these compounds and the pyrethroid, OP, and pyrrole insecticides do not share a common mode of action or elicit cross-resistance. Detailed tests are needed to fully understand the modes of action of the isolated compounds, although the octopaminergic and γ-aminobutyric acid (GABA) receptors have been suggested as novel target sites for some essential oil constituents by Kostyukovsky *et al*. (2002) and Priestley *et al*. (2003), respectively.

In conclusion, *A. heterotropoides* root-derived materials, particularly pellitorine and (–)-asarinin, could be useful as larvicides in the control of mosquito populations, particularly due to their activity against insecticide-resistant mosquito larvae. For the practical use of these materials as novel mosquito larvicides to proceed, further research is needed to establish their safety issues in relation to human health, nontarget aquatic organisms, and the aquatic environment. Historically, *A. heterotropoides* has been used as an analgesic and antitussive agent for the treatment of influenza, headache, rheumatic pain, and asthma (Tang, 1992). In addition, formulations for improving larvicidal potency and stability need to be developed.

1.3. Enhanced Toxicity of Binary Mixtures of Larvicidal Constituents from *Asarum heterotropoides* Root to Insecticide-Susceptible and -Resistant *Culex pipiens pallens*

INDRODUCTION

Species in the *Culex pipiens* complex are considered to be the primary vectors of West Nile virus in North America (Fonseca *et al*., 2004) and wuchererisais and epidemic encephalitis in some Asian countries (Ye, 1995; Lu, 1999; Rowland *et al*., 1999). The greatest problem with the mosquito species is their ability to rapidly evolve resistance to insecticides (Anonymous, 2011). Increasing levels of resistance to the commonly used insecticides have caused multiple treatments including overdoses, fostering serious environmental and human health concerns. Many of the insecticides currently used in South Korea have failed to control the mosquito species in the field, most probably because of the development of resistance (Lee *et al*., 1997; Kim *et al*., 2007). However, increasing public concern for the environmental effects of insecticides and human health effects (Hayes and Laws, 1991) becomes more critical on continued or repeated applications of conventional insecticides. These problems substantiate the need for the development of new improved alternatives and management strategies for mosquito control.

Various compounds (e.g., phenolics, terpenoids, and alkaloids) found in plants jointly or independently contribute to acute toxicity against various arthropod species (Wink, 1993; Lawless 2002; Ahn *et al.,* 2006; Isman, 2006). These plant-derived materials have

been suggested as alternative sources for mosquito larvicides largely because they constitute a potential source of bioactive chemicals that have been perceived by the general public as relatively safe and pose fewer risks to the environment with minimal impacts to animal and human health (Sukumar *et al*., 1991; Lawless, 2002; Ahn *et al*., 2006; Isman, 2006). They often act at multiple and novel target sites, thereby reducing the potential for resistance (Kostyukovsky *et al*., 2002; Priestley *et al*., 2003; Isman, 2006). Complex mixtures exerting synergistic or potentiating actions are considered to have a higher and long-lasting effect through various mechanisms than pure compound alone (Berenbaum, 1985, Hummelbrunner and Isman, 2001). (−)-Asarinin, α-asarone, methyleugenol, pellitorine, and pentadecane had good larvicidal activity against third instars from insecticide-susceptible *Cx. p. pallens*, *Ae. aegypti*, and *Oc. togoi*, as well as field-collected *Cx. p. pallens* as stated in Chapter 1.2. However, no information is available concerning the potential of binary mixtures of these compounds for managing mosquitoes, although joint toxic effects of insecticides with plant extracts on different mosquito species have been well-noted (Shaalan *et al*., 2005).

In this study, an assessment is made of the potential of pellitorine alone or in combination with (−)-asarinin, α-asarone, methyleugenol, and pentadecane at five tested ratios (1:1, 1:2, 1:3, 2:1, and 3:1) against third instar larvae from insecticide-susceptible and -resistant *Cx. p. pallens* for use as future commercial mosquito larvicides.

MATERIALS AND METHODS

1.3.1 Chemicals

(−)-Asarinin, α-asarone, methyleugenol, pellitorine, and pentadecane were obtained from *A. heterotropoides* root, as stated in Chapter 2.2. Triton X–100 was obtained from Shinyo Pure Chemicals (Osaka, Japan). All of the other chemicals used in this study were of reagent-grade quality and available commercially.

1.3.2 Mosquitoes

The insecticide-susceptible KS-CP strain and DJ-CP colony of *Cx. p. pallens*, as stated in Chapter 1, were used in this study. Adult mosquitoes were maintained on a 10% sucrose solution and blood fed on live mice. Larvae were reared in plastic trays (24 × 35 × 5 cm) containing 0.5 g of sterilized diet (40-mesh chick chow powder: yeast, 4:1 by weight). They were held at 27 ± 1°C and 65–75% relative humidity under a 14:10 h light:dark cycle.

1.3.3 Bioassay

A direct-contact mortality bioassay, as stated in Chapter 2.1, was used to evaluate the toxicity of pellitorine alone or in combination of (−)-asarinin, α-asarone, methyleugenol, and pentadecane at five tested ratios (1:1, 1:2, 1:3, 2:1, and 3:1) to third instars from KS-CP and DJ-CP of *Cx. p. pallens*. Bioassays were conducted from April to June 2010. Each compound and binary mixture in methanol was suspended in distilled water with Triton X–100 (20 μL/L). Groups of 20 mosquito larvae were separately put into paper

cups (270 mL) containing each tested material solution (250 mL). The toxicity of each tested material was determined with four to six concentrations ranging from 0.1 to 200 mg/L. Controls received methanol-Triton X–100 solution in distilled water. All treatments were replicated three times using 20 larvae per replicate. Treated and control larvae were held at the same conditions as those used for colony maintenance. Larvae were considered to be dead if they did not move when they prodded with fine wooden dowels 24 h post treatment. Because not all bioassays could be conducted at the same time, treatments were blocked over time with a separate control treatment included in each block. Freshly prepared solutions were used for each block of bioassays (Robertson and Preisler, 1992).

1.3.4 Data Analysis

Data were corrected for control mortality using Abbott's (1925) formula. Concentration–mortality data were subjected to probit analysis (SAS Institute, 2004). LC_{50} values of their treatments were considered to be significantly different from one another when their 95% confidence limits (CLs) failed to overlap. The co-toxicity coefficient (CC) and synergistic factor (SF) were calculated according to the methods of Sun and Johnson (1960) and Kalyanasundaram and Das (1985), respectively. Values of SF >1 indicate synergism and SF <1 indicate antagonism.

RESULTS

The toxicity of pellitorine alone or in combination with (−)-asarinin, α-asarone, methyleugenol, and pentadecane to the susceptible KS-CP larvae was evaluated using the direct-contact mortality bioassay (Table 23). Binary mixtures of pellitorine and (−)-asarinin (3:1 and 1:3 ratios) were significantly more toxic than either pellitorine or (−)-asarinin alone. The toxicity of the other three binary mixtures of pellitorine and (−)-asarinin (1:1, 1:2, and 2:1 ratios) did not differ significantly from that of pellitorine alone. Based on the CC and SF values, the four binary mixtures (except for 1:1 ratio) operated in a synergy pattern (CC, 250–390 and SF, 1.1–2.2). The toxicity of binary mixtures of pellitorine and α-asarone, methyleugenol, and pentadecane at all tested ratios were significantly less toxic than pellitorine alone. Mortality in the methanol-Triton X–100-water-treated controls was less than 2%.

Toxic effects of binary mixtures of pellitorine and each tested compounds on the insecticide-resistant DJ-CP larvae were likewise compared (Table 24). Binary mixture of pellitorine and (−)-asarinin (3:1 ratio) was significantly more toxic than either pellitorine or (−)-asarinin alone. The other four binary mixtures of pellitorine and (−)-asarinin (1:1, 1:2, 1:3, and 2:1 ratios) and pellitorine did not differ significantly in toxicity. As judged by the CC and SF values, the binary mixtures (except for 1:1 and 1:2 ratios) exhibited synergistic action (CC, 257–279 and SF, 1.1–2.1). The toxicity of binary mixtures of pellitorine and α-asarone, methyleugenol, and pentadecane were significantly lower than pellitorine alone.

Table 23. Toxicity to third instars from insecticide-susceptible KS-CP strain of *Culex pipiens pallens* of pellitorine alone or in combination with other compounds during a 24-h exposure

Treatment	Slope ± SE	$LC_{50,}$ mg/L (95% CL)	CC[a]	SF[b]
Pellitorine (PE) only	2.1 ± 0.27	2.08 (1.73–2.53)		
(−)-Asarinin (AS) only	2.1 ± 0.41	11.45 (9.30–14.60)		
PE + AS (1:1)	2.1 ± 0.27	2.15 (1.76–2.67)	164	0.9
PE + AS (1:2)	1.8 ± 0.27	2.00 (1.62–2.51)	305	1.1
PE + AS (1:3)	2.6 ± 0.42	1.38 (1.16–1.68)	390	1.5
PE + AS (2:1)	1.5 ± 0.27	1.53 (1.17–2.29)	250	1.4
PE + AS (3:1)	1.7 ± 0.27	0.95 (0.76–1.21)	275	2.2
α-Asarone (AR) only	2.5 ± 0.39	22.35 (19.00–26.21)		
PE + AR (1:1)	2.4 ± 0.32	17.57 (14.89–21.27)	22	0.1
PE + AR (1:2)	2.2 ± 0.31	16.42 (13.75–20.06)	43	0.2
PE + AR (1:3)	2.2 ± 0.33	15.79 (13.23–19.16)	41	0.2
PE + AR (2:1)	1.5 ± 0.39	8.06 (5.42–10.61)	49	0.3
PE + AR (3:1)	2.2 ± 0.28	4.84 (4.01–6.00)	56	0.4
Methyleugenol (ME) only	5.0 ± 0.66	53.36 (49.72–57.83)		
PE + ME (1:1)	2.5 ± 0.36	34.49 (30.04–40.72)	12	0.06
PE + ME (1:2)	2.7 ± 0.40	23.83 (20.60–27.73)	32	0.08
PE + ME (1:3)	2.1 ± 0.31	18.29 (15.11–23.14)	41	0.1
PE + ME (2:1)	2.1 ± 0.32	16.07 (13.35–13.94)	25	0.1
PE + ME (3:1)	1.6 ± 0.29	10.99 (8.28–13.94)	25	0.2
Pentadecane (PD) only	7.0 ± 1.13	97.46 (91.59–107.65)		
PE + PD (1:1)	4.7 ± 0.64	51.51 (47.80–55.88)	8	0.04
PE + PD (1:2)	2.6± 0.57	37.72 (32.61–46.00)	21	0.05
PE + PD (1:3)	2.9± 0.43	27.67 (24.21–32.38)	8	0.07
PE + PD (2:1)	1.8 ± 0.29	11.89 (9.37–14.76)	35	0.2
PE + PD (3:1)	1.7 ± 0.40	8.68 (6.32–11.21)	32	0.3

[a] Co-toxicity coefficient.

[b] Synergistic factor.

Table 24. Toxicity to third instars from field-collected DJ-CP colony of *Culex pipiens pallens* of pellitorine alone or in combination with other compounds during a 24-h exposure

Treatment	Slope ± SE	$LC_{50,}$ mg/L (95% CL)	CC[a]	SF[b]
Pellitorine (PE) only	2.4 ± 0.30	2.33 (1.96–2.80)		
(−)-Asarinin (AS) only	2.0 ± 0.41	12.61 (10.13–16.89)		
PE + AS (1:1)	1.5 ± 0.39	2.86 (2.12–4.21)	138	0.8
PE + AS (1:2)	1.9 ± 0.27	2.36 (1.90–3.04)	288	0.9
PE + AS (1:3)	1.9 ± 0.27	2.15 (1.75–2.69)	279	1.1
PE + AS (2:1)	1.3 ± 0.39	1.66 (1.18–3.12)	257	1.4
PE + AS (3:1)	2.4 ± 0.30	1.07 (0.91–1.29)	273	2.1
α-Asarone (AR) only	3.0 ± 0.41	23.82 (20.77–27.43)		
PE + AR (1:1)	2.3 ± 0.34	20.44 (17.18–25.45)	21	0.1
PE + AR (1:2)	2.3 ± 0.32	18.69 (15.70–23.10)	42	0.2
PE + AR (1:3)	1.7 ± 0.30	17.56 (14.13–23.00)	41	0.2
PE + AR (2:1)	2.1 ± 0.32	9.76 (7.53–14.79)	46	0.3
PE + AR (3:1)	2.2 ± 0.30	5.84 (4.84–7.39)	52	0.4
Methyleugenol (ME) only	6.3 ± 0.83	67.02 (63.29–71.90)		
PE + ME (1:1)	3.0 ± 0.41	38.39 (33.89–44.97)	12	0.06
PE + ME (1:2)	2.1 ± 0.33	24.60 (19.94–33.61)	35	0.09
PE + ME (1:3)	2.2 ± 0.33	21.31 (17.57–27.64)	40	0.1
PE + ME (2:1)	1.8 ± 0.31	22.22 (17.72–30.99)	21	0.1
PE + ME (3:1)	2.0 ± 0.30	15.50 (12.81–19.12)	20	0.2
Pentadecane (PD) only	5.6 ± 1.21	117.94 (107.46–144.42)		
PE + PD (1:1)	4.9 ± 0.67	57.37 (53.32–62.88)	8	0.04
PE + PD (1:2)	3.4 ± 0.49	43.37 (39.17–48.90)	21	0.05
PE + PD (1:3)	2.3 ± 0.34	32.65 (28.11–38.96)	27	0.07
PE + PD (2:1)	1.4 ± 0.26	13.92 (10.39–22.27)	33	0.2
PE + PD (3:1)	1.3 ± 0.25	9.73 (7.32–14.13)	32	0.3

[a] Co-toxicity coefficient.

[b] Synergistic factor.

DISCUSSION

The current findings clearly indicate that binary mixtures of pellitorine and (−)-asarinin (1:3, 2:1, and 3:1 ratios) exhibited synergistic action against both KS-CP and DJ-CP larvae, based on the CC and SF values. Particularly, 3:1 mixture was significantly more effective than either pellitorine or (−)-asarinin alone. This original finding indicates that these mixtures may hold promise for the development of novel and effective mosquito larvicides even against currently insecticide-resistant mosquito populations. Individual compounds are active at high concentration, which makes them uneconomical for practical use. George and Vincent (2005) reported that binary mixtures of petroleum ether extracts from *Annona squamosa* L. (Annonaceae) and *Pongamia glabra* Vent. (Fabaceae) against *Cx. quinquefasciatus* exhibited synergistic action at all tested ratios (3:1, 1:1, and 1:3). Most studies on the synergistic, antagonistic, and additive toxic effects of binary mixtures involving phytochemicals have been conducted on agricultural pests rather than disease vectors. It has been demonstrated that (*E*)-anethole acted synergistically with thymol, citronellal, and α-terpineol against *Spodoptera litura* (Fab.) larvae (Hummelbrunner and Isman 2001).

Investigations on the joint toxic action mechanisms of binary mixtures and the insecticide resistance mechanisms are of practical importance for mosquito control largely because they may give useful information on the most appropriate formulations to be adapted for their future commercialization and for future resistance management (Kim et al. 2006, Perumalsamy et al. 2010). Complex mixtures of a refined *Azadirachta*

indica A. Juss. (Meliaceae) seed extract are likely to be more durable with respect to insects evolving resistance in *Myzus persicae* (Sulzer) than pure azadirachtin alone (Feng and Isman 1995). However, available information on toxic effects of binary mixtures of phytochemicals on mosquitoes, particularly insecticide-resistant mosquitoes, is limited, although the enhanced toxicity of binary mixtures of some plant extracts with an insecticide against different mosquito species has been well noted (Shaalan et al. 2005). Joint toxic action mechanisms of binary mixtures of chemicals include that one may interfere with the other's activation, or with its detoxification reaction induced by enzyme systems such as cytochrome P450 monooxygenases, glutathione *S*-transferases, and/or esterases (leading to rapid detoxification or sequestration of a chemical), or with both in insects. The most plausible explanation for the enhanced toxicity of a binary mixture would be the hypothesis that one toxicant interferes with the enzymatic detoxification of the second toxicant, thereby potentiating its toxicity (Corbett 1974). Thangam and Kathiresan (1990) studied the toxicity of DDT, BHC, and malathion, and the effects of their synergism with leaf and flower extracts of *Bougainvillea glabra* Choisy (Nyctaginaceae) on *Culex sitiens* Wiedemann. They suggested that synergism might be due to plant extract inhibiting some factors, such as detoxifying enzymes in mosquito larvae. In the current study, (−)-asarinin has acted as a powerful synergist, enhancing the effectiveness of pellitorine against both KS-CP and DJ-CP larvae. Many of lignans, such as asarinin, found in plants contain methylenedioxyphenyl (MDP) substituents and compounds of this structure interfere with insect detoxification via cytochrome P450 (Berenbaum 1985) like a synergist piperonyl butoxide with MDP

moiety, a specific inhibitor of the enzyme. Hummelbrunner and Isman (2001) suggested that the inclusion of a number of plant constituents is more desirable in that the insecticidal spectrum of action is increased, because different species have different responses to individual constituents. However, detailed tests are needed to fully understand the exact synergy mechanism of the binary mixture of pellitorine and (−)-asarinin.

In conclusion, the binary mixtures of pellitorine and asarinin could be useful as larvicides in the control of mosquito populations, particularly in the light of their activity against insecticide-resistant mosquito larvae. For practical use of the binary mixture as novel larvicides to proceed, further research is needed to establish their human safety. In addition, their effects on nontarget aquatic organisms and the environment need to be established. Formulations for improving larvicidal potency and stability, thereby reducing costs, also need to be developed.

CHAPTER I I

Isolation and Ientification of Larvicidal Principles Identified in *Millettia pinnata* Seed against Insecticide-Susceptible *Culex pipiens pallens* and *Aedes aegypti* and Wild *Aedes albopictus*

2. Isolation and Ientification of Larvicidal Principles Identified in *Millettia pinnata* Seed against Insecticide-Susceptible *Culex pipiens pallens* and *Aedes aegypti* and Wild *Aedes albopictus*

INTRODUCTION

The yellow fever mosquitoes, *Aedes aegypti* (L.), the Asian tiger mosquito, *Aedes albopictus* (Skuse), and the northern house mosquito, *Culex pipiens pallens* (Coquillett), are serious disease vectoring insects because of the widespread distribution and abundance worldwide (Mullen, 2009). More than 2.5 billion people are at risk of dengue infection over 100 countries worldwide, and there may be 50–100 million dengue infections every year, including 22000 deaths annually, mostly among children (CDC, 2012). From 1999 to 2010, 30502 cases of human West Nile Virus disease (including 8991 neuroinvasive disease cases) were reported in the United States, resulting in 1216 deaths (CDC, 2012). In addition, the number of approved insecticides will likely be reduced in the near future in the United States by the US Environmental Protection Agency as reregistration occurs under the 1996 Food Quality and Protection Act (USEPA, 2012). Reregistration requirement is also a concern in other regions including in the European Union, where it is under the control of the Commission Regulation (EC) No 1048/2005 (Commission Regulation, 2005). Therefore, there is a critical need for the development of selective alternatives for the control of mosquitoes, with novel target sites in order to establish a rational resistance management strategy based on all available information on the extent and nature of resistance in mosquitoes because

vaccines for malaria (Richie, 2002) or dengue (WHO, 2011) are not yet available.

Biocides derived from plants, particularly higher plants, may provide potential alternatives for mosquito control largely because they constitute a potential source of bioactive chemicals that have been perceived by the general public as relatively safe and with less risk to the environment, and with minimal impacts to animal and human health (Sukumar *et al*., 1991; Shaalan *et al*., 2005; Isman, 2006) They often act at multiple and novel target sites (Kostyukovsky *et al*., 2002; Priestley *et al*., 2003; Isman, 2006) thereby reducing the potential for resistance (Wang *et al*., 2012). These potential new mosquitocides can be applied to mosquito breeding places in the same manner as the conventional insecticides. Much effort has been focused on them as potential sources of commercial mosquito control products, in part, because certain plant preparations meet the criteria of reduced risk pesticides (Isman, 2006). The Indian beech, *Millettia pinnata* (L.) Panigrahi, belongs to family Fabaceae. The seeds of the plant are known to have antiinflammatory, analgesic and antiulcerogenic activities (Singh, *et al*., 1996) while the flowers are used to treat diabetes (Akhtar and Akhtar, 1999). Very little information has been done to consider potential of Indian beech seed-derived materials to manage mosquito population. Laboratory evaluation revealed that the treatment of larvae of *Ae. aegypti* and *Cx. quinquefasciatus* with ethanol and aqueous extract of *Pongamia glabra* seed kernels significantly increased the larval mortality and developmental period proportionately with increase in the extract concentrations (Sagar and Sehgal, 1996; Sagar *et al*., 1999).

In this study, an assessment is made of the contact toxicity of the constituents from

the seeds of Indian beech to third instars from *Cx. p. pallens* and *Ae. aegypti* as well as wild colony of *Ae. albopictus*. The toxicities of the seed constituents were compared with those of fenthion and temephos for use as future commercial mosquito larvicides.

