A THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

Isolation and Identification of Larvicidal Constituents from *Cnidium monnieri* and *Magnolia denudata* against Four Mosquito Species and Their Potential Mode of Action

벌사상자 및 백목련 유래 화합물들의 4종 모기에 대한 살유충활성 과 작용메커니즘

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

The yellow fever mosquito, *Aedes aegypti* and the Asian tiger mosquito, *Aedes albopictus*, the malaria vector mosquito, *Anopheles sinensis*, and the northern house mosquito, *Culex pipiens pallens* mosquitoes transmit dengue fever, malaria, and West Nile virus diseases, respectively. 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 22,000 deaths annually, mostly among children. A recent study calculated that 3.97 billion people are at risk of dengue infection in 128 countries worldwide. From 1999 to 2010, 37,088 cases of human West Nile virus disease (including 16,196 neuroinvasive disease cases) were reported in the United States, resulting in 1,549 deaths. With global warming and increased international travel, a number of mosquitoes are distinctly increasing in incidence with a high occurrence of dengue fever all over the globe. Widespread insecticide resistance has been a major obstacle in the cost-effective integrated mosquito management program. There is a pressing need for the development of selective alternatives for the control of mosquitoes, with novel target sites to establish a rational management strategy and tactics because vaccines for malaria or dengue are not yet

available.

The aim of the study was to assess the contact toxicity of two coumarins from the fruits of *Cnidium monnieri* and 11 structurally related coumarins, the 17 constituents from *Magnolia denudata* seed hydrodistillation (MD-SHD), and honokiol, linoleic acid and palmitic acid from the seeds *M. denudata* to third instar larvae from insecticide-susceptible *Cx. p. pallens* (KS-CP strain) and *Ae. aegypti* as well as a wild *Cx. p. pallens* (YS-CP colony), *Ae. albopictus* and *An. sinensis*, using a direct-contact mortality bioassay. Quantitative structure-activity relationships (QSAR) of the test compounds are also discussed. In addition, the possible mode of larvicidal action of the constituents was elucidated using histologic and biochemical methods.

In this study, a direct-contact mortality bioassay was used to identify the larvicidal constituents of *C. monnieri* fruit. The larvicidal principles were identified as the linear furanocoumarin imperatorin and the simple coumarin osthole. Imperatorin (LC_{50}, 3.14 and 2.88 mg L^{-1}) was 1.9-, 3.7- and 4.2-fold and 2.4-, 4.5- and 4.6-fold more toxic than isopimpinellin, isoimperatorin, and osthole against susceptible *Cx. p. pallens* and *Ae. aegypti* larvae, respectively. Overall, all the compounds were less toxic than the larvicide temephos (0.011 and 0.019 mg L^{-1}). The toxicity of these compounds was virtually identical against larvae from the two *Culex* strains, even though YS-CP larvae were resistant to fenthion (resistance ratio (RR), 390), deltamethrin (RR, 164), cyfluthrin (RR, 14) and temephos (RR, 14). This finding indicates that the coumarins and the insecticides do not share a common mode of action. QSAR indicates that chemical structure and alkoxy substitution and length of the alkoxyl side chain at C8 position are essential for imparting toxicity. The molecular weight, hydrophobic parameter, and molecular refraction are negatively

related to the observed coumarin toxicities.

An assessment is made of the contact toxicity assess of the 17 constituents from MD-SHD to third instar larvae from insecticide-susceptible *Cx. p. pallens* and *Ae. aegypti* as well as wild *Ae. albopictus* and *An. sinensis*. Results were compared with those of the four conventional insecticides fenitrothion, fenthion, temephos, and deltamethrin. The efficacy of four liquid formulations (10, 20, 30 and 50 mg L^{-1} liquids) containing the seed hydrodistillation was compared with that of the commercial larvicide temephos 200 g L^{-1} emulsifiable concentrate (EC) because the larvicide has low toxicity to mammals and aquatic organisms and is less persistent in the environment. 2, 4-Di-*tert*-butylphenol was the most toxic constituent (LC_{50}, 1.98–3.90 mg L^{-1}), followed by linoleic acid (7.19–10.49 mg L^{-1}) towards four mosquito species larvae. High toxicity was also produced by nerolidol, (±)-limonene, α-terpinene and γ-terpinene (LC_{50}, 9.84–36.42 mg L^{-1}). The toxicity of these compounds was virtually identical towards four mosquito species larvae, even though *An. sinensis* larvae were resistant to deltamethrin and temephos. The MS-SHD 50 mg L^{-1} liquid resulted in 92–100% control towards four mosquito species larvae while commercial temephos 200 g L^{-1} emulsifiable concentrate was almost ineffective towards *An. sinensis* larvae (30% mortality).