MATERIALS AND METHODS

2.1. General Instrumental Analyses

^{1}H and ^{13}C NMR spectra were recorded in $CDCl_3$ on a Bruker AVANCE 600 spectrometer (Karlsruhe, Germany) using tetramethylsilane as an internal standard, and chemical shifts are given in δ (ppm). UV spectra were obtained in ethanol or acetone on a Kontron UVICON 933/934 spectrophotometer (Milan, Italy), and mass spectra on a Jeol JMS-DX 303 spectrometer (Tokyo, Japan). Merck silica gel (0.063–0.2 mm) (Darmstadt, Germany) was used for column chromatography. Merck pre-coated silica gel plates (Kieselgel 60 F_{254}) were used for analytical thin-layer chromatography (TLC). An Agilent 1200 series high-performance liquid chromatograph (HPLC) (Santa Clara, CA) was used for isolation of active principles.

2.2. Chemicals

Oleic acid, elaidic acid, arachidonic acid, octadecanamide, and behenic acid were purchased from Tokyo Chemical Industry (Tokyo, Japan). Triton X–100 was obtained from Shinyo Pure Chemicals (Osaka, Japan). Fenthion (98.4% purity) and temephos (97.3% purity) were purchased from Supelco (West Chester, PA) and Riedel (Seelze, Germany). All of the other chemicals of were of reagent-grade quality and available commercially.

2.3. Mosquitoes

The insecticide-susceptible strains of *Cx. p. pallens* and *Ae. aegypti* were used in this

study. Larvae of wild mosquitoes were collected near rice paddy fields and cowsheds in Daejeon (South Korea) in early August 2011. The larvae were immediately transferred to an insect rearing room (Seoul National University). Species identification revealed that the adults from the collected larvae belonged to *Ae. albopictus*. Adults and larvae were reared under the same conditions as those stated in Chapter 1. The wild mosquitoes were reared for four generations to ensure sufficient numbers for testing.

2.4. Plant Material

The seeds of Indian beech were collected from the foothill at Western Ghats (Coimbatore, Tamil Nadu, India) in September 2009. A voucher specimen (MP–01) was deposited in the Research Institute for Agriculture and Life Science, Seoul National University.

2.5. Extraction and Isolation

Air-dried seeds (130 g) of Indian beech were pulverized and extracted with methanol (2 × 3 L) at room temperature for 1 day and filtered. The combined filtrate was concentrated under vacuum at 40°C to yield ~23.21 g of a pale yellowish tar. The extract (20 g) was sequentially partitioned into hexane- (3.22 g), chloroform- (2.96 g), ethyl acetate- (0.50 g), butanol- (1.03 g), and water-soluble (12.29 g) portions for subsequent bioassay (Fig. 30). The organic solvent-soluble portions were concentrated to dryness by rotary evaporation at 40°C, and the water-soluble portion was freeze-dried. For isolation of active principles, 50 mg/L of each Indian beech seed-derived material was tested in a direct-contact mortality bioassay as described previously (Kim *et al*., 2008).

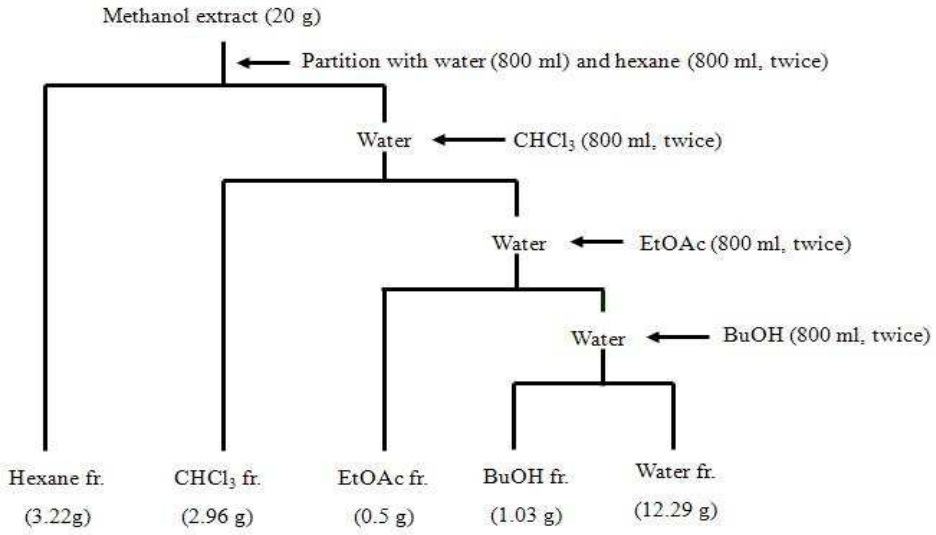

Fig. 30. Solvent fractionation of methanol extract of *M. pinnata.*

The chloroform-soluble fraction (2.96 g) was most biologically active and was recrystallized in methanol at –4°C to afford compound **1** (46 mg). Compound **1** (Fig. 31) was purified by HPLC (7.8 mm i.d. × 300 mm Waters μBondapak C18 (Milford, MA) using a mobile phase of methanol and water (6:4 by volume), at 340 nm in flow rate 1 mL/min. The remaining portion (2.50 g) was chromatographed on a 70 × 5.5 cm silica gel (300 g) column by elution with a gradient of chloroform and methanol [(100:0 (1 L), 99:1 (2 L), 98:2 (2 L), 95:5 (2 L), 90:10 (2 L), 70:30 (1 L), and 0:100 (1 L) by volume] to provide 28 fractions (each about 250 mL). Column fractions were monitored by TLC on silica gel plates developed with chloroform and methanol (98:2 by volume) mobile phase. Fractions with similar R_f values on the TLC plates were pooled. Spots were

detected by spraying with 2% H_2SO_4 and then heating on a hot plate. Active fractions 7 to 11 (40 mg) were pooled and purified by preparative TLC plates developed with chloroform and methanol (98:2 by volume) to provide compound **2** (3 mg, R_f = 0.61). Compound **2** (Fig. 32) was purified by HPLC (7.8 mm i.d. × 300 mm Waters μBondapak C18 using a mobile phase of methanol and water (6:4 by volume), at 260 nm in flow rate 1 mL/min.

The active hexane-soluble fraction (5 g) was chromatographed on a 70 × 5.5 cm silica gel (300 g) column by elution with a gradient of hexane and ethyl acetate [(10:1 (2.2 L), 9:1 (2 L), 7:3 (2 L), 5:5 (1 L), and 3:7 (1 L) by volume] and finally with methanol (1 L) to provide 48 fractions (each about 250 mL). Column fractions were monitored by TLC on silica gel plates developed with hexane and ethyl acetate (7:3 by volume) mobile phase. Fractions with similar R_f values on the TLC plates were pooled, as stated above. Three active fractions 4 to 8 (I, 1.24 g), 9 to 12 (II, 590 mg), and 16 to 22 (III, 1.02 g) were obtained. Fraction I was recrystallized in hexane at –4°C to afford compound **3** (78 mg). Compound **3** (Fig. 33) was purified by HPLC (7.8 mm i.d. × 300 mm Waters μBondapak C18 using a mobile phase of methanol and water (6:4 by volume), at 205 nm in flow rate 1 mL/min. Fraction II was purified by preparative TLC plates developed with (hexane:ethyl acetate, 7:3 by volume) to yield compound **4** (85 mg, R_f = 0.61). Compound **4** (Fig. 34) was purified by HPLC (7.8 mm i.d. × 300 mm Waters μBondapak C18 using a mobile phase of methanol and water (6:4 by volume), at 260 nm in flow rate 1 mL/min. Fraction III was rechromatographed on a 70 × 5.5 cm silica gel (300 g) column by elution with a gradient of hexane and ethyl acetate [(10:1

(2.2 L), 9:1 (2 L), 7:3 (2 L), 5:5 (1 L), and 3:7 (1 L) by volume] and finally with methanol (1 L) to provide 25 fractions (each about 150 mL). A preparative HPLC was used for separation of the constituents from the active fractions 9 to 14 (III-1, 45 mg) and 18 to 22 (III-2, 75 mg). The column was a 7.8 mm i.d. × 300 mm Waters μBondapak C18 with a mobile phase of methanol and water (6:4 by volume) at a flow rate of 1 mL/min. Chromatographic separation was monitored using a UV detector at 260 nm. Finally, two active principles **5** (12 mg) from fraction III-1 and **6** (24 mg) from fraction III-2 were isolated at a retention time of 19.30 (Fig. 35) and 31.22 min (Fig. 36), respectively.

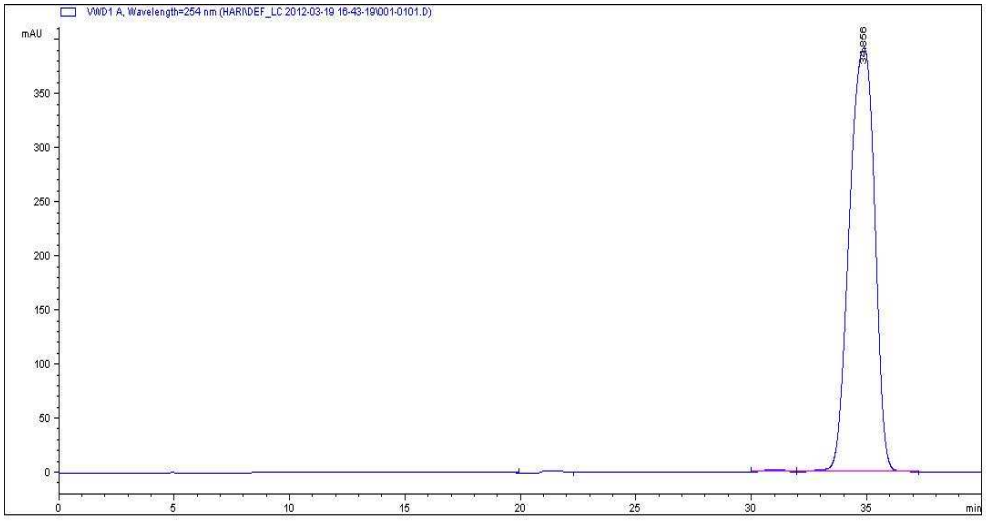

Fig. 31. HPLC chromatogram of compound **1**.

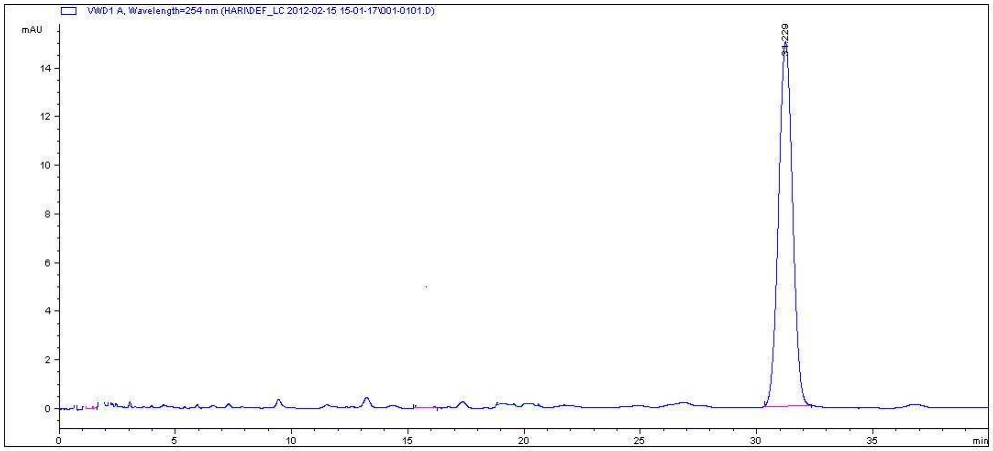

Fig. 32. HPLC chromatogram of compound **2**.

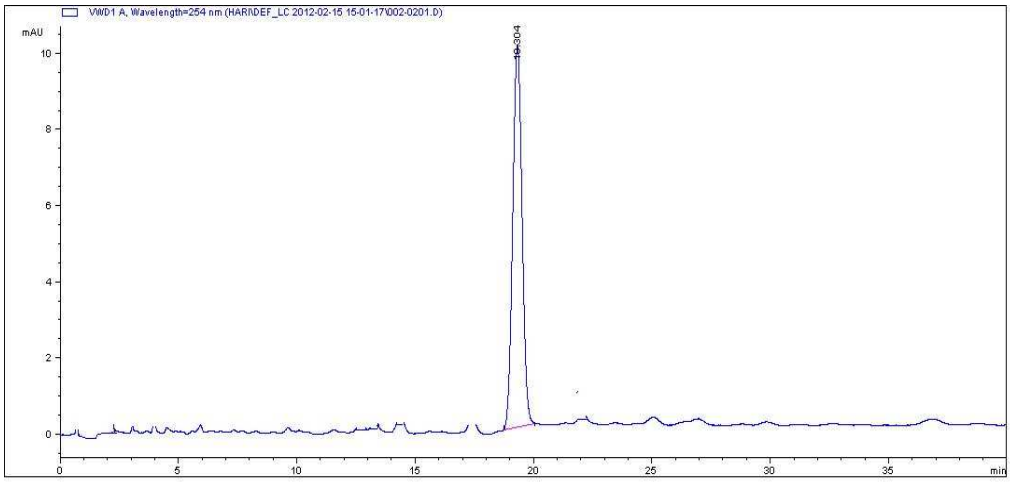

Fig. 33. HPLC chromatogram of compound **3**.

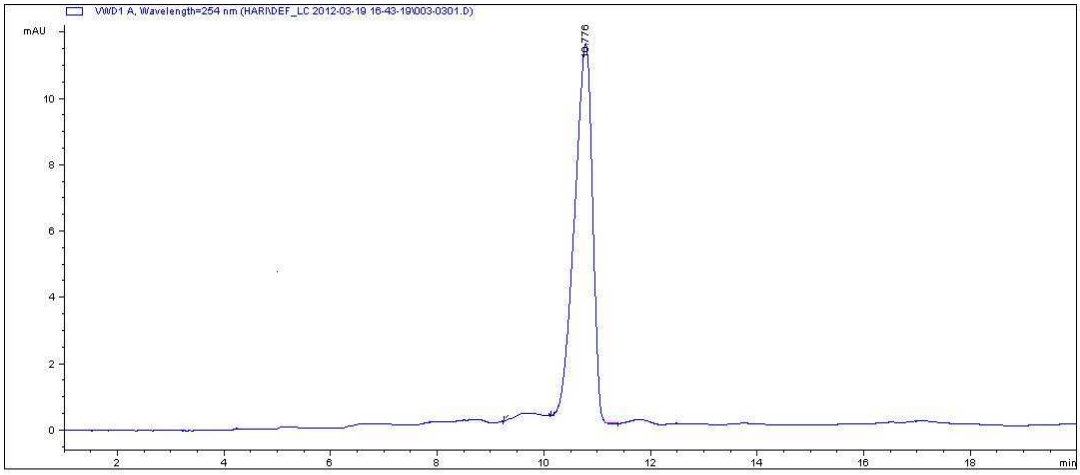

Fig. 34. HPLC chromatogram of compound **4**.

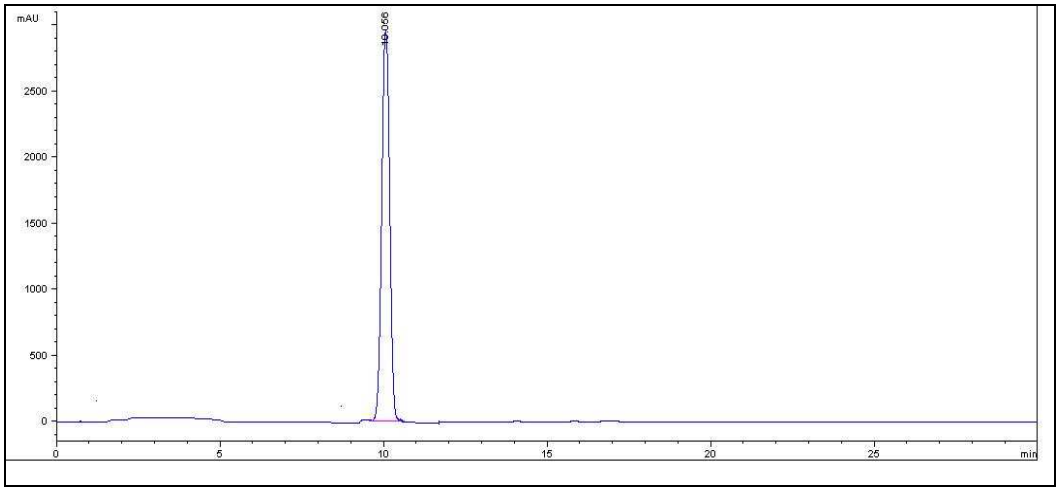

Fig .35. HPLC chromatogram of compound **5**.

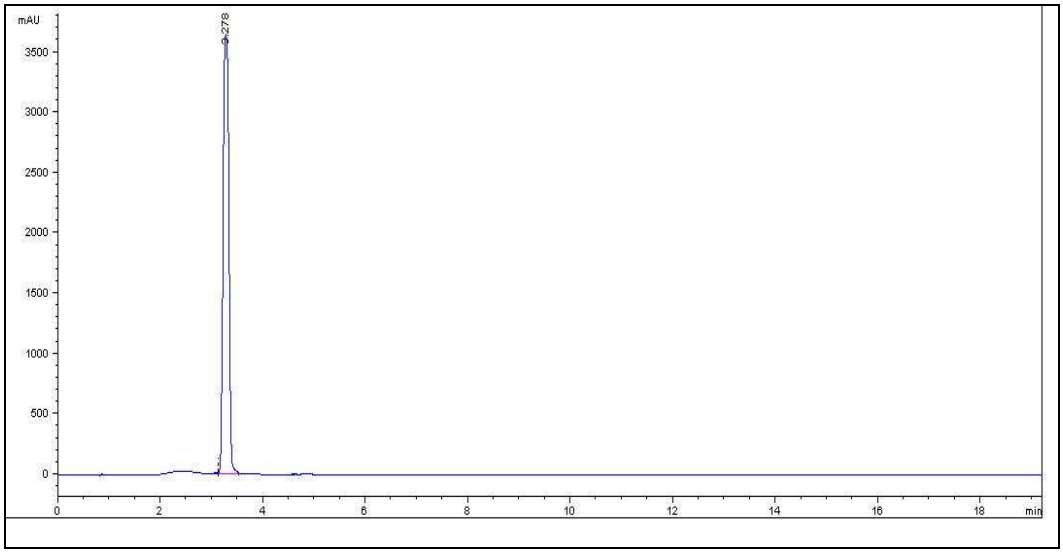

Fig. 36. HPLC chromatogram of compound **6**.

2.6. Steam Distillation

Air-dried seed (500 g) of *M. pinnata* was pulverized and subjected to steam distillation at 100°C for 2 h using a Clevenger-type apparatus. The volatile oil was dried over anhydrous sodium sulfate and stored in a sealed vial at 4°C until use. The yield of the distillate was 1.87% based on dried weight of the plant.

2.7. Gas Chromatography (GC)

A Shimadzu GC 2010 gas chromatograph (Kyoto, Japan), equipped with splitless injector, was used to separate and detect the constituents of *M. pinnata* steam distillate. Analytes were separated with a 30 m × 0.25 mm i.d. (d_f = 0.25 μm) DB−5 MS bonded-phase fused-silica capillary column (J&W Scientific, Ringoes, NJ). The flow velocity of

the helium carrier gas was 1.1 ml/min. The oven temperature was kept at 50°C (5 min isothermal) and programmed to 280°C at a rate of 5°C/min, then isothermal at 280°C for 10 min. The injector temperature was 280°C. The steam distillate constituents were identified by co-elution of authenticated samples following co-injection.

2.8. Gas Chromatography-Mass Spectroscopy (GC-MS)

GC-MS analysis was performed using a Shimadzu GC 2010 gas chromatograph-Shimadzu QP 2010 mass spectrometer. The capillary column and temperature conditions for the GC-MS analysis were the same as described above for GC analysis. Helium carrier gas was used at a column head pressure of 8.89 psi (61.3 kPa). The ion source temperature was 200°C. The interface temperature was kept at 290°C, and mass spectra were obtained at 70 eV. The sector mass analyzer was set to scan from 50 to 650 amu every 0.50 s. Chemical constituents were identified by comparison of mass spectrum (NIST, Version 2.0, 2008).

2.9. Bioassay

A direct-contact mortality bioassay, as stated in Chapter 1 was used to evaluate the toxicity of test materials to third instars from the susceptible mosquitoes. Each compound in acetone (for karanjin) or methanol (for the other compounds) was suspended in distilled water with Triton X–100 (20 μL/L). Groups of 20 mosquito larvae were separately put into paper cups (270 mL) containing each test compound solution (250 mL). The toxicity of each test compound was determined with four to six concentrations ranging from 0.1 to 200 mg/L. Fenthion and temephos served as standard

references and were similarly formulated as the test materials. Controls (i.e. no material or insecticide) consisted of the methanol- or acetone-Triton X–100 solution in distilled water. All treatments were replicated three times using 20 larvae per replicate.Treated and control larvae were held at the same conditions as those used for colony maintenance. Larvae were considered to be dead if they did not move when prodded with fine wooden dowels 24 h posttreatment. Because all bioassays could not be conducted at the same time, treatments were blocked over time with a separate control treatment included in each block. Freshly prepared solutions were used for each block of bioassays (Robertson *et al*., 1992).

2.10. Data Analysis

Concentration-mortality data were subjected to probit analysis (SAS Institute, 2004). The LC_{50} values for each species and their treatments were considered to be significantly different from one another when their 95% confidence limits failed to overlap.

RESULTS

2.1. Chemical Constituents of Indian Beech Seed Steam Distillate

M. pinnata seed steam distillate was composed of eight major constituents by comparison of mass spectral data and by GC with authentic sample co-injection (Fig. 37). The major constituents, karanjin, karanjachromene, and octadecanamide, oleic acid, palmitic acid and elaidic acid comprised 23.5, 18.6, and 6.66% of the steam distillate, respectively. *M. pinnata* seed steam distillate GC-MS identified compounds were confirmed by the co-injection with steam distillate (Fig. 38). As a result of co-injection (Table 25), 8 compounds were identical which is present in the steam distillate of *M. pinnata* seed.

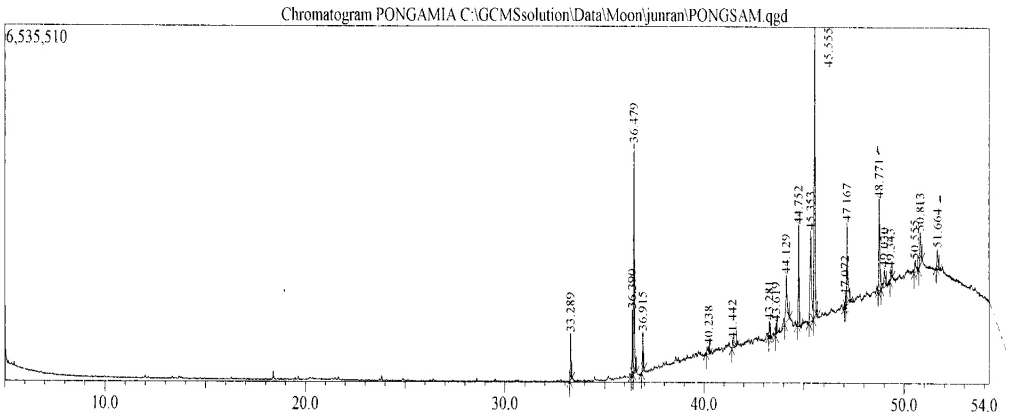

Fig. 37. GC-MS chromatogram of staeam distillate from *Millettia pinnata.*

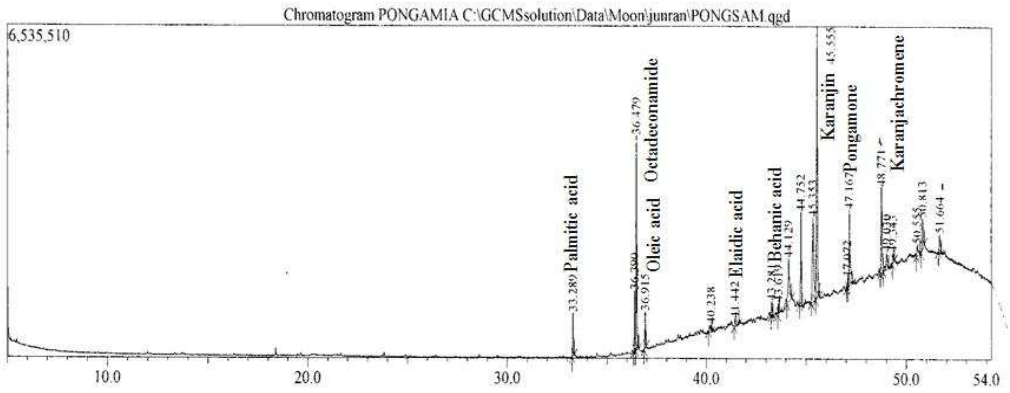

Fig. 38. Co-injection of identified compounds from staeam distillate from *Millettia pinnata*

Table 25. Chemical constituents of *Millettia pinnata* seed steam distillate identified by gas chromatography and gas chromatography-mass spectrometry (GC-MS)

Compound[a]	RT[b] (min)	% Area
Hexadecanoic acid	33.28	3.78
Octadecanamide	36.47	18.6
Oleic acid	36.9	3.18
Elaidic acid	41.44	0.65
Behenic acid	43.21	1.29
Karanjin	45.55	23.5
Pongamone	47.16	6.66
Karanjachromene	48.77	3.33

[a] Tentative identifications from mass spectral data.

[b] Retention time.

2.2. Bioassay-Guided Fractionation and Isolation

Fractions obtained from the methanol extract of Indian beech seed were bioassayed against third instars from *Cx. p. pallens*, *Ae. aegypti*, and *Ae. albopictus* by the direct contact application (Tables 26-28). Significant differences in larvicidal activity in fractions of the extract were observed, and they were used to identify peak activity fractions for the next step in the purification. After 24 h of exposure, the chloroform-soluble fraction was significantly toxic than the hexane- and ethyl acetate-soluble fractions. Weak and no toxicity were produced by the butanol- and water-soluble fractions, respectively. There was no mortality in acetone- or methanol-Triton X–100 solution-treated controls for any of the species in this study.

Table 26. Toxicity of each solvent fraction-derived from methanol extract of Indian beech seeds against third instars from *Culex pipiens pallens* during a 24 h exposure

Material	LC_{50}, mg/L (95% CL[a])	Slope ± SE	χ^2	*P*-value
Methanol extract	24.19 (20.67–28.59)	2.5 ± 0.40	1.28	0.999
Hexane-soluble fr.	19.81(17.35–22.66)	2.9 ± 0.30	8.32	0.821
Chloroform-soluble fr.	12.40(10.39–14.96)	2.2 ± 0.22	5.97	0.946
Ethyl acetate-soluble fr.	73.27 (69.24–78.23)	6.1 ± 0.89	2.93	0.998
Butanol-soluble fr.	95.95 (92.07–100.91)	8.5 ± 1.10	1.35	1.000
Water-soluble fr.	>200			

[a] CL denotes confidence limit.

Table 27. Toxicity of each solvent fraction-derived from methanol extract of Indian beech seeds against third instars from *Aedes aegypti* during a 24 h exposure

Material	LC_{50}, mg/L (95% CL[a])	Slope ± SE	χ^2	*P*-value
Methanol extract	27.70 (24.53–31.82)	3.3 ± 0.46	1.79	0.997
Hexane-soluble fr.	21.42 (18.82–24.51)	2.9 ± 0.31	7.58	0.869
Chloroform-soluble fr.	14.58 (10.64–17.81)	2.0 ± 0.38	6.99	0.998
Ethyl acetate-soluble fr.	75.29 (71.27–80.50)	6.3 ± 0.91	1.89	0.999
Butanol-soluble fr.	97.60 (93.54–103.04)	8.4 ± 1.20	1.96	0.999
Water-soluble fr.	>200			

[a] CL denotes confidence limit.

Table 28. Toxicity of each solvent fraction-derived from methanol extract of Indian beech seeds against third instars from *Aedes albopictus* during a 24 h exposure

Material	LC_{50}, mg/L (95% CL[a])	Slope ± SE	χ^2	*P*-value
Methanol extract	61.30 (54.75–69.79)	3.1 ± 0.42	3.09	0.997
Hexane-soluble fr.	54.70 (50.57–60.10)	5.3 ± 0.68	6.34	0.785
Chloroform-soluble fr.	43.83 (40.01–48.40)	3.9 ± 0.46	4.89	0.997
Ethyl acetate-soluble fr.	77.21 (73.36–82.28)	7.1 ± 0.95	2.60	0.999
Butanol-soluble fr.	134.58 (126.14–146.18)	5.5 ± 0.79	2.04	0.999
Water-soluble fr.	>200			

[a] CL denotes confidence limit.

All the chloroform column fractions were assayed against third instars of *Cx. p. pallens* (Table 29) and *Ae. aegypti* (Table 30). Of six fractions, fraction C22 was most toxic. Similarly, hexane column fractions were assayed against third instars of *Cx. p. pallens* (Table 31) and *Ae. aegypti* (Table 32). Of six fractions, fraction H5 was most toxic.

Table 29. Lethality of chloroform-soluble subfraction-derived from silica gel column chromatography against third instars of *Culex pipiens pallens*

Fraction	Mortality (%) (± SE)		
	Concentration (mg/L)		
	10	25	50
C1	10 ± 0.0cd	25 ± 1.7c	95 ± 1.7c
C21	3 ± 1.7d	17 ± 1.7c	18 ± 1.7d
C22	87 ± 1.7a	98 ± 1.7a	100a
C23	0e	0d	55 ± 2.9b
C24	0e	0d	23 ± 1.7a
C25	0e	0d	0e
C26	0e	0d	0e

Table 30. Lethality of chloroform-soluble subfraction-derived from silica gel column chromatography against third instars of *Aedes aegypti*

Fraction	Mortality (%) (± SE)		
	Concentration (mg/L)		
	10	25	50
C1	0e	17 ± 1.7c	93 ± 3.3c
C21	0e	0d	17 ± 1.7c
C22	82 ±1.7a	95 ± 1.7a	100a
C23	0e	0d	42 ± 4.4b
C24	0e	0d	22 ± 1.7a
C25	0e	0d	0e
C26	0e	0d	0e

Table 31. Lethality of hexane-soluble subfraction-derived from silica gel column chromatography against third instars of *Culex pipiens pallens*

Fraction	Mortality (%) (± SE)		
	Concentration (mg/L)		
	10	25	50
H1	0e	d	17 ± 1.7d
H2	0e	d	75 ± 2.9cd
H3	72 ± 1.7a	93 ± 1.7a	100a
H4	0e	0d	57 ± 1.7
H5	87 ± 1.7a	98 ± 1.7a	100a
H6	0e	0d	0e

Table 32. Lethality of hexane-soluble subfraction-derived from silica gel column chromatography against third instars of *Aedes aegypti*

Fraction	Mortality (%) (± SE)		
	Concentration (mg/L)		
	10	25	50
H1	0e	0d	8 ± 1.7d
H2	0e	0d	72 ± 1.7cd
H3	62 ± 1.7a	92 ± 1.7a	100a
H4	0e	0d	42 ± 1.7
H5	87 ± 1.7a	97 ± 1.7a	100a
H6	0e	0d	0e

Direct-contact mortality bioassay-guided fractionation of *M. pinnata* seed extract afforded six active principles identified by spectroscopic analyses, including MS and NMR. The six active principles were karanjin (**1**), karanjachromene (**2**), (9*Z*)-octadecenoic acid (**3),** hexadecanoic aicd (**4**), pongamol (**5**), and pongarotene (**6**) (Fig. 60).

The compound **1** was obtained as white crystal and identified as karanjin (**1**) by spectroscopic analysis, including EI-MS (Fig. 39), ^{1}H NMR (Fig. 40), ^{13}C NMR (Fig. 41), and DEPT (Fig. 42). The EI-MS revealed a molecular ion at *m*/*z* 292 $[M]^{+}$ and its ^{13}C NMR spectra showed 18 carbons including a methyl group in the molecule suggesting the molecular formula $C_{18}H_{12}O_4$. The interpretations of proton and carbon signals of compound **1** were largely consistent with those of Katekhaye *et al*. (2012).

Karanjin (**1**) was identified on the basis of the following evidence: white crystal; UV (acetone): λ_{max} = 215, 340. EI-MS (70 eV), *m/z* (rel. int.): 292 $[M]^+$ (100), 273 (12), 263 (7), 160 (66), 149 (7), 125 (12), 97 (19), 61 (49). ^{1}H NMR ($CDCl_3$, 600 MHz) and ^{13}C NMR ($CDCl_3$, 150 MHz) are given in Table 33.

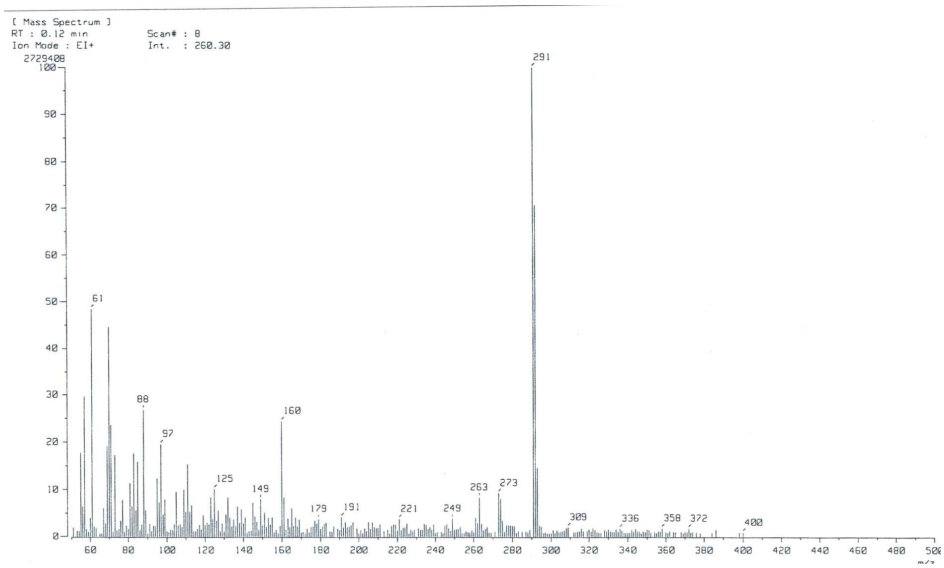

Fig. 39. EI-MS spectrum of compound **1**.

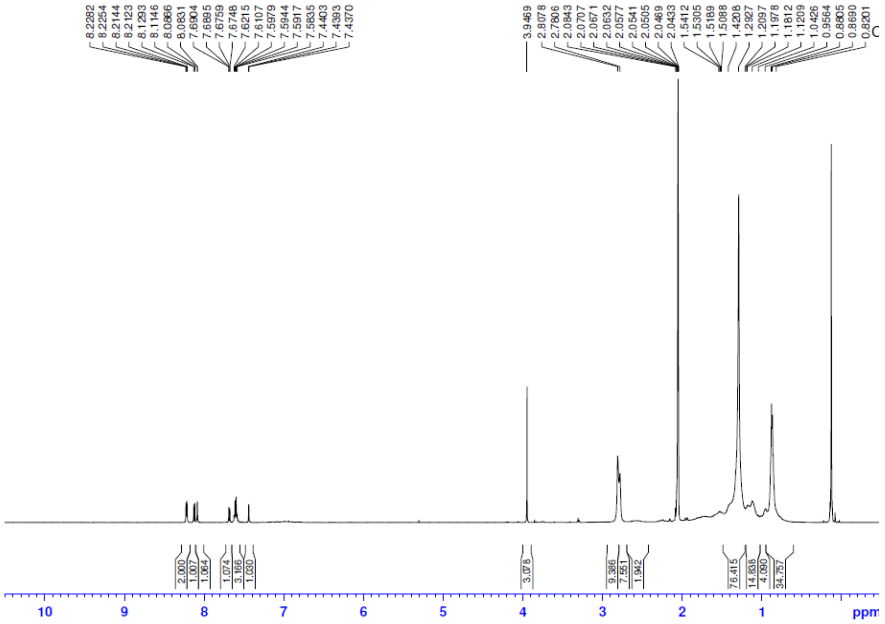

Fig. 40. [1]H NMR spectrum of compound **1**.

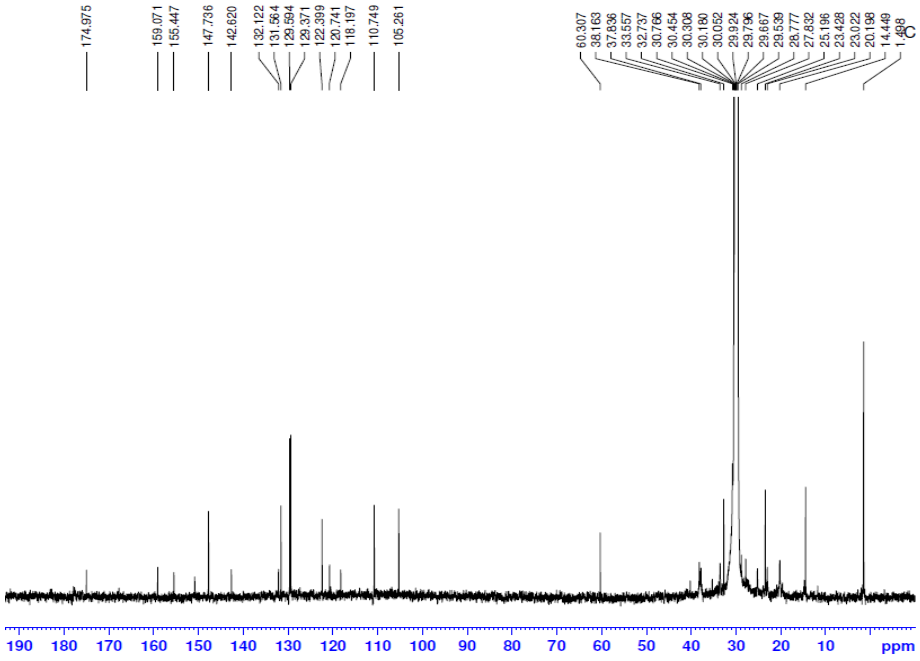

Fig. 41. [13]C NMR spectrum of compound **1**.

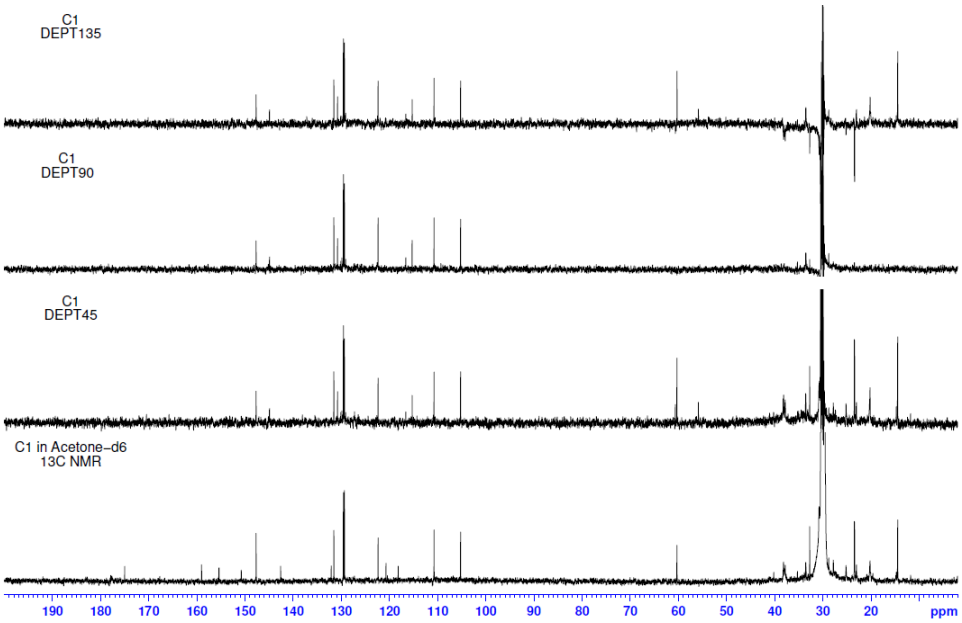

Fig. 42. DEPT spectrum of compound **1**.

Table 33. ^{1}H NMR (600 MHz) and ^{13}C NMR (150 MHz) spectral data of compound **1**

Position	Partial structure	δ_C (ppm)	δ_H (ppm)	δ_C (ppm)	δ_H (ppm) Katekhaye *et al*., 2012
1	C	159.1		158.2	
2	CH	142.6	8.21, m	145.4	8.22, (J = 8.5 Hz)
3	C	118.2		117.0	
4	CH	105.3	7.43, d (J = 1.38 Hz)	104.3	7.18, d (J = 1.0 Hz)
5	C	151.0		149.9	
6	C	155.4		155.0	
7	C	174.9		175.0	
8	C	132.2		130.9	
9	CH	110.7	6.85, d (J = 8.16 Hz)	109.9	7.57, d (J = 8.5 Hz)
10	C	120.7		119.7	
11	C	131.5		141.5	
12	CH	122.4	7.67, d (J = 8.82 Hz)	121.9	7.78, d (J = 2.0 Hz)
13	CH	128.0	7.59, m	128.0	8.17, m
14	CH	127.9	7.59, m	128.0	7.56, m
15	CH	129.3	7.59, m	128.6	7.57, m
16	CH	129.5	8.11 d (J = 8.82 Hz)	128.6	8.13,m
17	CH	130.5	8.08, d (J = 2.10 Hz),	130.6	7.58, m
18	CH3	60.3	3.94, s	60.2	3.93, s

The compound **2** was obtained as white crystal and identified as karanjachromene (**2**) by spectroscopic analysis, including EI-MS (Fig. 43), ^{1}H NMR (Fig. 44), ^{13}C NMR (Fig. 45), and DEPT. The EI-MS revealed a molecular ion at *m*/*z* 334 $[M]^{+}$ and its ^{13}C NMR spectra showed 21 carbons including a methyl group in the molecule suggesting the molecular formula $C_{21}H_{18}O_4$.