In *M. denudata* seed, the larvicidal principles were identified as the lignin honokiol and the fatty acids linoleic acid, and palmitic acid. Honokiol (LC_{50}, 6.32, 6.51, 6.13 and 7.37 mg L^{-1}) was the most toxic compound against susceptible *Cx. p. pallens*, *Ae. aegypti*, *Ae. albopictus*, and *An. sinensis* larvae, respectively. The lignin compound was 1–1.2 and 5–14.8 times more toxic than linoleic acid and palmitic acid, respectively. Overall, all the compounds were less toxic than either fenthion or temephos. The toxic effect of honokiol

alone or in combination with linoleic acid and palmitic acid (1:1, 1:2, 1:3, 2:1, and 3:1 ratios) to third instar larvae from insecticide-susceptible and -resistant *Ae. albopictus* and *An. sinensis* was evaluated using a direct-contact mortality bioassay. Binary mixture of honokiol and linoleic acid (2:1 ratio) was significantly more toxic against insecticide-susceptible *Ae. albopictus* (LC_{50}, 2.11 mg L^{-1}) and resistant *An. sinensis* (2.19 mg L^{-1}) than either honokiol or linoleic acid alone. The binary mixture of honokiol and linoleic acid (2:1 ratio) merit further study as potential larvicides for the control of insecticide-resistant mosquito populations.

In acetylcholinesterase (AChE) inhibition assay, no potent inhibition was observed (IC_{50} $>1 \times 10^{-3}$) in isolated and identified compounds imperatorin osthole from *C. monnieri* fruits. However, the selected two linear furanocoumarins (osthole, 7-ethoxycoumarin), two simple coumarins (imperatorin, isoimperatorin), one angular furanocoumarins (angelicin) were caused decrease in cAMP levels, indicating that the mechanism of insecticidal action of these coumarins might be due to interference with the octopaminergic system. Honokiol and linoleic acid, with good AChE inhibitory activity against third instar larvae of *Ae. aegypti* have been sought out from the seed of *M. denudata*, and have been proved to have potential for development of natural insecticides. In histopathological study, osthole and imperatorin were shown, compound penetration and delocalized or demolished of internal cell organelles of larval body. Transmission electron microscope (TEM) pictures clearly indicated that damaged organelles after 24 hours of LC_{50} treatment.

C. monnieri fruit- and *M. denudata* seed-derived materials merit further study as potential mosquito larvicides for the control of insecticide-resistant mosquito populations in light of global efforts to reduce the level of highly toxic synthetic insecticides in the

aquatic environment.

Key words: Insecticide, mosquito, *Culex pipiens pallens*, *Anopheles sinensis*, *Aedes aegypti*, *Aedes albopictus*, botanical insecticide, essential oil, binary mixture, synergy, *Magnolia denudate* seed, *Cnidium monnieri* fruit, acetylcholinesterase inhibition, adenylate cyclase, octopamine, cyclic AMP immunoassay, histopathology.

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LIST OF ABBREVIATIONS

AChE	Acetylcholinesterase
CC	Co-toxicity coefficient
DEPT	Distortionless enhanced by polarization transfer
EI-MS	Electron impact-mass spectroscopy
GC	Gas chromatography
GC-MS	Gas chromatography-mass spectroscopy
HPLC	High performance liquid chromatography
NMR	Nuclear magnetic resonance
OPs	Organophosphorus insecticides
PCR	Polymerase chain reaction
RH	Relative humidity
ROK	Republic of Korea
RR	Resistance ratio
SF	Synergistic factor
TEM	Transmission electron microscope
TMS	Tetramethylsilane
TLC	Thin layer chromatography
WHO	World Health Organization