Karanjachromene (**2**): white crystal; UV (EtOH): λ_{max} = 260, 320; EI-MS (70 eV), *m*/*z* (rel. int.): 334 $[M]^{+}$ (64), 319 (100), 291 (21), 263 (19), 175 (33), 160 (13), 127 (5), 97 (17), 71 (21). ^{1}H NMR ($CDCl_3$, 600 MHz) and ^{13}C NMR ($CDCl_3$, 150 MHz) are given in Table 34. The interpretations of proton and carbon signals of compound **2** were largely consistent with those of Koysomboon *et al*. (2006).

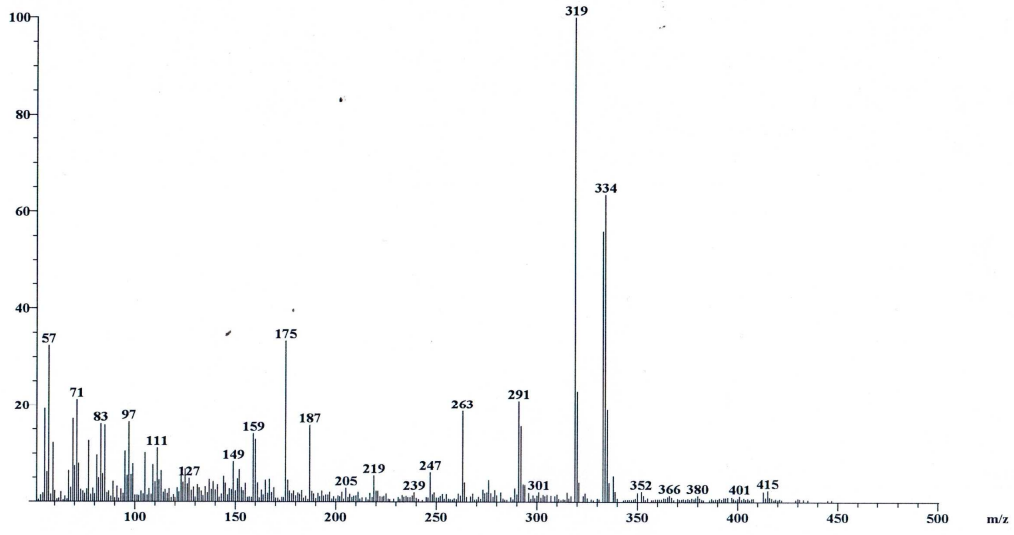

Fig. 43. EI-MS spectrum of compound **2**.

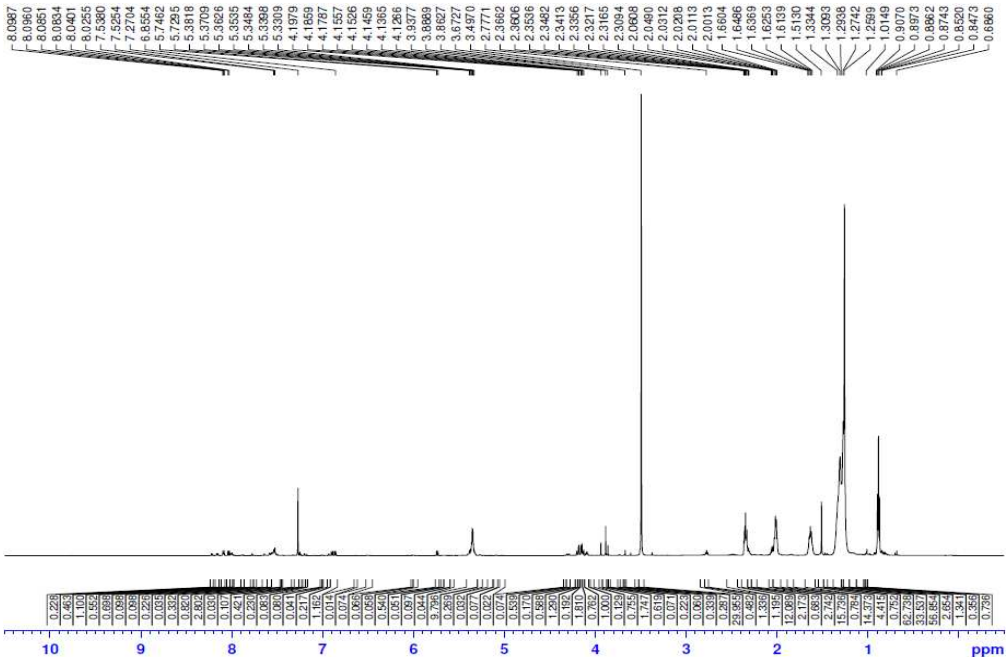

Fig. 44. ^{1}H NMR spectrum of compound **2**.

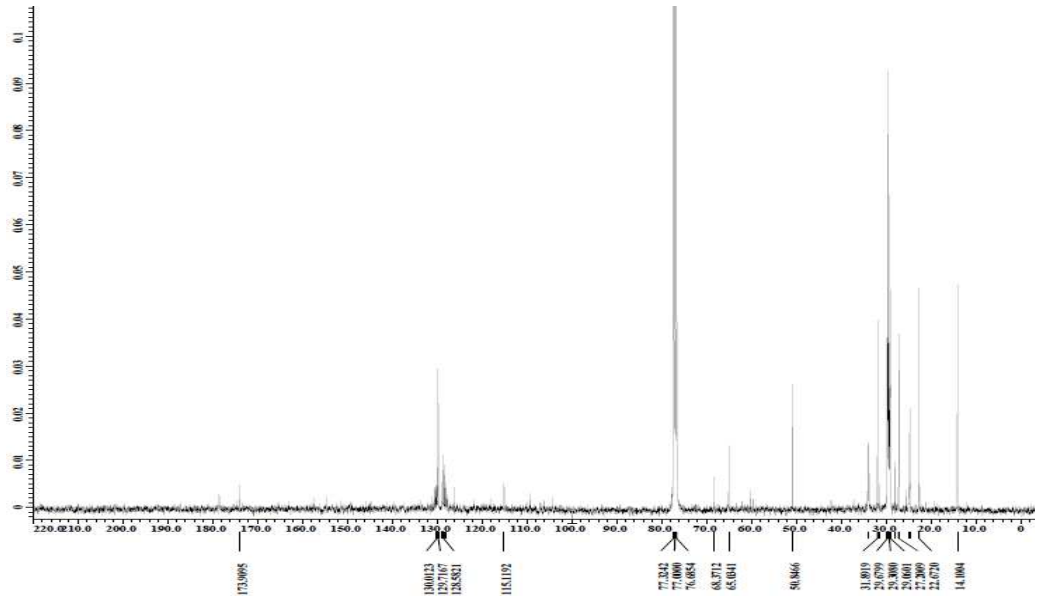

Fig. 45. ^{13}C NMR spectrum of compound **2**.

Table 34. ^{1}H NMR (600 MHz) and ^{13}C NMR (150 MHz) spectral data of compound **2**

Position	Partial structure	δ_C (ppm)	δ_H (ppm)	δ_C (ppm)	δ_H (ppm) Koysomboon *et al*., 2006
1	C	157.3		157.3	
2	C	154.7		151.3	
3	C	157.4		154.6	
4	C	68.4		77.7	
5	C	174.7		174.6	
6	C	151.4		141.2	
7	C	109.1		109.1	
8	C	105.4		109.4	
9	C	131.1		131.1	
10	CH	115.5	5.74, d, (*J* = 11.16 Hz)	115.0	5.74, d (*J* = 5.0 Hz)
11	CH	126.7	7.55, m	125.9	6.89, d (*J* = 5.0 Hz)
12	CH	127.8	8.05, d (*J* = 8.76 Hz)	128.2	8.08, m
13	CH	130.6	7.65, m	131.1	7.63, m
14	CH	118.0	6.85, dd (*J* = 7.56, 10.02 Hz)	117.9	6.86, d (*J* = 8.50 Hz)
15	CH	128.2	8.01, m	128.2	8.03, d (*J* = 8.50 Hz)
16	CH	130.5	7.60, m	130.5	7.63, m
17	CH	130.2	7.60, m	130.2	7.63, m
18	CH	128.3	8.10, d (*J* =6.54 Hz)	128.2	8.08, m
19	CH3	65.0	3.88, s	60.1	3.88, s
20	CH3	22.6	2.05, m	20.8	1.51, s
21	CH3	22.5	1.51, s	20.8	1,51, s

The compound **3** was obtained as white powder or crystal and identified as octadecenoic acid (**3**) by spectroscopic analysis, including EI-MS (Fig. 46), ^{1}H NMR (Fig. 47), ^{13}C NMR (Fig. 48). The EI-MS revealed a molecular ion at *m*/*z* 282 $[M]^{+}$ and its ^{13}C NMR spectra showed 18 long carbonated chain including a hydroxyl group in the molecule suggesting the molecular formula $C_{18}H_{34}O_2$.

Octadecenoic acid (**3**): white crystal; UV (EtOH): λ_{max} = 205; EI-MS (70 eV), *m*/*z* (rel. int.): 282 $[M]^{+}$ (2.5), 264 (13.8), 220 (3.2), 180 (3.5), 111 (21.2), 97 (44.3), 69 (75.7). ^{1}H NMR ($CDCl_3$, 600 MHz) and ^{13}C NMR ($CDCl_3$, 150 MHz) are given in Table 35. The interpretations of proton and carbon signals of compound **3** were largely consistent with those of Yang *et al*. (2010).

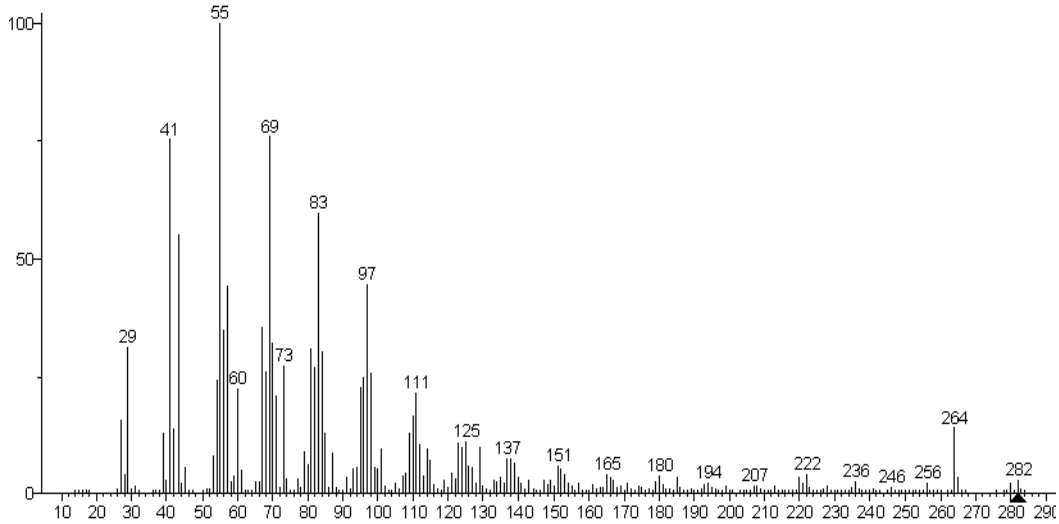

Fig. 46. EI-MS spectrum of compound **3**.

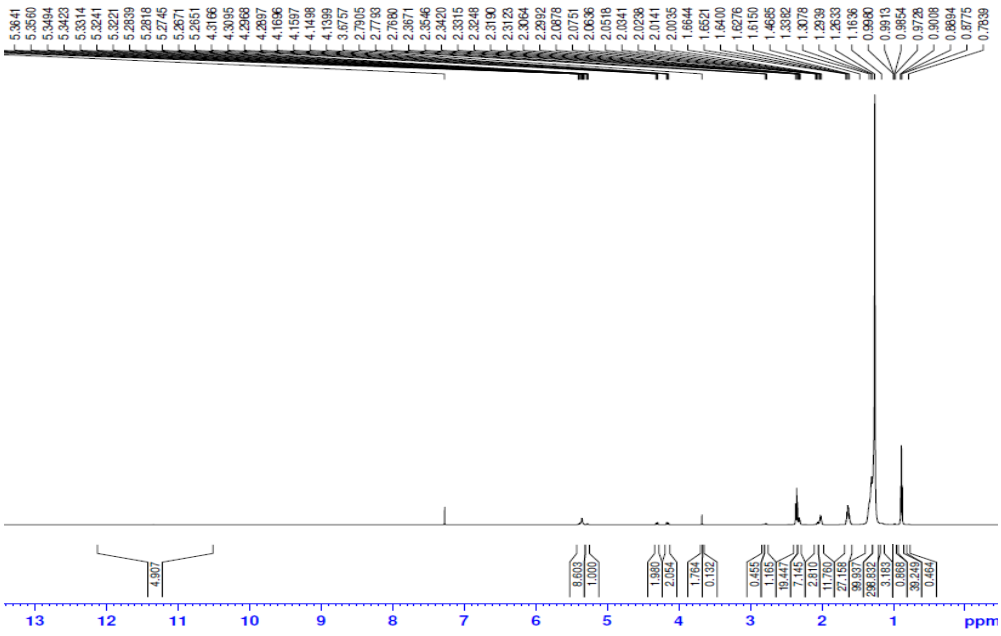

Fig. 47. ^{1}H NMR spectrum of compound **3.**

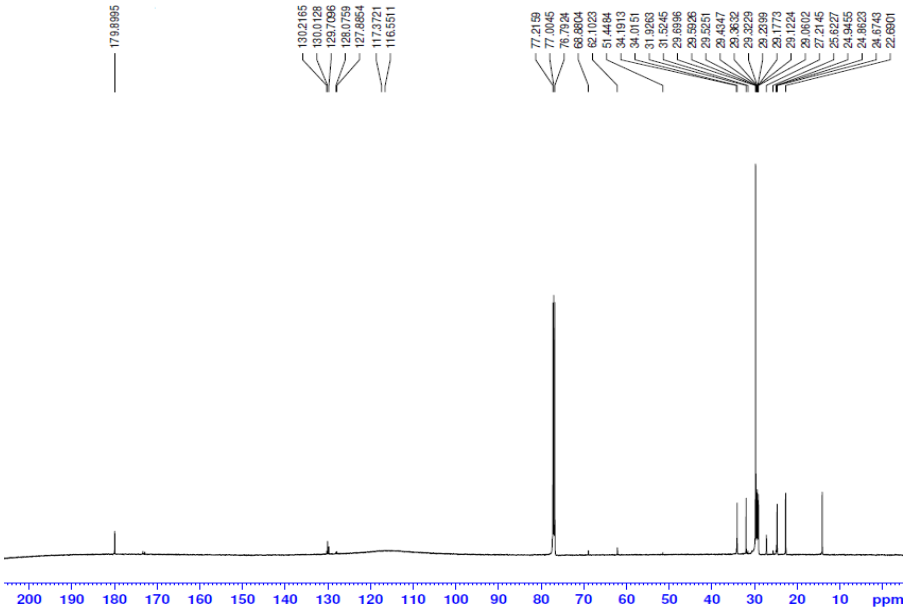

Fig. 48. ^{13}C NMR spectrum of compound **3**.

Table 35. ^{1}H NMR (600 MHz) and ^{13}C NMR (150 MHz) spectral data of compound **3**

Position	Partial structure	δ_C (ppm)	δ_H (ppm)	δ_C (ppm)	δ_H (ppm) Yang *et al*., 2010
1	C	180.6		173.9	
2	CH	130.2	5.29, m	129.8	5.27
3	CH	130.0	5.34, m	129.8	5.34
4	CH2	34.2	2.35, s	34.3	2.50
5	CH2	27.3	2.03, m	27.2	2.02
6	CH2	27.3	2.05, m	27.2	2.04
7	CH2	24.8	1.64, m	24.9	1.96
8	CH2	29.6	1.29, m	29.7	1.29
9	CH2	29.6	1.29, m	29.7	1.29
10	CH2	29.0	1.29, m	29.7	1.29
11	CH2	29.4	1.30, s	29.5	1.31
12	CH2	29.4	1.29, m	29.5	1.28
13	CH2	29.4	1.29, m	29.5	1.28
14	CH2	29.5	1.29, m	29.6	1.28
15	CH2	29.3	1.29, m	29.3	1.28
16	CH2	31.9	1.29, m	34.3	1.28
17	CH2	22.7	1.33, s	22.4	1.34
18	CH3	14.1	0.88, s	14.2	0.90
	OH		11.3		11.4

The compound **4** was obtained as white crystal and identified as hexadecanoic aicd (**4**) by spectroscopic analysis, including EI-MS (Fig. 49), ^{1}H NMR (Fig. 50), ^{13}C NMR (Fig. 51). The EI-MS revealed a molecular ion at *m*/*z* 256 $[M]^{+}$ and its ^{13}C NMR spectra showed 16 long carbonated chain including a hydroxyl group in the molecule suggesting the molecular formula $C_{16}H_{32}O_2$.

Hexadecanoic aicd (**4**): white crystal; UV (Acetone): λ_{max} = 210; EI-MS (70 eV), *m*/*z* (rel. int.): 256 $[M]^{+}$ (100), 241 (4.3), 213 (19.5), 185 (10.3), 129 (32), 97 (20.1), 73 (58.3), 57 (45.7). ^{1}H NMR ($CDCl_3$, 600 MHz) and ^{13}C NMR ($CDCl_3$, 150 MHz) are given in Table 36. The interpretations of proton and carbon signals of compound **4** were largely consistent with those of Laura *et al*. (2000).

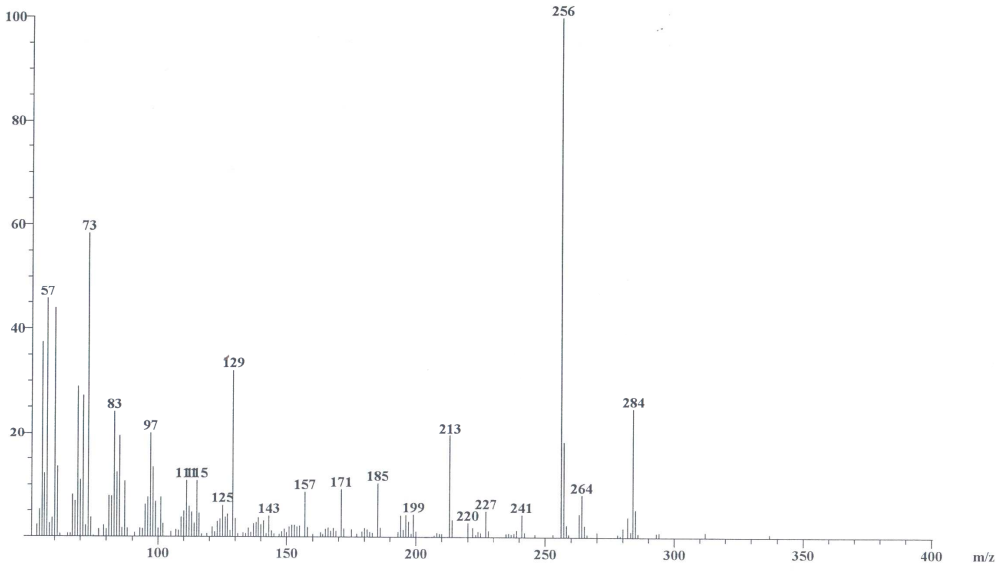

Fig. 49. EI-MS spectrum of compound **4**.

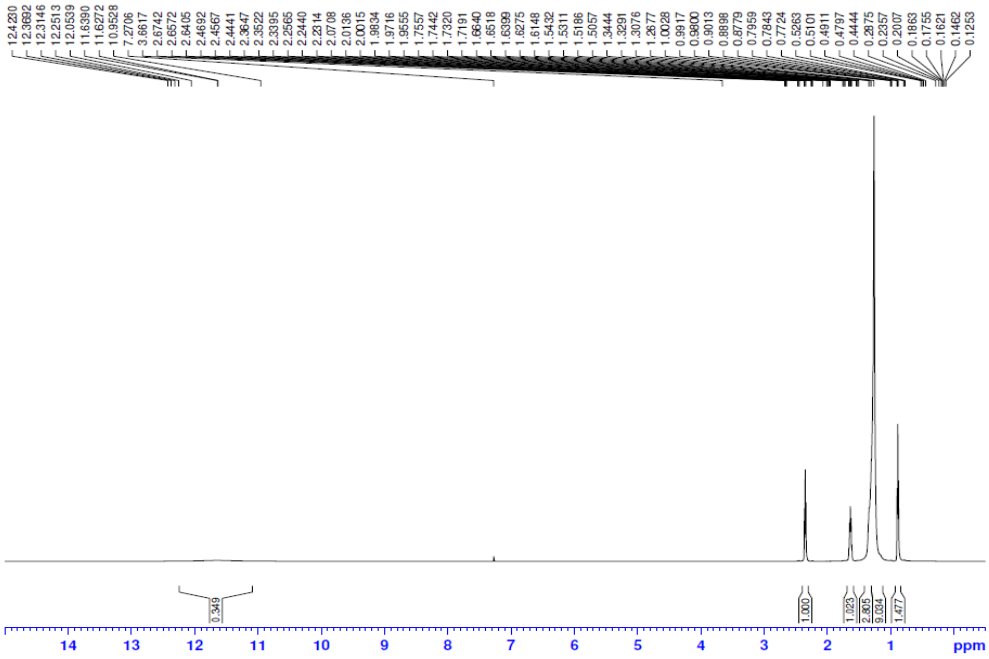

Fig. 50. [1]H-NMR spectrum of compound **4**.

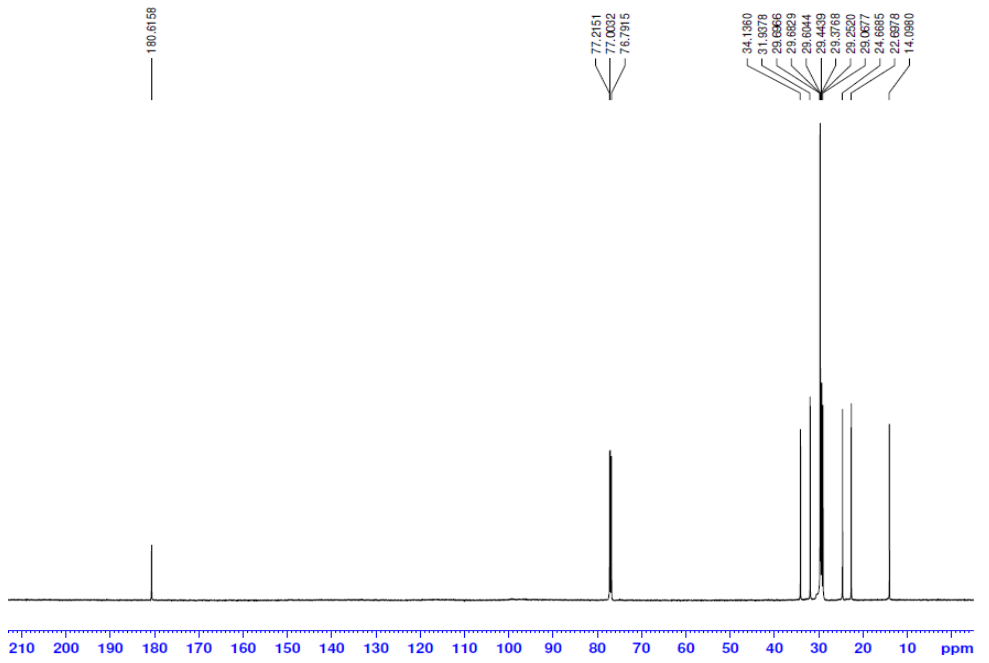

Fig. 51. [13]C-NMR spectrum of compound **4**.

Table 36. ^{1}H NMR (600 MHz) and ^{13}C NMR (150 MHz) spectral data of compound **4**

Position	Partial structure	δ_C (ppm)	δ_H (ppm)	δ_C (ppm)	δ_H (ppm) Laura *et al*., 2000
1	C	179.8			180.1
2	CH2	34.2	2.35, s	2.12	34.7
3	CH2	27.3	2.03, m	2.01	26.4
4	CH2	27.3	2.05, m	2.02	26.5
5	CH2	24.8	1.64, m	1.50	24.9
6	CH2	29.6	1.29, m	1.24	29.2
7	CH2	29.6	1.29, m	1.24	29.2
8	CH2	29.0	1.29, m	1.24	29.2
9	CH2	29.4	1.30, s	1.50	28.9
10	CH2	29.4	1.29, m	1.24	28.9
11	CH2	29.4	1.29, m	1.24	28.9
12	CH2	29.5	1.29, m	1.24	29.5
13	CH2	29.3	1.29, m	1.24	29.3
14	CH2	31.9	1.29, m	1.24	32.4
15	CH2	22.7	1.33, s	1.50	25.3
16	CH3	14.1	0.88, s	0.54	16.5
	OH		11.6		11.4

The compound **5** was obtained as white powder or needle and identified as pongamol (**5**) by spectroscopic analysis, including EI-MS (Fig. 52), ^{1}H NMR (Fig. 53), ^{13}C NMR (Fig. 54), and DEPT (Fig. 55). The EI-MS revealed a molecular ion at *m*/*z* 294 $[M]^{+}$ and its ^{13}C NMR spectra showed 18 carbons including a methyl group in the molecule suggesting the molecular formula $C_{18}H_{14}O_4$.

Pongamol (**5**): white powder or needle; UV (EtOH): λ_{max} = 260, 350. EI-MS (70 eV), *m*/*z* (rel. int.): 294 $[M]^{+}$ (52), 276 (19), 263 (24), 207 (22), 179 (62), 160 (100), 148 (97), 105 (19), 75 (91). ^{1}H NMR ($CDCl_3$, 600 MHz) and ^{13}C NMR ($CDCl_3$, 150 MHz) are given in Table 37. The interpretations of proton and carbon signals of compound **5** were largely consistent with those of Parmar *et al*. (1989).

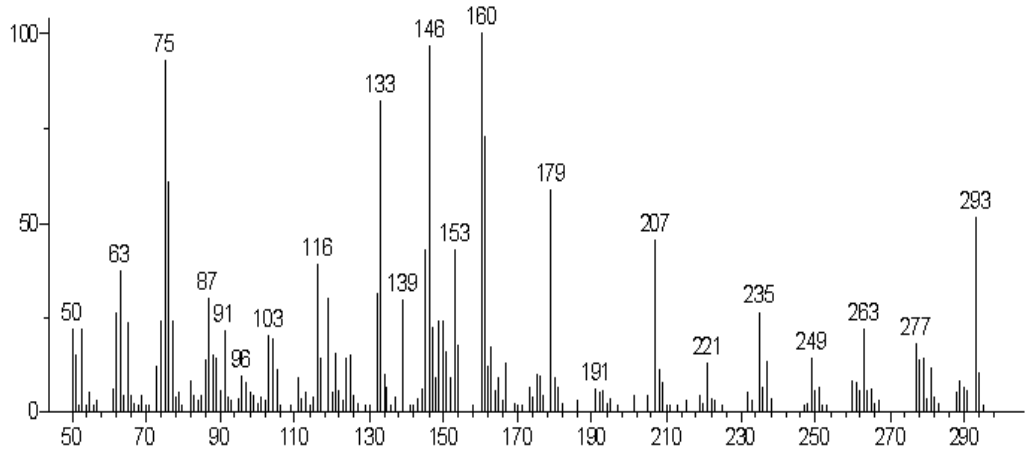

Fig. 52. EI-MS spectrum of compound **5**.

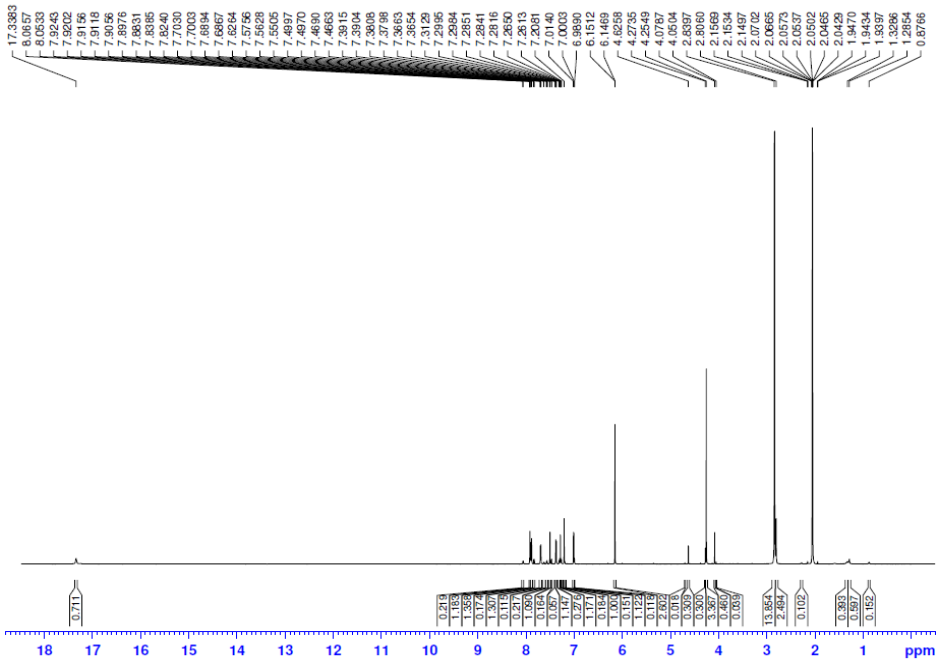

Fig. 53. [1]H NMR spectrum of compound **5**.

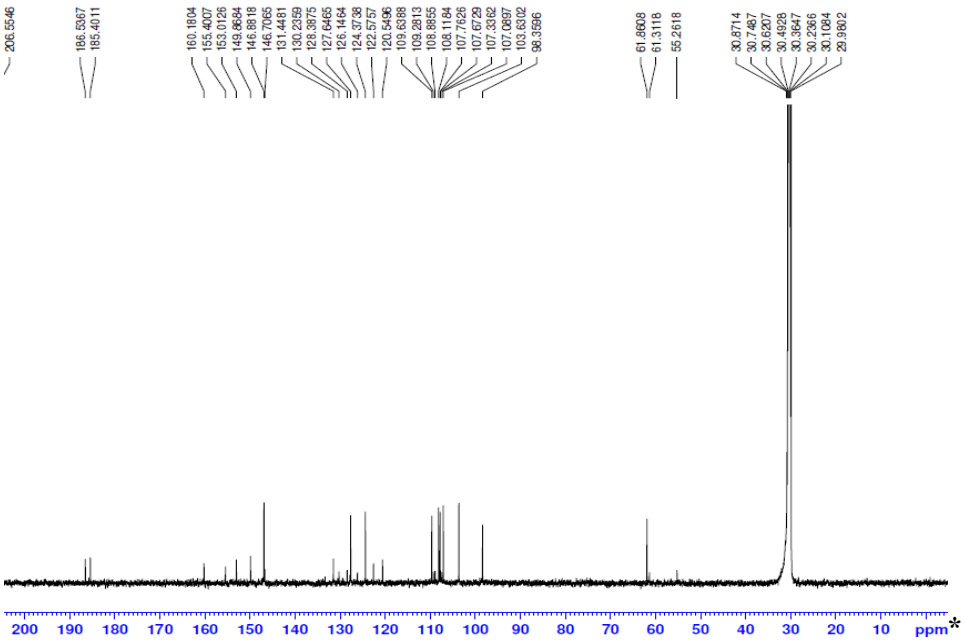

Fig. 54. [13]C NMR spectrum of compound **5**.

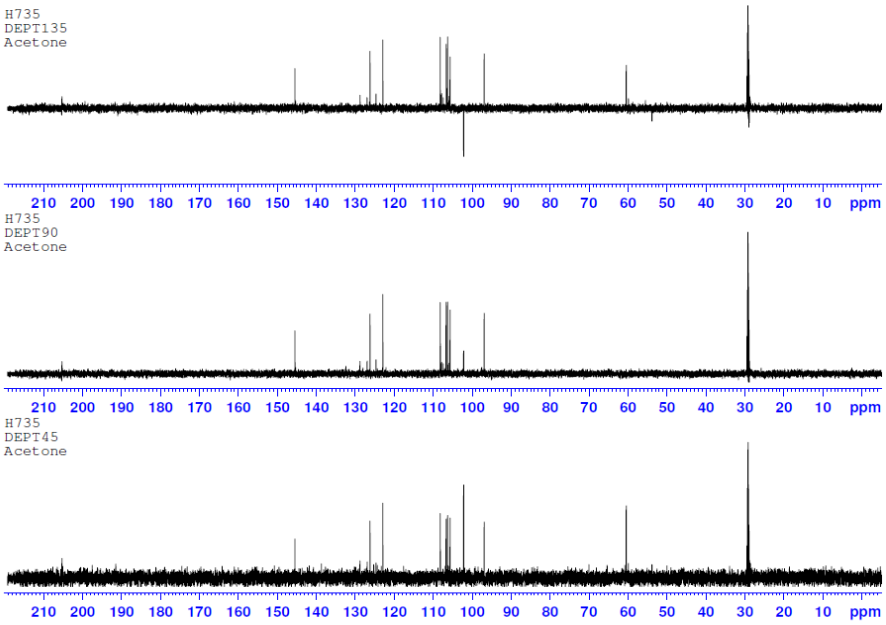

Fig. 55. DEPT spectrum of compound **5**.

Table 37. ^{1}H NMR (600 MHz) and ^{13}C NMR (150 MHz) spectral data of compound **5**

Position	Partial structure	δ_C (ppm)	δ_H (ppm)	δ_C (ppm)	δ_H (ppm) Parmar *et al*., 1989
1	CH	146.8	7.92, m	144.8	7.94, m
2	C	160.1		158.9	
3	CH	107.8	7.01, d (*J* = 8.22 Hz),	119.6	6.96, d (*J* = 2.0 Hz),
4	C	108.1		107.1	
5	C	153.0		152.8	
6	CH	103.6	7.15, s	105.2	7.16, s
7	C	120.5		122.1	
8	C	130.2		135.7	
9	CH	124.3	7.49 d (*J* = 1.62 Hz),	127.1	7.48, m
10	CH	122.5	7.26, d (*J* = 2.22 Hz),	127.1	7.28, d (*J* = 9.0 Hz),
11	CH	127.6	7.36 d (*J* = 0.54 Hz),	128.6	7.48, m
12	CH	127.6	7.36 d (*J* = 0.54 Hz),	128.6	7.48, m
13	CH	128.3	7.85, s	132.0	7.85, d (*J* = 9.0 Hz),
14	CH	126.1	7.68, d (*J* = 6.54 Hz),	126.5	7.60, d (*J* = 2.0 Hz),
15	C	186.5		186.2	
16	C	185.4		184.3	
17	CH3	61.8	4.25, s	61.7	4.12, s
18	CH	98.3	7.68, d (*J* = 6.54 Hz),	97.8	7.94, m
	OH		17.20, s		16.92, s

The compound **6** was obtained as colorless solid and identified as pongarotene (**6**) by spectroscopic analysis, including EI-MS (Fig. 56), ^{1}H NMR (Fig. 57), ^{13}C NMR (Fig. 58), and DEPT (Fig. 59). The EI-MS revealed a molecular ion at *m*/*z* 290 $[M]^{+}$ and its ^{13}C NMR spectra showed 18 carbons in the molecule suggesting the molecular formula $C_{18}H_{10}O_4$.

Pongarotene (**6**): colorless solid; UV (EtOH): λ_{max} = 260, 330. EI-MS (70 eV), *m*/*z* (rel. int.): 290 $[M]^{+}$ (10), 205 (33), 187 (100), 176 (4), 160 (64), 145 (9), 131 (22), 121 (11), 69 (20). ^{1}H NMR ($CDCl_3$, 600 MHz) and ^{13}C NMR ($CDCl_3$, 150 MHz) are given in Table 38. The interpretations of proton and carbon signals of compound **6** were largely consistent with those of Simin *et al*. (2002).

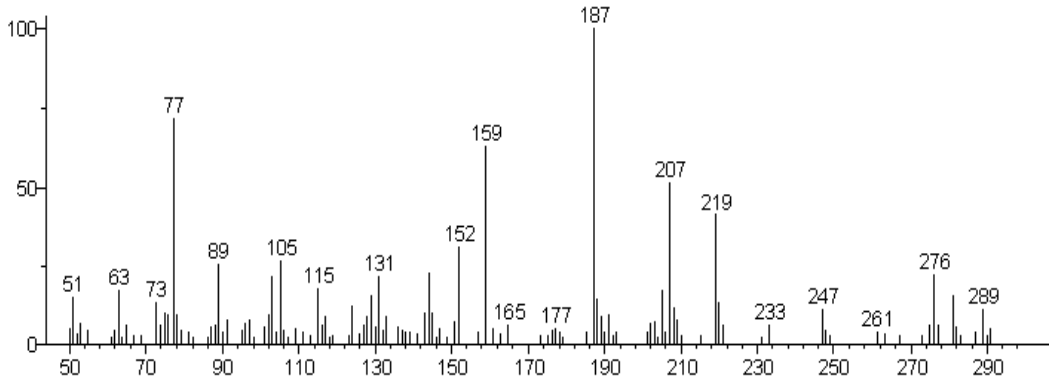

Fig. 56. EI-MS spectrum of compound **6**.

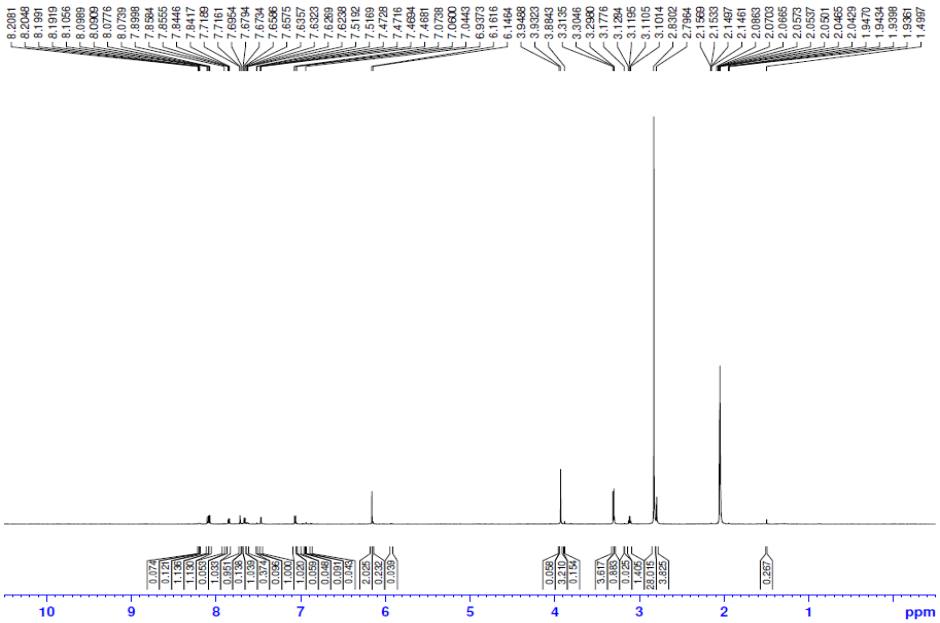

Fig. 57. ^{1}H NMR spectrum of compound **6**.

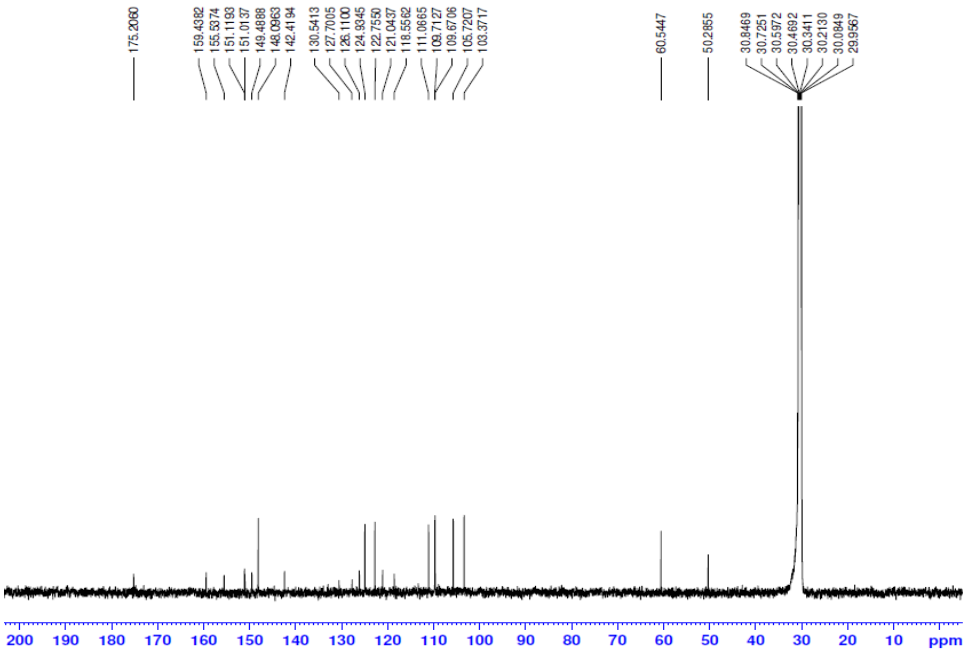

Fig. 58. ^{13}C NMR spectrum of compound **6**.