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INTRODUCTION

Insect-transmitted disease remains a major source of illness and death worldwide. Mosquitoes alone transmit diseases to more than 700 million persons annually (Taubes, 1997). The northern house mosquito, *Culex pipiens pallens* (Coquillett), the yellow fever mosquito, *Aedes aegypti* (Linnaeus), the Asian tiger mosquito, *Aedes albopictus* (Skuse), and the malaria vector mosquito, *Anopheles sinensis* (Wiedemann), are widespread, which transmit a number of diseases, such as dengue fever, Japanese encephalitis, filariasis, and malaria that are increasing in prevalence, particularly in tropical and subtropical zones. Malaria ranks amongst the world's most prevalent tropical infectious diseases. Worldwide it causes over a million deaths annually, the majority among African children (WHO, 2008). Lee *et al*., (2007) suggested that *An. kleini*, *An. pullus*, and *An. sinensis* are vectors of malaria found in Korea.

The control of mosquito-borne diseases is the use of insecticides, and many synthetic agents have been developed and employed in the field with considerable success. However, one major drawback with the use of chemical insecticides is that they are non-selective and could be harmful to other organisms in the environment. Due to the unawareness of high dose and continuous insecticide application, particular insecticides might cause development of resistance. 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 use of OP and carbamate insecticides

will likely be reduced in the near future in the United States (US) by the US Environmental Protection Agency (EPA) as re-registration under the 1996 Food Quality and Protection Act (USEPA, 2010).

These problems substantiate the need of the development of selective mosquito control alternatives to establish an efficient resistance management strategy based on all available information on the extent and nature of resistance. Plants, particularly higher plants, have been suggested as alternative sources for arthropod control products 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 on animal and human health, and often act at multiple and novel target sites, thereby reducing the potential of resistance (Sukumar *et al*., 1991; Ahn *et al*., 2006; Isman, 2006). 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 the behavioral efficacy such as repellence and feeding deterrence, and the 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 insecticides 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). In addition, AChE responsible for neurotransmitter degradation at the cholinergic nerve synapse, is the target of both OP and carbamate

insecticides. 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. 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, standard systems are established for evaluation of insecticide resistance of malaria vector mosquito *Anopheles sinensis*, collected from rice paddy fields in Osong (Chungbuk, ROK), and *Culex pipiens pallens*, from paddy fields and cowsheds in Daejeon (Chungnam, ROK). An assessment is made of the contact toxicity of two coumarins from the fruits of *Cnidium monnieri* and 11 structurally related coumarins to third instar larvae from insecticide-susceptible *Cx. p. pallens* (KS-CP strain) and *Aedes aegypti* as well as a wild colony of *Cx. p. pallens* (YS-CP colony) resistant to various insecticides. In addition, another assessment is made of the contact toxicity assess of the 17 constituents from *Magnolia denudata* seed hydrodistillation (MD-SHD) and isolated constituents from methanolic extracts were assayed against larvae of mosquito species. Quantitative structure-activity relationship (QSAR) of the most toxic constituent 2, 4-di-*tert*-butylphenol (DTBP) and its 13 analogues were also discussed. Moreover, synergistic effect of isolated compounds binary mixture from *M. denudata* assayed against larvae from insecticide-susceptible and -resistant *Aedes albopictus* and *An. sinensis*. The possible mode of action and delivery of isolated active principles were also examined.

LITERATURE REVIEW

1. Mosquitoes

Mosquitoes are unquestionably the most important vectors of diseases. There important species groups of mosquitoes worldwide are *Anopheles gambiae*, *Culex pipiens* complexes, and *Aedes* subgenus *Stegomyia*. Two of the *An. gambiae* species are *An. gambiae* and *An. arabiensis* (Mullen *et al*., 2002). Both prefer to bite humans, but *An. gambiae* is more important vector of malaria and lymphatic filariasis because they feed almost exclusively on humans (anthropophilic). *Cx. pipiens* complex is the most ubiquitous group of closely related domestic and peridomestic species. *Culex quinquefasciatus* and *Cx. p. pipiens*, representing the southern and northern mosquito populations, which are ubiquitous in tropical and temperate regions, respectively. The genus *Culex* and the genus *Aedes* are comprised of many related species. The species that this study is most concerned with are *Cx. p. pipiens*, common in the US and ROK known for being a vector of several human pathogens, such as the West Nile virus which causes febrile illness in migratory birds, as well as in humans (Ragona *et al*., 2000). Several brightly marked *Aedes* species are medically important, including the Asian tiger mosquito, *Aedes albopictus*, and mosquito, *Aedes aegypti*. They are the primary vector of both dengue fever and yellow fever viruses (Gubler, 1998). The former was largely confined to Asia and *Ae. aegypti* occurred in tropical and subtropical rural settings. However, *Aedes albopictus* has spread from northern Japan to at least 28 other countries around the world, largely through the international trade in used tires (Reiter *et al*., 1987). In addition, in the southern United States, *Ae. albopictus* has replaced *Ae. aegypti* as the predominant mosquito in artificial containers in suburban