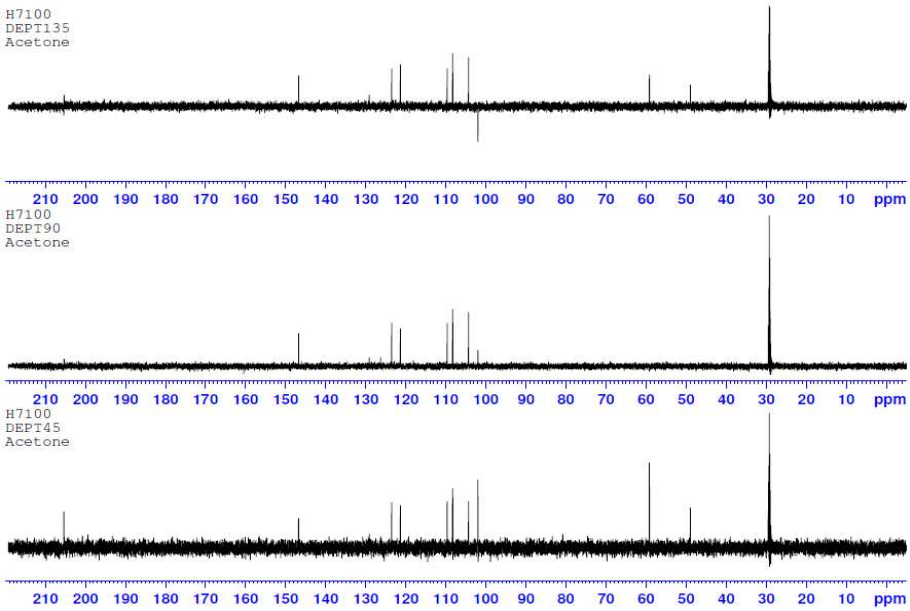

Fig. 59. DEPT spectrum of compound **6**.

Table 38. ^{1}H NMR (600 MHz) and ^{13}C NMR (150 MHz) spectral data of compound **6**

Position	Partial structure	δ_C (ppm)	δ_H (ppm)	δ_C (ppm)	δ_H (ppm) Simin *et al*., 2002
1	C	159.0		157.9	
2	CH	142.4	7.67, d (*J* = 3.6 Hz)	145.7	7.48, m
3	C	111.6		117.4	
4	CH	103.3	7.06, d (*J* = 8.28 Hz)	104.2	7.20, m
5	C	149.4		149.9	
6	C	148.0		146.9	
7	C	155.5		155.8	
8	CH2	60.4	6.16, s	68.4	5.31, s
9	C	175.2		174.7	
10	CH	109.6	7.47, m	109.9	7.48, m
11	C	124.9		124.6	
12	C	118.5		117.0	
13	C	121.1		122.2	
14	CH	126.1	8.20, m	121.6	8.23, d (*J* = 8.80 Hz)
15	CH	119.5	7.74, d (*J* = 1.68 Hz)	119.8	7.75, d (*J* = 2.2 Hz)
16	CH	127.7	7.84, dd (*J* = 1.74, 1.76 Hz)	128.7	7.53, dd (*J* = 8.8, 0.8 Hz)
17	CH	130.5	8.07, dd (*J* = 2.22, 14.5Hz),	131.3	7.89, dd (*J* = 6.3, 2.6 Hz)
18	CH	122.7	7.74, d (*J* = 1.68 Hz)	122.0	7.22, d (*J* = 2.2 Hz)

Structures of isolates from *M. pinnata* seed, karanjin (**1**), karanjachromene (**2**), oleic acid (**3**), hexadecanoic acid (**4**), pongamol (**5**) and pongarotene (**6**) are given in Fig. 60.

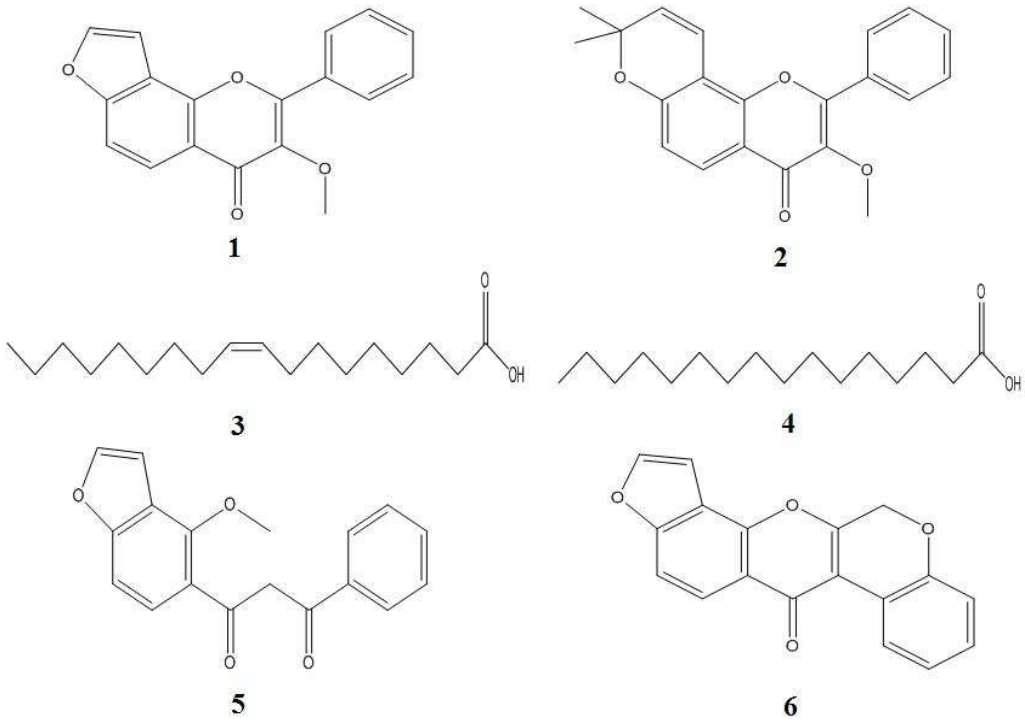

Fig. 60. Structures of isolated compounds from *M. pinnata* seed.

2.3. Larvicidal Activity of Test Compounds

The toxicity of the six isolated flavonoids and temephos to third instars from susceptible *C. p. pallens* was evaluated by a direct-contact mortality bioassay (Table 39). Responses varied according to compound tested. Based on 24 h LC_{50} values, karanjin (**1**) (14.61 mg/L) was the most toxic compound, followed by (9*Z*)-octadecenoic acid (**3**) (18.07 mg/L) and karanjachromene (**2**) (18.74 mg/L). The LC_{50} of pongamol (**5**) and pongarotene (**6**) is 23.95 and 25.52 mg/L. The toxicity of hexadecanoic acid (**4**) was the lowest of any of the constituents examined. Overall, all of the constituents were less toxic than either fenthion or temephos.

Table 39. Toxicity of *Millettia pinnata* seed-derived constituents and two mosquito larvicides to third instars of insecticide-susceptible *Culex pipiens pallens* during a 24h exposure

Compound	LC_{50}, mg/L (95%CL[a])	Slope ± SE	χ^2
Karanjin	14.61 (12.11-.17.81)	2.2±0.30	1.41
Karanjachromene	18.74 (16.25-21.18)	3.3±0.42	1.72
Pongamol	23.95 (20.68-27.34)	2.6±0.33	1.25
Pongarotene	25.52 (22.14-29.16)	2.6±0.34	1.48
Oleic acid	18.07 (15.55-20.52)	3.2±0.41	1.80
Palmitic acid	34.50 (36.36-40.72)	3.6±0.48	1.32
Fenthion	0.0031 (0.0025-0.0037)	1.9±0.17	5.23
Temephos	0.021 (0.017-0.025)	1.2±0.17	4.57

[a] CL denotes confidence limit.

Toxic effects of all compounds on third instars from susceptible *A. aegypti* were likewise compared (Table 40). As judged by 24 h LC_{50} values, karanjin (**1**) (16.13 mg/L) was most toxic and was less toxic than temephos. The LC_{50} of (9*Z*)-octadecenoic acid (**3**), karanjachromene (**2**), and pongamol (**5**) was between 18.45–25.76 mg/L. These constituents were significantly more toxic than pongarotene (**6**) and hexadecanoic acid (**4**).

Table 40. Toxicity of *Millettia pinnata* seed-derived constituents and two mosquito larvicides to third instars from *Aedes aegypti* using direct-contact mortality bioassay during a 24 h exposure

Compound	LC_{50}, mg/L (95% CL[a])	Slope ± SE	SR[b]	χ^2
Karanjin	16.13 (13.61-.18.99)	2.2±0.26	1.1	1.02
Karanjachromene	20.57 (17.69-23.57)	2.9±0.40	1.1	2.03
Pongamol	25.76 (22.44-29.35)	2.7±0.34	1.0	1.48
Pongarotene	37.61 (33.96-41.50)	3.6±0.44	1.5	4.88
Oleic acid	18.45 (15.75-21.08)	3.0±0.40	1.0	1.71
Palmitic acid	42.96 (39.25-46.37)	4.6±0.62	1.2	1.48
Fenthion	0.0043 (0.0035–0.0054)	1.9±0.28	1.4	4.29
Temephos	0.045 (0.040–0.051)	3.5±0.44	2.2	1.96

[a] CL denotes confidence limit.

[b] Susceptibility ratio = LC_{50} of *Ae. aegypti* larvae/LC_{50} of *Cx. p. pallens* larvae.

Against third instars from wild *Ae. albopictus* (Table 41), (9*Z*)-octadecenoic acid (**3**) (24 h LC_{50}, 18.79 mg/L) was the most toxic compound, followed by karanjin (**1**) (35.26 mg/L). The LC_{50} of karanjachromene (**2**), pongamol (**5**), and pongarotene (**6**) was between 52.97–64.97 mg/L. Hexadecanoic acid (**4**) was least toxic. Overall, all the constituents were less toxic than temephos (Table 47).

Table 41. Toxicity of *Millettia pinnata* seed-derived constituents and two mosquito larvicides to third instars from field-collected *Aedes albopictus* using direct-contact mortality bioassay during a 24 h exposure

Compound	LC_{50}, mg/L (95% CL[a])	Slope ± SE	RR[b]	χ^2
Karanjin	35.26 (31.01-39.54)	3.1±0.41	2.4	3.36
Karanjachromene	52.97 (46.52-60.17)	2.7±0.41	2.8	5.45
Pongamol	56.14 (49.50-64.69)	2.8±0.33	2.3	8.42
Pongarotene	64.97 (57.47-72.53)	3.1±0.43	2.5	3.64
Oleic acid	18.79 (16.23-21.33)	3.2±0.41	1.1	2.25
Palmitic acid	85.61 (78.31-93.69)	4.5±0.63	2.5	2.47
Fenthion	0.0048 (0.0040-0.0060)	2.2±0.29	1.5	5.51
Temephos	0.050 (0.044-0.056)	3.8±0.44	2.3	0.48

[a] CL denotes confidence limit.

[b] Resistance ratio = LC_{50} of *Ae. albopictus* larvae/LC_{50} of *Cx. p. pallens* larvae.

2.4. Larvicidal Activity of Steam Distillate Constituents

The toxicity to third instars of *Cx. p. pallens* of *M. pinnata* seed steam distillate and four test compounds was evaluated by comparing the LC_{50} values estimated from the direct contact application (Table 42). Based on 24 h LC_{50} values, elaidic acid ($LC_{50,}$ 28.22 mg/L) was the most toxic compound than octadecanamide ($LC_{50,}$ 30.62 mg/L), arachidonic acid ($LC_{50,}$ 54.91 mg/L) and behenic acid ($LC_{50,}$ 68.76 mg/L) respectively.

Table 42. Toxicity of *Millettia pinnata* seed steam distillate constituents to third instars of insecticide-susceptible *Culex pipiens pallens* during a 24 h exposure

Compound	LC_{50}, mg/L (95%CL[a])	Slope ± SE	χ^2	*P*-value
Elaidic acid	28.22 (25.55-31.09)	3.9±0.42	7.38	0.881
Octadecanamide	30.62 (28.08-33.35)	4.6±0.50	9.22	0.755
Arachidonic acid	54.91(50.05-60.31)	3.9±0.45	13.1	0.439
Behenic acid	68.76 (64.54-73.28)	6.5±0.84	7.42	0.685

[a] CL denotes confidence limit.

The larvicidal activity of *M*. *pinnata* seed steam distillate and four test compounds were observed against third instars of *Ae*. *aegypti* was likewise examined (Table 43). As judged by 24 h LC_{50} values, elaidic acid ($LC_{50,}$ 32.16 mg/L) was the most toxic compound, followed by octadecanamide ($LC_{50,}$ 41.30 mg/L), arachidonic acid ($LC_{50,}$ 60.51 mg/L), and behenic acid ($LC_{50,}$ 86.83 mg/L), respectively.

Table 43. Toxicity of *Millettia pinnata* seed steam distillate constituents to third instars of insecticide-susceptible *Aedes aegypti* during a 24 h exposure

Compound	LC_{50}, mg/L (95%CL[a])	Slope ± SE	χ^2	*P*-value
Elaidic acid	32.16 (29.15-35.62)	3.8±0.44	3.13	0.997
Octadecanamide	41.30 (38.87-43.88)	6.6±0.68	5.12	0.972
Arachidonic acid	60.51 (55.09-67.01)	3.8±0.45	15.4	0.283
Behenic acid	86.83 (81.18-92.83)	6.1 ±1.2	4.07	0.944

[a] CL denotes confidence limit.

Toxic effects on third instars of *Ae*. *albopictus* in direct contact application of *M*. *pinnata* seed steam distillate and four test compounds were observed (Table 44). Based on 24 h LC_{50} values, strong larvicidal activity was observed in elaidic acid ($LC_{50,}$ 66.35 mg/L) followed by octadecanamide ($LC_{50,}$ 69.89 mg/L), arachidonic acid ($LC_{50,}$ 95.01 mg/L), and behenic acid (105.41 mg/L), respectively.

Table 44. Toxicity of *Millettia pinnata* seed steam distillate constituents to third instars of wild *Aedes albopictus* during a 24 h exposure

Compound	LC_{50}, mg/L (95%CL[a])	Slope ± SE	χ^2	*P*-value
Elaidic acid	66.35 (58.70-74.21)	3.1±0.43	3.86	0.992
Octadecanamide	69.89 (61.18-79.52)	2.7±0.42	3.35	0.996
Arachidonic acid	95.01 (86.64-105.01)	3.5±0.38	6.12	0.986
Behenic acid	105.41 (98.0-113.97)	4.9 ±0.59	9.56	0.728

[a] CL denotes confidence limit.

DISCUSSION

Certain plant preparations can be developed into products suitable for integrated vector management because they can be selective, biodegrade to nontoxic products, and have few harmful effects on nontarget organisms and the environment (Sukumar *et al*., 1991; Shaalan *et al*., 2005; Isman, 2006). They also can be used in conjunction with biological control (Isman, 2006). Sukumar *et al*., (1991) has pointed out that the most promising botanical mosquito control agents are plants in the families Asteraceae, Cladophoraceae, Lamiaceae, Meliaceae, Oocystaceae, and Rutaceae. Many plant preparations manifest toxicity to different mosquito species larvae (Sukumar *et al*., 1991; Shaalan *et al*., 2005; Wang *et al*., 2006; Park *et al*., 2002), and have been proposed as potential alternatives to the conventional larvicides. In the lab study, a methanol extract of Indian beech seeds exhibited good larvicidal activity against three mosquito species, although this plant belongs to the family Fabaceae.

Phytochemicals, such as phenolics, terpenoids, and alkaloids, jointly or independently contribute to physiological efficacy such as acute toxicity and developmental disruption against various arthropod species (Ahn *et al*., 1991; Shaalan *et al*., 2005). For example, the isobutylamide alkaloids pellitorine, guineensine, pipercide, and retrofractamide A possess potent toxicity to larvae of *Cx*. *pipiens pallens*, *Ae*. *aegypti*, and *Oc*. *togoi* (Park *et al*., 2002). Indian beech contains abundant flavonoid metabolites such as chromenoflavones, flavones, furanochalcones, furanoflavones, furanoflavonols, and pyranochalcones (Arote *et al*., 2010; Meera *et al*., 2003). In the present study, the larvicidal principles of Indian beech seed were determined to be the

furanoflavonoid karanjin (**1**), the pyranoflavonoid karanjachromene (**2**), the dihydrochalcone flavonoid pongamol (**5**), the rotenoid flavonoid pongarotene (**6**), the unbranched alkenic carboxylic acid (9*Z*)-octadecenoic acid (**3**), and the saturated unbranched carboxylic acid hexadecanoic acid (**4**). Karanjin, karanjachromene, and (9*Z*)-octadecenoic acid were more pronounced in larvicidal activity than pongamol, pongarotene, and hexadecanoic acid. This original finding indicates that the Indian beech seed-derived materials may hold promise for the development of novel and effective mosquito larvicides toward mosquito populations. Karanjin is commercialized as an insecticide/acaricide (20 g/L emulsifiable concentrate) for the control of mites, scales, and chewing and sucking insect pests in a wide range of agricultural crops and ornamentals (Copping and Duke, 2007).

Investigations on the modes of action and the resistance mechanisms of natural insecticidal products are of practical importance for mosquito control because it may give useful information on the most appropriate formulations and delivery means to be adapted for their future commercialization and for future resistance management. The characteristic of insecticide resistance is inherited, and in most cases it has proved to be due to unitary genetic factors (gene alleles) for resistance. The resistance allele may be either recessive (as in certain DDT-resistances), or dominant (as in OP-resistance), or codominant, the resistant susceptible hybrids being intermediate (as in dieldrin-resistance (Brown, 1986). Major mechanisms of resistance to insecticides currently available to control mosquitoes are target site insensitivity that reduces sodium channel sensitivity to pyrethroid insecticides or acetylcholinesterase sensitivity to OP and

In conclusion, the Indian beech seed-derived products containing the flavonoids and carboxylic acids described could be useful as larvicides in the control of mosquito populations. For practical use of the products as novel mosquito larvicides to proceed, further research is needed to establish their human safety, although historically Indian beech has been used as a crude drug for the treatment of tumors, piles, skin diseases, itches, abscess ulcers, rheumatic joints, and diarrhea (Meera *et al*., 2003). In addition, their effects on nontarget aquatic organisms including larvivorous fishes and the aquatic environment need to be established. Lastly, formulations for improving larvicidal potency and stability and for reducing adverse effects also need to be developed.

CHAPTER III

Acetylcholinesterase Inhibition and Histopathological Effects of Phytochemicals on the Midgut Epithelium: Possible Mode of Action against Mosquito Larvae

1. Actylcolineesterase (AChE) Inhibition of Phytochemicals

2. Histopathological Effects of Pellitorine

3.1. Acetylcholinesterase (AChE) Inhibition of Phytochemicals

INTRODUCTION

Informations on the mode of action of synthetic insecticides or naturally occurring insecticides are very important because they could give important clues as to the cause of resistance development in pests, particularly that involving target insensitivity, and thereby helps in designing countermeasures to avoid resistance or reverse the development of resistance. Possessing a different mode of action would make these compounds more valuable for further commercial development as they would be particularly beneficial in areas with documented insecticide resistance. These products also would be novel in that they were developed for vector control. Furthermore, they have the potential of being more readily acceptable by the public because of their origins from natural sources.

Resistance to insecticides in mosquitoes has been documented to occur throughout the world in a variety of important species (Hemingway and Ranson, 2000; Xu *et al*., 2006). With only two primary modes of action available for control of mosquitoes *(i.e.* the gated sodium channel and inhibition of AChE), the development of resistance in an area can critically affect vector control. Finding suitable chemicals with alternative modes of action is of urgent concern among vector control personnel. All of these chemical classes rely on three primary target sites within the insect vector: the gated

sodium channel (pyrethroids and DDT), inhibition of GABA receptors (cyclodienes), and the inhibition of AChE (organophosphates and carbamates) (Ware, 1991).

In this study, AChE inhibitory activity of *A. heterotropides* root- and Indian beech seed-derived constituents against third instars of *Ae. aegypti* was evaluated using a microplate AChE assay.

MATERIALS AND METHODS

3.1 Chemicals and Reagents

A. heterotropides root- and Indian beech seed-derieved constituents and screening of naturally derived product (25 compounds) were used in this study: 3-carene, eugenol, safrole, α-terpineol, toluene, α-pinene, **c**itronellal, myristic acid, bornel, linalool, (-)camphene, γ-terpinene, β-pinene, carvacrol, (±)camphor, 1,8 cineole purchased from Sigma-Aldrich (St.Louis, MO, USA); Other compounds isolated from the plants. Limonene, ρ-cyamene terpinen-4-ol β-caryophyllene α-phellandrene α-thujone menthol limonene oxide thymol undecane purchased from Wako (Osaka, Japan), Acetylthiocholine iodide(ATChI), 5,5'-dithiobio-2-nitro-benzoic acid (DTNB), serine salicylate, tetrabutylammonium phosphate and bovine serum albumin (BSA) were purchased from Sigma-Aldrich (St.Louis, MO).

3.2 Acetylcholinesterase (AChE) Assay

Third instars of *Ae. aegypti* were used in all experiments. Whole bodies of the larvae (14.5 mg fresh weight/1 mL buffer) were homogenized in 2 mL of ice-cold 0.1 M phosphate buffer (pH 8.0) using a Teflon glass tissue homogenizer. After filtering through cheese cloth, the homogenate was centrifuged at 1000 *g* at 4°C for 5 min. The supernatant was used as the AChE preparation. Protein content was determined by the Bradford dye method (1976) using BSA as the standard. Microplate AChE assay was carried out following the method of Hemingway *et al*., (1998) adapted from Ellman *et al*., (1961). The reaction mixture consisted of 50 μL of the crude enzyme preparation,

150 μL of 0.1 M phosphate buffer, 20 μL of 3 mM DTNB in phosphate buffer (pH 7.0), and 1 μL of various concentrations of each test compound in ethanol. The reaction mixture was incubated at 30°C for 5 min and 20 μL of 32 mM ATChI was then added to the mixture. After incubation for 30 min, the reaction was stopped by adding 20 μL of 5 mM eserine salicylate. The absorbance was recorded at 412 nm using a Molecular Devices VersaMax microplate reader (Sunnyvale, CA). All bioassays were repeated three times in triplicates.