and rural environments (Juliano, 1998). The other genus *Anopheline* mosquito is one of the most important vectors of tropical diseases. *Anopheles* was first introduced as a genus of mosquitoes in 1818 (Meigen *et al*., 1869). All human malaria is transmitted by this genus *Anopheles*. Malaria ranks amongst the world's most prevalent tropical infectious diseases. Worldwide, there were about 198 million cases of malaria in 2013 (with an uncertainty range of 124 million to 283 million) and an estimated 584,000 deaths (with an uncertainty range of 367,000 to 755,000) reported in 2014, the majority among African children (WHO, 2014). The genus *Anopheles* currently includes 467 formally named species that are divided between seven subgenera (Manguin *et al*., 2013; Harbach, 2014).

Eight species of *Anopheline* mosquitoes have been described in the ROK (Rueda *et al*., 2006). *An. sinensis* is known as the most important vivax malaria vector, and plays important roles in malaria transmission (Chai, 1999). There is still some debate regarding the primary vector species for malaria in Korea (Chai, 1999). However, recently, *Anopheles pullus* and *Anopheles 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 the ROK, based on the finding that higher proportions of *An. kleini* and *An. pullus*.

2. Medical importance of mosquitoes

Some of the more common diseases are air-borne, or diseases transmitted through contact, however, some are transmitted through vectors. Vectors are animals that are able to transport a disease from one host to another. The most common vectors are mosquitoes and rodents, which are more commonly found in warm climate areas (Gubler *et al*., 2001).

Mosquitoes are responsible as a pest because of the biting activity and transmission ability to carry and transmit arthropodborne diseases, such as malaria, yellow and dengue fever, encephalitis, and lymphatic filariasis (Becker *et al*., 2010). Due to their multiple blood-sucking behaviors, they are able to acquire the pathogens or parasites in the vector that can be transmitted to people. Since only the female mosquitoes take a blood meal, only female mosquitoes spread these diseases. Once a mosquito becomes infected, it remains so for life, which is normally only a few weeks. During their lifetime, mosquitoes may take several times blood meals. Mosquito bites can also cause skin irritation through an allergic reaction to the mosquito's saliva. Mosquitoes are more important as biological vectors including protozoan diseases, i.e., malaria, filarial diseases such as dog heartworm, and viruses such as dengue, encephalitis and yellow fever (Bonnefoy *et al*., 2008). Among the more than 520 known arthropodborne viruses, which can cause human diseases and have already registered in the International Catalogue of Arthropod-Borne Viruses (Karabatsos, 1985), approximately at least 100 are pathogenic and are associated with humans (Bustamante *et al*., 2010). The majority of arbovirus species are included four families Flaviviridae, Togaviridae, Bunyaviridae, and Rhabdoviridae (Monath, 1988).

Mosquito-borne diseases are important public health problems in most tropical countries. Positive serology has been found in the population for dengue fever (Schwartz *et al*., 2013), West Nile fever (Petersen *et al*., 2013), filariasis (Ichimori *et al.*, 2014), Sindbis (Wang *et al*., 2012), and Chikungunya virus infections (Leparc-Goffart *et al*., 2014). 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 diseases occur due to the direct injection of the saliva 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).

Malaria is one of the most prevalent and infectious human diseases transmitted by mosquitoes. It is the only protozoan disease transmitted by the bite of mosquitoes and it is inoculated by infected *Anopheles spp*. The understanding of mosquito vector biology and ecology derived from field research and laboratory studies was directed toward understanding the bionomics of *Anopheles* vectors and was useful as a determinant for designing adequate measures to control malaria (Gabaldon, 1983). Due to increasing tourism worldwide, an increasing number of sporadic cases are imported in countries outside of endemic areas (Giacomini *et al*., 1997). The parasites multiply inside the red blood cells, which then break open within 48 to 72 hours, 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 symptoms occur in cycles of 48 to 72 hours. Malaria is endemic in 100 countries and territories and affects 300–500 million people with 2.7 million deaths annually (WHO, 1995). 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 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. 86% of the persons 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 (Newman, 2012).