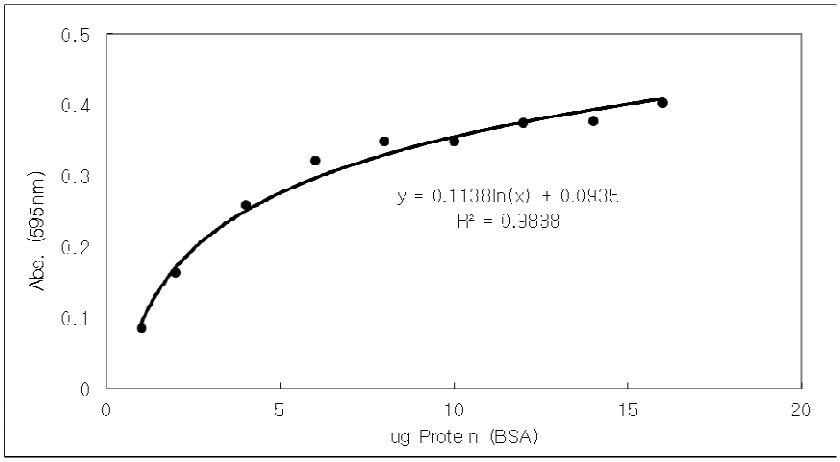

Fig. 61. Protein standard curve.

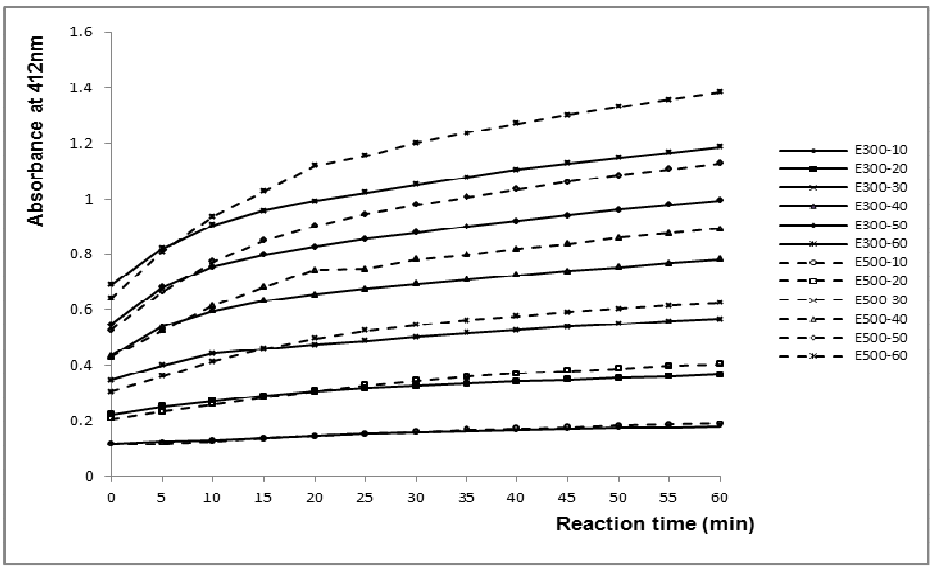

Fig. 62. Reaction intensity of various concentration of AChE enzyme concentration standard curve.

RESULTS

AChE enzyme inhibiton of plant derived natural products was assayed against third instar larvae of *Aedes aegypti*. The compounds from *Asarum heterotropoides* root isolated compounds and the compounds from *Millettia pinnata* seed (Table 45) was assayed against the mosquito larvae. Based on IC_{50} values, strong AChE inhibition was produced by (9*Z*)-octadecenoic acid, pongarotene, pongamol, and karanjachromene (3.3–5.9 mM). Low AChE inhibition was observed with karanjin (IC_{50}, 28.4 mM) and hexadecanoic acid (39.6 mM). But the compounds from *A. heterotropoides* root derived compounds showed low AChE inhibition.

Table 45. Acetylcholinesterase inhibitory activity of isolated principles from *Asarum heterotropoides* and *Millettia pinnata* against third instars of *Aedes aegypti*

Compound	$IC_{50,}$ mM
Pongamol	3.3
Pongarotene	3.9
Palmitic acid	4.2
Karanjachromene	5.9
Karanjin	28.4
Oleic acid	39.6
Asarone	55.2
Pellitorine	52.3
Methyleugenol	62.3
Pentadecane	64.5
Asarinin	75.2

DISCUSSION

Investigations on the modes of action of bioinsecticides may provide useful information for the development of biorational insecticides with novel target sites and for future resistance management (Shaalan *et al*., 2005; Isman, 2006). Certain terpenoids inhibit AChE from housefly and Madagascar roach (Grundy *et al*., 1985) and head louse (Picollo *et al*., 2008). Ryan and Byrne (1988) reported a relationship between insecticidal and electric eel AChE inhibitory activities of terpenoids, whereas no direct correlation between insect toxicity and AChE inhibition by terpenoids was also reported (Grundy *et al*., 1985; Picollo *et al*., 2008). Although not yet proven, the octopaminergic and γ-aminobutyric acid receptors have been also suggested as novel target sites for some essential oil constituents by Kostyukovsky *et al*. (2002) and Priestley *et al*. (2003) respectively. In this study, no correlation was found between contact toxicity and AChE inhibition. The toxicity of pellitorine, asarinin, and asarone from *A. heterotropoides* against *Ae. aegypti* larvae did not differ significantly, but the inhibition of AChE was very low. This result indicates that AChE was not the major site of action for isolates. Likewise the toxicity of karanjin, (9*Z*)-octadecenoic acid, and karanjachromene to *Ae. aegypti* larvae did not differ significantly but karanjin was 8.6- and 4.8-fold less pronounced at inhibiting AChE than (9*Z*)-octadecenoic acid and karanjachromene, respectively. Strong AChE inhibition was also obtained from pongamol and pongarotene, although these constituents were significantly less toxic than karanjin. These results indicate that AChE was not the major site of action for karanjin. Detailed tests are needed to fully understand the exact mode of action of these compounds.

3. 2. Histopathological Effects of Pellitorine

INTRODUCTION

The biological control of immature stages appears to be the most powerful means of reducing mosquito populations. Because of environmental and consumer concerns, the use of nonsynthetic insecticides is becoming more popular, and research at this level has developed in two main directions. On histopathological study in insect response to a variety of toxic substances, the midgut and anal gills is the main target organ for many xenobiotics, which include not only dietary substances from plants (Steinly and Berenbaum, 1985) but also bacterial endotoxins produced by *B. thuringiensis* and *B. sphaericus* (Rey *et al*., 1998a). This is not surprising, owing to the major role of the midgut as well as anal gills in absorption. However, the central question about the direct/indirect involvement of natural products in the midgut pathology remains open because, in Diptera, the permeability to natural products of the peritrophic envelope is not yet established, contrary to the case of Lepidoptera larvae, where the primary determinant of the deleterious effects of tannins is their chemical transformation in the gut (Barbehenn and Martin, 1994).

Many studies have been done, both on the biocidal effects of these insecticides among different taxa (Feitelson *et al*., 1992) and on the pathology of the midgut as the target organ of *B. thuringiensis* (Gill *et al*., 1992) and *B. sphaericus* endotoxins (Davidson and Titus, 1987). However, because of the emergence of resistance to these insecticidal bacteria (Stone *et al*., 1991; Tabashnik, 1994), numerous secondary

compounds from plants are being studied for use as new effective, ecofriendly biocides (Pathak and Dixit, 1988; Chockalingam *et al*., 1990; Govindachari *et al*., 1996; Jayaprakasha *et al*., 1997). Of these phyochemicals, tannins are extremely diverse. Very few studies are peformed on those aspects in mosquito larvae. Rey *et al*. (1999) observed at various concentrations of tannic acid posttreatment intervals in third instars of *Cx. pipiens* clearly indicate variably dramatic degenerative response of the midgut through a sequential epithelial disorganization which may or may not irremediably increase according to the sensitivity of the taxa, as also observed after *B. thuringiensis* intoxication (Rey *et al*., 1998a). In this study, the histopathological effects of pellitorine on *Ae. aegypti* larvae were elucidated.

MATERIALS AND METHODS

3.2.1. Chemicals and Reagents

Pellitorine was isolated from root of *A. heterotropoides*. The pyrethroid insecticide deltamethrin (99% purity) was purchased from Supelco (West Chester, PA). The reagents and chemcials such as glutaraldehyde, osmium tetroxide, sodium cacodylate buffer, propylene oxide, epon or elastic material, ortho periodic acid methylene blue, and uranyl acetate were of reagent-grade quality and available commercially.

3.2.2 Transmission Electron Microscopy (TEM)

The compound pellitorine (5 mg/L) was treated against third instar larvae of *Ae. aegypti* using a direct-contact mortality bioassay, as stated in Chapter 1. After 24 h, both untreated and treated samples were centrifuged at 3000 × *g* at 4°C for 5 min. The bacterial pellet was primarily fixed in Karnovsky's fixative (2 % glutaraldehyde and 2% paraformaldehyde in 0.05 M sodium cacodylate buffer pH 7.2). The samples were incubated at 4°C in darkness for 2–4 h. After fixing, the samples were washed three times with 0.05 M sodium cacodylate buffer at pH 7.2. Osmium tetroxide (1%) was used as a post-fixating agent and the samples were allowed to fix by placing them at 4°C for 2 h. After secondary fixation, they were washed three times with same buffer and distilled water. The samples were then dehydrated using ethyl alcohol in increasing concentrations up to 100% for 15 min. Then samples were dried in a critical point dryer. Finally, they were dried using hexamethyldisilazane (HMDS). Specimens were sectioned on a Leica UC6 ultra-microtome, stained in uranyl acetate and Reynolds lead

citrate and viewed on a Philips CM120 (FEI Co. Eindhoven, Netherlands) at 80 kV. Images were collected on an Olympus-SIS MegaView III (Olympus-SIS Corp., Lakewood, CO) digital camera.

RESULTS AND DISCUSSION

3.2.1. Light Microscopy Observation of Midgut Region

A microscopic picture shows the anterior midgut narrow and then broadens to a tube-like 'anterior midgut' (Am), which represents clear alimentary canal region in untreated thirs instars of *Ae. aegypti* (Fig. 63A). However, complete damage of the gut was occurred after the treatment of pellitorine (Fig. 63B). Fig. 112 shows microscopic picture of the thorax region of third instars of *Ae. aegypti* without (A) or with treatment with pellitorine (B). The figures clearly indicated that damages of the thorax region were occred by the treatment of pellitorine.

Fig. 63. Light microscopic picture of anterior midgut of *Ae. aegypti* larvae without (A) or with treatment with pellitorine (B).

Fig. 64. Light microscopy picture of thorax and anterior midgut of *Ae. aegypti* larvae without (A) or with treatment with pellitorine (B).

3.2.2. Histopathological Structural Region of the Anterior and Posterior Midgut

The isolates compounds patricualry pellitorine showed very good larvicidal activity. At a concentration of 5 mg/L, pellitorine was targeted mainly on midgut epithelium and anal gills, indicating variably dramatic degenerative response of the midgut through a sequential epithelial disorganization. Anterior midgut region of compounds treated cells are shown in Fig. 65 b. The compound was destroyed all clear cells and also degeneration of dark cells, particularly cells were surrounded in the nucleus and cytoplasmic cells which were destroyed completely. After 24 h of treatment, the anterior midgut was almost entirely necrosed, bearing only residues inside the plasma membranes on the basal lamina.

The midgut of dipteran larvae has been subdivided into two different regions, each including one characteristic cell type (Rey *et al*., 1998a). The anterior midgut included tall cells with clear cytoplasm (clear cells: Clements, 1992), extending along one-third of the midgut. Depending on their stage of development, clear cells displayed different degrees of apical swelling into the gut lumen, reducing intercellular contacts with the neighboring cells and degeneration of the nuclei and brush border, as shown, for example, in control anterior midgut larvae of *Ae. aegypti* (Fig. 65 a) were shown clear cells and undamaged. Apical parts of adjacent untreated dark cells are showing long microvilli, dense cytoplasm with mitochondria and polysomes, and normal junctionnal complex.

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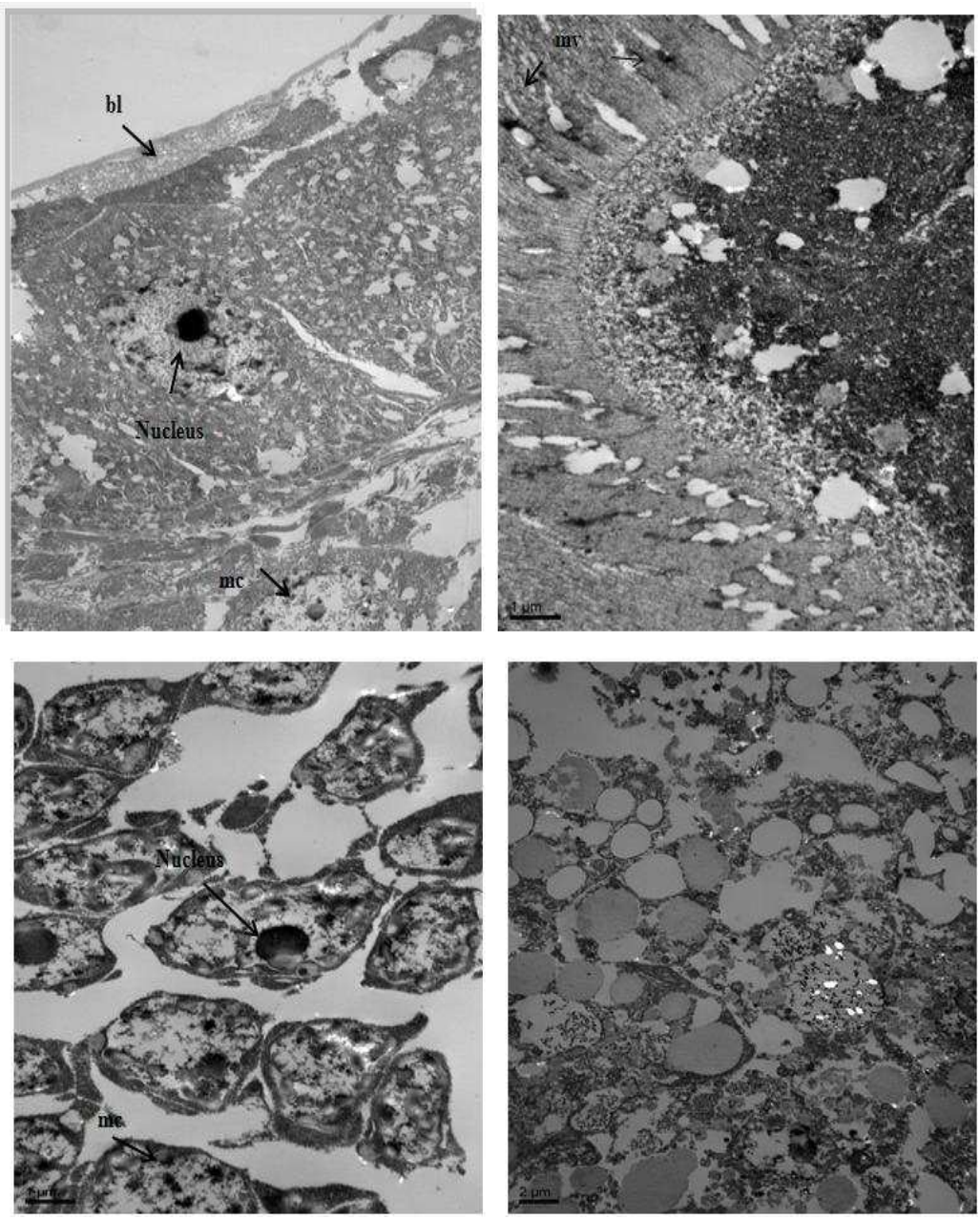

Fig. 65 (a). Anterior mid-gut region of untreated third instars of *Aedes aegypti*.

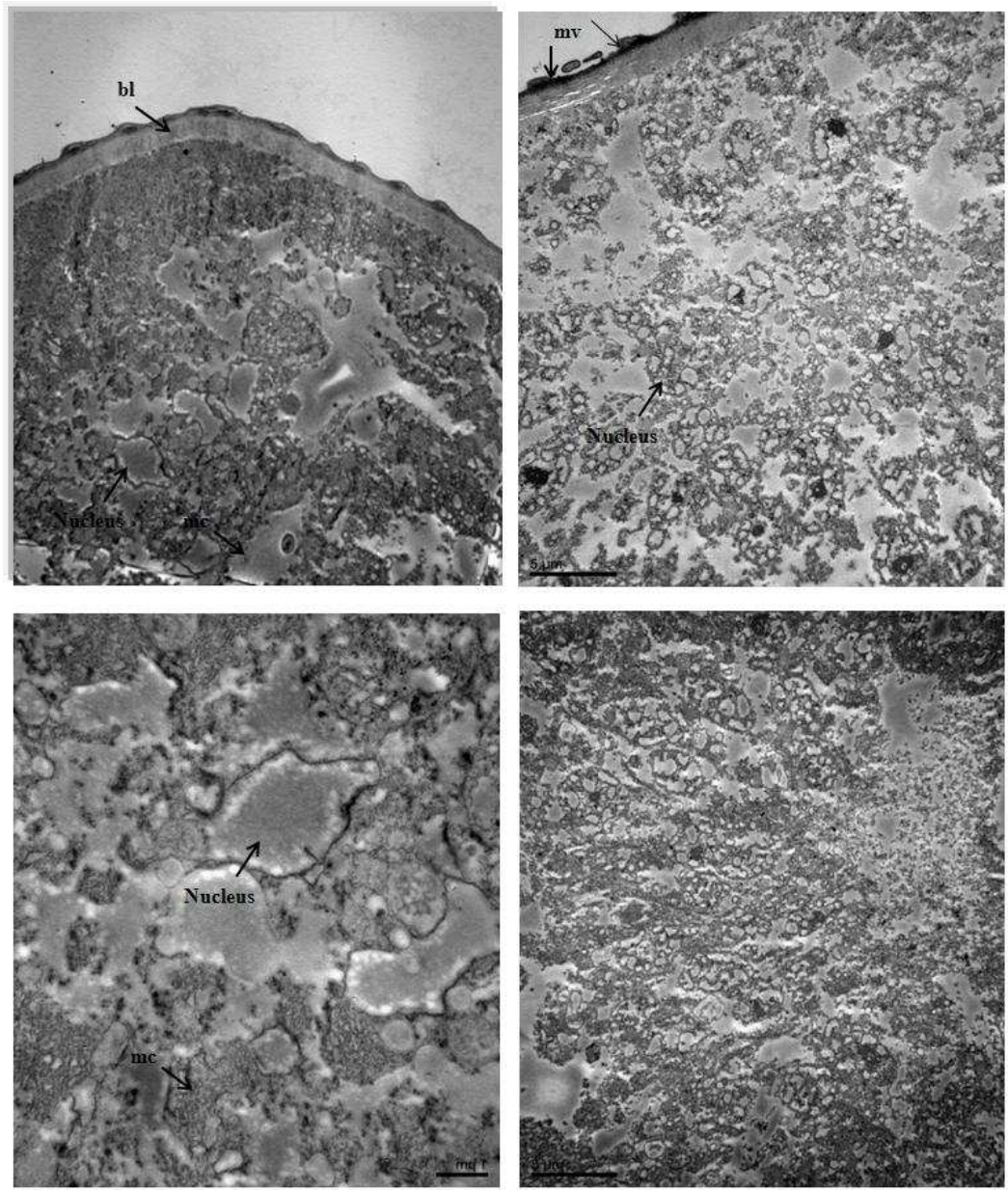

Fig. 65 (b). Anterior mid-gut region of third instars of *Aedes aegypti* treated with pellitorine

The posterior midgut, extending along two-thirds of the gut, was characterized by low epithelial cells with electron-dense cytoplasm (Clements, 1992; Rey *et al*., 1999). Dark cells (polysomes) showed normal intercellular contacts along the whole lateral plasma membranes, lumen, a well-developed brush border, and a normal adhesive basal lamina, as observed in control sections (Fig. 66A). Posterior midgut region of pellitorine-treated cells is shown in Fig. 66B. The compound destroyed all clear cells and also caused degeneration of polysomes cells. In particular, cells of basal lamina and lumen regions were completely destroyed. After 24 h of treatment, the posterior midgut was almost entirely damaged, bearing only residues inseide the intestinal lumen cells.

The region of anal gills showed well cleared sectioned region containing anal grandular cells, which show well developed inside cuticle region (Fig. 67A). After 24 h treatment of pellitorine (Fig. 67B), it has shown complete damage of all grandular cells, because of cuticle damage caused by pellitorine.

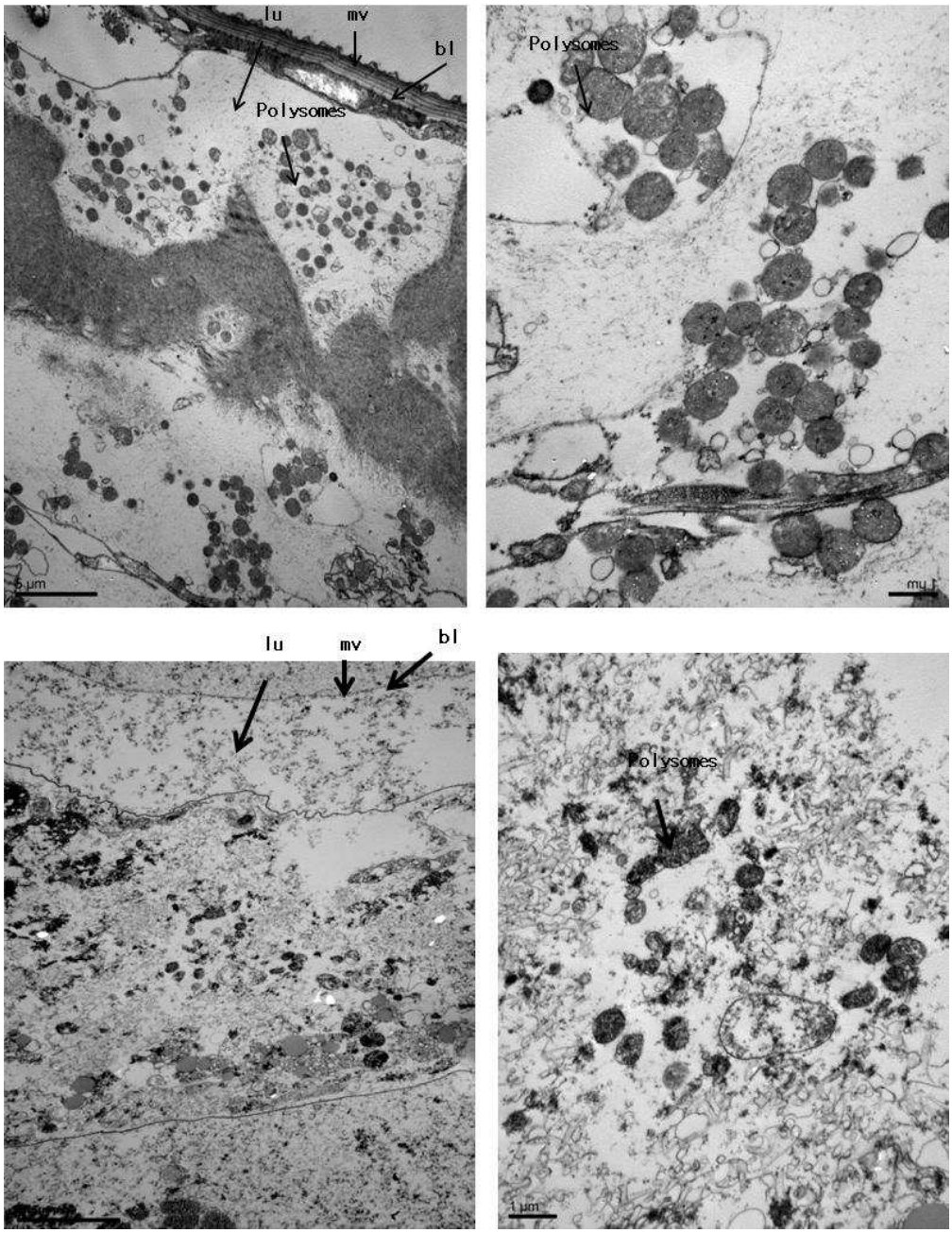

Fig. 66. Posterior mid-gut region of third instars of *Aedes aegypti* without (A) or with treatment with pellitorine (B).

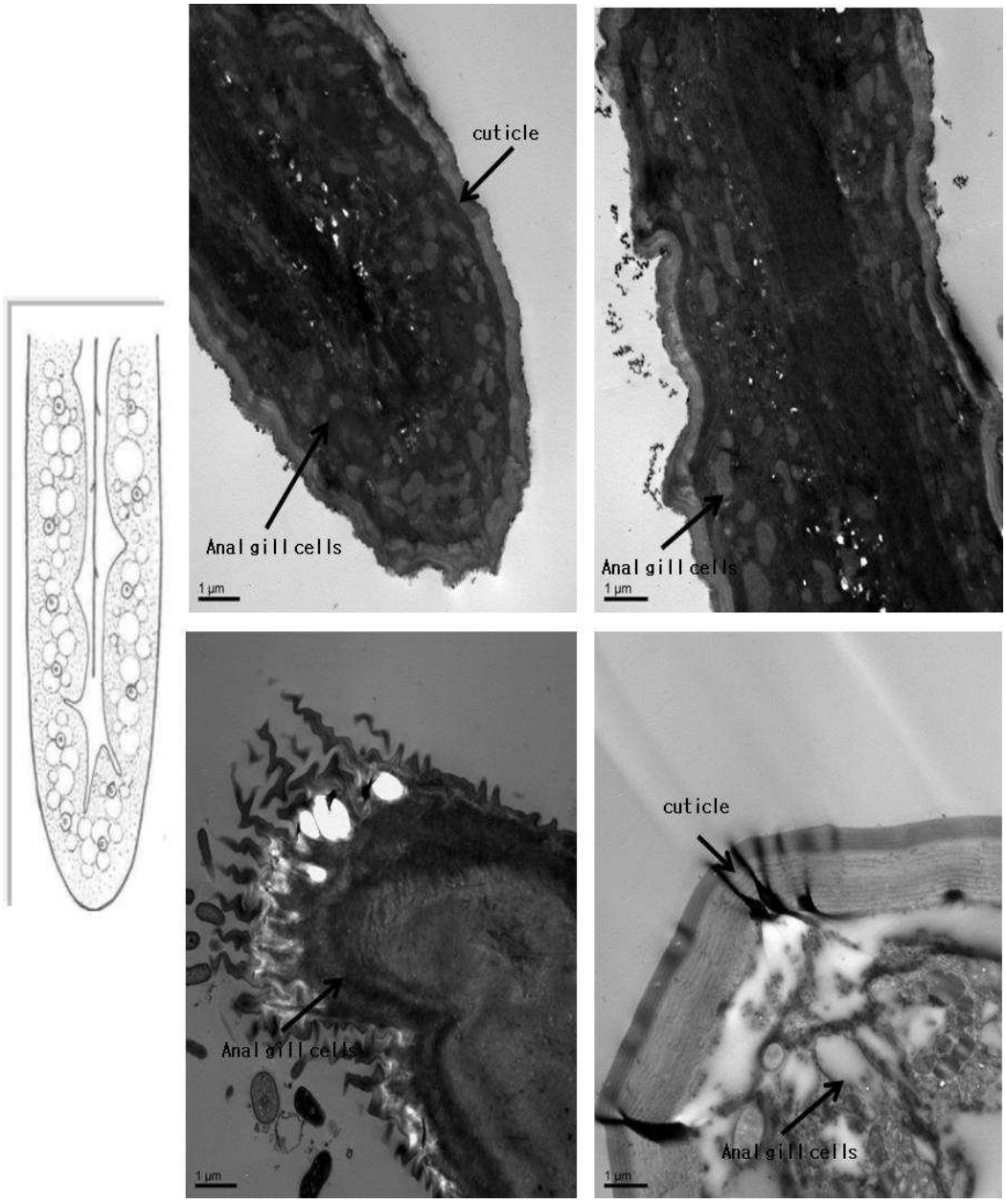

Fig. 67. Anal gills region of third instars of *Aedes aegypti* without (A) or with treatment with pellitorine (B).

In this study, the present observations at pellitorine posttreatment clearly indicate variably dramatic degenerative response of the midgut through a sequential epithelial disorganization which may or may not irremediably increase according to the sensitivity. In the same manner, the differential impact of tannins on the two different regions of the midgut clearly attests to the differences in vulnerability between clear and dark cells, which have been correlated with their morphofunctional status (Rey *et al*., 1998a). Pellitorine showed very strong toxicity and caused dramatic degenerative response in the cell organelles, which indicates pellitorine acts as potential larvicides which helps to improve further study about specific target site and mode of delivery to target insects.

CONCLUSION

The primary screening method for potential larvicides is not universally used before time, funds and energy are expended in detailed examinations of phytochemicals that are useless to vector control from a practical point of view. For the purpose of efficiently testing large numbers of samples within a biodiscovery process, mosquitocidal bioassays may occur at five stages (Shaalan *et al.,* 2005). Stage 1 screening should be undertaken to determine the general potency of a crude botanical extract as a larvicide (dose of 10 mg/L), where 100% mortality is indicative of potential efficacy. Stage 2 screening involves determination of lethal and effective concentrations for 50% of the test population (LC_{50} and EC_{50}) and is carried out using a range of doses to determine the exact efficacy of potential larvicides as acute or chronic toxicants and and the tage 3 screening involves mosquitocidal bioassay guided fractionation to identify highly active fractions and compounds, should only be carried out on the most promising phytochemicals such as those showing an LC_{50} of <1 mg/L or those exhibiting a wide range of combined effects. Stage 4 involves the determination of effective field application rates in simulated field trials and/or small-scale field trials. Stage 5 involves consideration of possible joint-action effects and aims to identify synergistic mixtures that can enhance control activities and minimise the development of insecticide resistance. It quite possible that a less active phytochemical, potentially discarded in the previous stages, could possess exceptional synergistic qualities in combination with other synthetic or natural mosquitocidal agents (Shaalan *et al.,* 2005).

In this study, *A*. *heterotropoides* root steam distillate and its constituents, particularly 3-carene, (+)-limonene, α-phellandrene, (–)-β-pinene, safrole, γ-terpinene, and terpinolene, (LC_{50}, 14.20–19.20 ppm), and *A. heterotropoides* root-derived materials, particularly pellitorine (2.08–2.38 mg/L) and (–)-asarinin (10.49–16.49 mg/L), *M. pinnata* seed-derived materials, particularly karanjin (LC_{50}, 14.26 ppm), karanjachromene (LC_{50}, 18.74 ppm), and oleic acid (LC_{50}, 18.07 ppm) fail to meet the stage 3 criteria (LC_{50}, < 1 mg/L) set by Shaalan *et al*. (2005). However, these constituents showed promise activity against insecticide-resistant mosquito larvae.

In coclusion, *A*. *heterotropoides* and *M. pinnata* derived materials merit further study as potential larvicides for the control of mosquito populations in the light of global efforts to reduce the level of highly toxic synthetic mosquito larvicides in aquatic environments.

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Larvicidal Activity of Constituents Identified in *Asarum heterotropoides* and *Millettia pinnata* against Four Mosquito Species and Their Potential Mode of Action

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국문 초록

모기는 전세계적으로 뎅그열, 황열, 말라리아 사상충증, 다양한 유형의 뇌염 등을 포함하는 열대·아열대성의 심각한 질병들을 매개하는 위생해충으로서 공중 위생보건분야에서 가장 중요하게 생각되고 있다. 모기유충의 방제를 위하여 유기인제, 프리메스린, 기타 살충제, 곤충 성장 조절제, 미생물살충제와 같은 살충제를 사용하고 있으나, 지속적이고 반복적인 사용으로 인하여 자연적인 생물학적 제어 시스템을 저해하고, 저항성 모기의 출현을 야기하였으며, 환경과 비대상 수서생물 및 인간의 건강에 문제가 나타남에 따라 대체 방제제의 탐색 및 개발이 요구되었다. 인간 삶과 질의 향상 그리고 환경문제의 해결을 위하여 기존에 사용되는 화학 살충제를 선택적 방제가 가능하고 자연상태에서 생분해되어 환경에 안전한 대안 살충제로의 대체가 진행되고 있으며, 이러한 대안 살충제는 대부분 식물에서 유래되고 있다.

따라서 현재 모기와 같은 해충의 제어를 위하여 친환경적이고, 비용에 있어서도 효율적인 식물 기반 제품의 사용이 불가피하게 요구된다. 현재 식물의 이차대사산물을 이용하는 제품과 같은 식물체 유래 제품은 잠재적으로 해충방제관리의 대체제로 새롭게 주목을 받고 있다. 본 연구에서는 세신(*Asarum heterotropoides*) 근경 정유와 메탄올 추출물의 구성성분 독성평가와 인도 식물 *Millettia pinnata* (L.) Panigrahi (formerly known as *Pongamia pinnata*)의 종자 유래 물질을 4종의 모기 유충에 대해 각각 생물검정을 실시하였다. 또한 분리된 활성물질을 동정하고, 가능한 메커니즘에 관하여 조사하였다.

세신 근경 정유 화합물의 3종의 모기 빨간집모기(*Culex pipiens pallens*), 에집트숲모기(*Aedes aegypti*), 토고숲모기(*Ochlerotatus togoi)* 3령 유충에 대한 독성을 직접-접촉 생물검정법을 이용하여 실시하였다. 결과는 fenthion과 temephos로 비교하였다. 세신 뿌리 정유는 반수치사농도(LC_{50}) 기준으로 (LC_{50}, 21.07–27.64 ppm)의 좋은 활성을 나타내었다. 높은 독성 화합물은 safrole (LC_{50}, 8.22–16.10 ppm), terpinolene (11.85–15.32 ppm), γ-terpinene (12.64–17.11 ppm), (–)-β-pinene (12.87–18.03 ppm), (+)-limonene (13.26–24.47 ppm), 3-carene (13.83–19.19 ppm), 그리고 α-phellandrene (13.84–23.08 ppm)이었으며, 비교 대상인 fenthion (LC_{50}, 0.023–0.029), temephos (0.016–0.020)보다는 낮은 독성을 보였다. 이

결과는 세신 근경 정유 및 그 구성 화합물이 잠재적인 모기 유충방제제로 이용될 수 있는 가능성을 시사한다.

세신 근경에서 동정한 (–)-asarinin, α-asarone, methyleugenol, pellitorine, 그리고 pentadecane의 독성평가를 살충제-감수성 빨간집모기[*Culex pipiens pallens* (KS-CP strain)], 에집트숲모기(*Aedes aegypti),* 토고숲모기*(Ochlerotatus togoi)* 그리고 다양한 살충제에 저항성을 가지는 야외 빨간집모기[*Culexx pipiens pallens* (DJ-CP colony)]의 모기 3령기 유충을 이용하여 실시하였으며, 중합효소 연쇄반응을 확인하였다. 결과는 사용되고 있는 모기 유충 살충제 fenthion 그리고 temephos와 비교하였다. 살충제-감수성 빨간집모기[*Culex pipiens pallens* (KS-CP strain)], 에집트숲모기(*Aedes aegypti*), 토고숲모기(*Ochlerotatus togoi*) 모기 3종의 3령기 유충에 대하여 Pellitorine (LC_{50}, 2.08, 2.33, 2.38 ppm)은 (–)-asarinin, α-asarone, 그리고 methyleugenol보다 각각 5.5–25.6배 4.5–24.7배, 그리고 6.9–24.6배 높은 독성을 보였으며, pentadecane은 가장 낮은 독성을 보였다. 전체적으로 모든 화합물은 fenthion과 temephos보다는 낮은 독성을 보였다. 세신 근경 유래물질 중 특히 (–)-asarinin과 pellitorine은 살충제 저항성 모기 개체수 조절과 수생 환경에 노출된 높은 독성의 합성 살충제의 수준을 줄이기 위한 세계적인 노력에 부합하는 추가적인 연구가 필요함을 시사한다.

Pellitorine을 독립적으로 사용하였을 때와 pellitorine을 (–)-asarinin, α-

asarone, methyleugenol, pentadecane와 1:1, 1:2, 1:3, 2:1, 그리고 3:1 비율로 혼합한 이진 화합물로 처리하였을 때 살충제-감수성 빨간집모기(KS-CP strain), 살충제-저항성 빨간집모기(DJ-CP colony) 각각의 3령기 유충에 대한 독성평가를 직접-접촉 생물검정을 통해 실시하였다.

Pellitorine과 asarinin (3:1비율) 이진 혼합물은 KS-CP 유충과 DJ-CP 유충에서 각각 0.95ppm, 1.07ppm으로 상당히 높은 독성을 보였으며, pellitorine [KS-CP (2.08 ppm), DJ-CP(2.33 ppm)]과 asarinin(11.45, 12.61 ppm)을 단독적으로 사용하였을 때보다 독성이 높음을 확인하였다. 기타 비율의 혼합물과 pellitorine은 모두 비슷한 독성을 나타내었다. 공동독성계수(co-toxicity, CC)와 상승 인자(synergistic factor, SF)를 바탕으로 확인하였을 때 3개 비율의 이진 혼합물(1/3, 2/1, 3/1)에서 상승패턴[KS-CP (CC, 250-390/ SF, 1.4-2.2); DJ-CP (CC, 257-279 그리고 SF, 1.1-2.1)]을 확인할 수 있었다. Pellitorine과 (-)-asarinin 이진 혼합물은 살충제 저항성 모기 유충 개체수 조절에 사용할 수 있는 가능성을 보여준다.

인도 식물인 *Millettia pinnata* 종자에서 유래한 활성 물질의 독성 평가를 살충제-감수성 빨간집모기, 에집트숲모기 그리고 야생 흰줄숲모기에 대하여 직접-접촉법 생물검정법을 이용하여 평가하였다. 결과는 모기 방제에 사용되는 fenthion, temephos와 비교하였다. *Millettia pinnata*의 활성물질을 분광분석법을 이용하여 탐색한 결과 karanjin (**1**), karanjachromene (**2**), pongamol (**3**), pongarotene (**4**), 올레산 (**5**), and 팔미트산 (**6**)으로

확인하였다. 24시간 노출한 반수치사농도(LC_{50})를 바탕으로 생물검정 결과 karanjin (14.61 and 16.13 mg/L)이 가장 높은 독성을 보였으며, 다음으로 올레산 (18.07 and 18.45 mg/L) 그리고 karanjachromene (18.74 and 20.57 mg/L) 순으로 높은 활성을 보였다. 에집트숲모기와 빨간집모기에서 이 구성 성분은 fenthion ($LC_{50,}$ 0.0031과 0.0048 mg/L) 또는 temephos (0.021과 0.050 mg/L)보다는 낮은 독성을 보였다. pongamol ($LC_{50,}$ 23.95와 25.76 mg/L), pongarotene (25.52와 37.61 mg/L), 그리고 팔미트산 (34.50 and 42.96 mg/L)은 낮은 독성을 나타내었다. 올레산 ($LC_{50,}$ 18.79 mg/L)은 흰줄숲모기에서 가장 높은 독성을 나타내었으며, 나머지 성분(LC_{50}, 35.26-85.61 mg/L)은 낮은 활성을 나타내었다. *M. pinnata* 종자 유래 활성 물질, 특히 karanjin, karanjachromene, 그리고 올레산의 경우 살충제 저항성 모기 개체 수 조절과 수생 환경에 노출된 높은 독성의 합성 살충제의 수준을 줄이기 위한 세계적인 노력에 부합하는 추가적인 연구가 필요함을 시사한다.

식물유래 물질의 살충메커니즘의 연구는 모기방제를 위해 실제적으로 중요하다. 첫째로 살충메커니즘에 대한 지식은 인간 또는 비대상 생물에 대한 독성을 이해하는데 중요하다. 두번째로 유사한 메커니즘에 작용하는 화합물의 개발에 있어 기반지식으로 활용될 수 있다. 마지막으로 해충 저항성 발현의 원인에 대한 중요한 단서를 제공하는 동시에 해충의 저항성을 줄이거나, 발현을 억제하는 대책에 도움을 줄 수 있다. 따라서 선발된 화합물에 대한 살충메커니즘을 밝히기 위해 아세틸콜린에스터라제 (AChE)의

저해를 조사하고, 조직병리학적으로 화합물 처리시 모기유충 위장의 변화를 관찰하였다.

에집트숲모기 3령기 유충을 이용한 AChE 저해 실험에서 반수저해농도(IC_{50})를 확인하였을 때 분리·확인된 화합물(IC_{50}>×10^{-7})은 ACh 저해를 확인할 수 있었으나, 상용화된 살충제 dichlorvos (DDVP) (IC_{50}>4×10^{-7})보다 강한 저해는 확인할 수 없었다. 조직병리학적인 연구에서는 pellitorine을 처리하였을 때 유충 내의 세포소기관이 관통, 치환 그리고 파괴되는 것을 확인할 수 있었다. 투과전자현미경(Transmission electron microscope, TEM) 사진을 통해 처리 24시간 후 세포소기관의 손상을 확실히 확인할 수 있었다.

주요어: 지하집모기, 에집트숲모기, 흰줄숲모기, 토고숲모기, 식물유래 살충제, 세신, 정유, pellitorine; (−)-asarinin; binary mixture, 상승효과, *Millettia pinnata,* karanjin, karanjachromene, 올레산; 아세틸콜린에스터라제 저해, 조직병리학

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