Dengue fever is a mosquito-borne infection found in tropical and subtropical regions around the world. In recent years, transmission has increased predominantly in urban and semi-urban areas and has become a major international public health concern. Typical symptoms include fever, headache, a characteristic skin rash, and muscle and joint pains (Rigau-Pérez *et al*., 1998; Gubler *et al*., 2014). Severe dengue fever can result in seizures, abnormal bleeding, and shock (Clark *et al*., 2015). Dengue viruses (family 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) (Gubler *et al*., 1995). The incidence of dengue has grown dramatically around the world in recent decades. Over 2.5 billion people, over 40% of the population of the world, are now at the risk of dengue (WHO, 2012). WHO (2012) currently estimates there may be 50–100 million dengue infections are recorded worldwide every year. Cases across the Americas, Southeast 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 (Murugananthan *et al*., 2014). 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 has been conducted in Connecticut since the virus was first detected 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 (family Flaviviridae) is one of the most widely spread arboviruses in Africa and Asia. The vectors are mosquitoes of the genus *Culex* (*Cx. pipiens*, *Cx. restuans*, *Cx. salinarius*, *Cx. quinquefasciatus*, and *Cx. 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. West Nile 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 West Nile has expanded from the Old World into the Americas, resulting in disease outbreaks in humans, domestic animals, and birds. An infected mosquito can bite any animal, but not all animals will become ill. As the reservoir host of these viruses, birds are most often infected, but other animals can be infected and become ill as well. The virus could infect humans either from a local transmission cycle involving local birds or by infected migrating birds. West Nile infection should be considered in the differential diagnosis of acute encephalitis. As no human immunization is currently available, mosquito population control and use of repellents are the only methods to prevent outbreaks (Hayes *et al*., 2006).

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 (semi-domestic). As *Ae. aegypti* is the classical vector and *An. albopictus* is a serious potential vector (Miller, 1989; Mondet, 1996) for yellow fever, an outbreak for this typical hemorrhagic fever could occur if a traveler contracts the disease overseas. Yellow fever virus has its origins in Africa and was introduced to the Americas as early as the 15th century, most likely by infected mosquitoes, including *Ae. aegypti*, breeding in water containers on ships trafficking slaves. 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 World Health Organization (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 is a viral infection of the membranes around the brain, which 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. tritaeniorhynchus*. The culicine mosquitoes that transmit Japanese encephalitis 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 (Van den Hurk *et al*., 2009). Japanese encephalitis is the most important cause of viral encephalitis in Asia (mostly Far East but also India and Singapore) (Campbell *et al*., 2011). It causes 50,000 cases per year and it resulted, for example, 45,000 cases and 4,300 deaths in 1990 (WHO, 1995). Recently, Japanese encephalitis cases occurred in areas where it had not been recognized previously (Australia, Nepal, and Papua New Guinea) (Spicer, 1997; Zimmerman, 1997). Avian vertebrates are natural hosts and infected pigs represent an additional amplification factor for the virus (Isselbacher, 1994). Japanese encephalitis (incubation period, 4–15 days; case-fatality rate, 10–40%) does not always produce an encephalitis syndrome but can also cause lower motor neuron, 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 (family Togaviridae) has primates as the reservoir. It is transmitted mostly by *Aedes* mosquitoes, *Ae. aegypti* and *Ae. albopictus*. However, *Cx. tritaeniorhynchus* can be an additional vector in Southeast Asia. Similarly to yellow fever virus, the virus is maintained among nonhuman primates living in Africa (sylvatic cycle) and 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 (Sebastian *et al*., 2009). Chikungunya is an acute febrile illness with sudden onset of fever and joint pains, particularly affecting the hands, wrists, ankles, and feet (Kalantri *et al*., 2006). Most patients recover after a few days but in some cases the joint pains may persist for weeks,

months, or even longer (Gear *et al*., 1975). There is no direct person-to-person transmission (Chen *et al*., 2010).

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* (Cobbold, 1864), *Onchocerca volvulus* (Bickel, 1982), and *Mansonella streptocerca*. The lymphatic group includes *Wuchereria bancrofti* (Cobbold, 1877; Seurat, 1921), *Brugia malayi* (S. L. Brug, 1927), and *Brugia timori* (Partono *et al*. 1977). The body-cavity group includes *Mansonella perstans* (Manson, 1891) and *Mansonella ozzardi.* Natural vectors for *W. bancrofti* are *Cx. pipiens* mosquitoes in urban settings and *Anopheline* and *Aedes* mosquitoes in rural areas (Ramzy *et al*., 2006). Mosquitoes of the genus *Mansonia* are accessory vectors. The common clinical manifestations of lymphatic filariasis are asymptomatic microfilariae, chyluria, lymphatic inflammation, and obstruction culminating in hydrocele and elephantiasis (Ottesen, 1992). Infection is usually acquired in childhood, but the painful and profoundly disfiguring visible manifestations of the disease occur later in life (Witt *et al*., 2001). Whereas acute episodes of the disease cause temporary disability, lymphatic filariasis leads to permanent disability (Taylor *et al*., 2010). Filariasis is the most widely distributed human filarial parasite throughout the tropics and subtropics and it affects an estimated 120 million persons worldwide (WHO, 1992). The 73 million are due to *W. bancrofti* with the largest number in India (Michael *et al*., 1996). 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 (Chu *et al*., 2010).

Approximately 65% of those infected live in the WHO Southeast Asia Region, 30% in the African Region, and the remainder in other tropical areas (Taylor *et al*., 2010).

Table 1. Estimates of the global burden of disease caused by major vector-borne diseases

Disease	Vector species	Agent (family)	Distribution
Malaria	*Anopheles spp*.	Prozoans of the *Plasmodium* genus	Africa, Latin America, Asia, and Oceania
Yellow fever (YF)	*Aedes aegypti* *Ae. albopictus* *Ae. scutellaris* *Ae. africanus* *Ae. bromeliae*	YF virus Flaviviridae	Africa, Central and South America
Dengue fever	*Ae. aegypti* *Ae. albopictus* *Ae. scutellaris* *Ae. polynesiensis* *Ae. pseudoscutellaris* *Ae. rotumae*	Dengue virus 1-4	Southeast Asia, India, Central and South America
West Nile fever	*Culex spp*.	West Nile virus (Flaviviridae)	Africa, Middle East, Europe, India and Indonesia
Japanese encephalitis	*Culex spp*.	Japanese encephalitis virus (Flaviviridae)	Southeast Asia, East Asia

3. Mosquito management

An effort of global strategy for malaria control has had some success, but this effort lost momentum and the disease has recovered much of its range. Probable reasons for this return include deforestation, decline of vector control program, ecological change, irrigation and other agricultural practices, resistance to insecticides, urbanization and the impacts of civil strife, war and natural disasters rather than climate change (Reiter, 2000).

Although there may have been times when mosquito management was carried out on the basis of intuition, local anecdotal experience, or of dogma the discipline is now becoming increasingly evidence-based. Such research on mosquito management has been carried out. Mosquito management plans generally include monitoring of mosquito populations and mosquito-transmitted diseases. Mosquito and vector control should base on scientifically planned management tactics and control strategies that reduce the abundance of target pests in a timely manner that incorporates the principles of Integrated Pest Management (Resh, 2010).

Biological control is typically focused against the aquatic stages of the mosquito cycle. Some organisms have proved to be effective against mosquito larvae such as larvivorous fish (*Gambusia* and *Poecilia*), which are practical and economically viable (McLaughlin *et al*., 1984), although the efficacy of alternative species continues to be evaluated (Sekar, 1986). One important issue is the risks to native ecosystems of introducing a non-native 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 killifish in salt marshes, or certain species of minnows in freshwater wetlands, can substantially assist with mosquito control (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. *Bacillus thuringiensis* (Bt), a family of bacteria which make insecticidal proteins, accounts for 90–95% of the insect biocontrol market (Goldberg and Margalit, 1977; Debarjac and Larget-Thiery, 1984). *Bacillus thuringiensis* subsp. *israelensis* (Bti) synthesizes intracellular crystal inclusions by sporulation that contains multiple protein components of 134, 125, 67, and 27 kDa (Jenkin *et al*., 1997; Kogan, 1998). When ingested by mosquito larvae, they alter gut permeability killing the larvae. They are believed to pose a minimal risk to non-target species (Delfosse, 2005). Cry toxins bind to specific protein receptors in the microvilli of the mosquito midgut cells (Cech *et al*., 1983). Though the high efficacy and specificity of *Bacillus sphaericus* (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. In the past, several efforts have been made to develop bacterial formulations using different culture media with varying degrees of efficacy (Adams *et al*., 2002; Vidyarthi *et al*., 2001).

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. 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 adult mosquitoes 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 (Arnó *et al*., 2011; Bryan *et al*., 1979; Hanazato, 2001; Relyea, 2005).

4. Mode of action

Studies on the mode of action of insecticides or phytochemicals are very important from several points of view (Metcalf, 1967). Firstly, such knowledge is needed to understand the health hazards of these chemicals to humans and other non-target 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.

The secondary metabolites potentially encountering toxic substances with relatively nonspecific effects on a wide range of molecular targets (Hariprasad *et al*., 2015). These

targets range from proteins (enzymes, receptors, signaling molecules, ion channels, and structural proteins), nucleic acids, biomembranes, and other cellular components (Rattan, 2010). There are several classes of chemicals have been implicated in transmission at various insects synapsis including acetylcholine (ACh). Acetylcholine is the transmitter at central nervous system (CNS) 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 affected by the enzyme AChE, which catalyzes hydrolysis of the ester bond. Insecticide molecules bind to the AChE sites in the synaptic junction, preventing the naturally released ACh for being removed and recycled from the junction. Ach continues to activate receptors, keeping their channels open thereby depolarizing the postsynaptic neuron. This leads ultimately to the death of the insect (Bisset *et al*., 1990). 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). Neurotoxicity of pyrethroids has been attributed to their activity on the nervous system. The types of ion channels that have been demonstrated to be the major target of insecticides are quite limited. Only the sodium channels and the $GABA_A$ receptor-chloride channel complex are known to be affected by neuroactive insecticides with only a few exceptions including some that act on the ACh receptor-channel complex (Narahashi, 1976). The pyrethroids appear to be acting at virtually every part of the insect nervous system: on sensory neurons (Roeder and Weiant, 1946), on interneurons (Narahashi, 1971), on motor neurons (Yeager and Munson, 1945), and on neurosecretion (Singh and 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 ACh receptors, Na^{+}/Ca^{2+} exchangers, receptors for gamma-aminobutyric acid (GABA) (Rossignal, 1991; Bloomquist, 1996), ACh-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 neurohemal organs that lie outside of the ventral nerve cord and paired ganglia (Arendt *et al*., 1999). The CNS is considered the ganglia, connectives, and commissures from the brain to the terminal abdominal ganglia.

The GABA receptors belong to a superfamily of neurotransmitter receptors that also includes the nicotinic ACh receptors. These receptors are formed by the oligomerization of five subunits around a central transmitter-gated ion channel (Khakh *et al*., 2000). 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 permethrin, resmethrin, and *S*-bioallethrin had no effect (Gammon *et al*., 1983). 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). Cyclodiene insecticide resistance is found to be associated with change in GABA receptor/chloride ion channel in *Ae. aegypti* and *Drosophila melanogaster* (Ffrench-Constant *et al*., 1994). The mode of action of chemicals was summarized in Table 2.

Table 2. Mode of action and target site of commonly used insecticide classes

Chemical class/group	Active ingredients	Mode of action	Targeted site
Carbamates	Aldicard, Bendiocarb, Carbofuran, Promecarb, Methiocarb	Acetyl cholinesterase inhibitor	Nerve synapse
Organophosphate	Acephate, Chlorpyrifos, Disulfoton, Fenthion, Monocrotophos, Phorate	Acetyl cholinesterase inhibitor	Nerve synapse
Pyrethrins	Bifenthrin, Cypermethrin, Fluvalinate, Permethrin	Sodium channel modulators	Axon of nerve
Neonicotinoids	Acetamiprid, Clothiamidin, Imidacloprid, Thiacloprid	Acetylcholine receptor agonist	Nerve post-synapse
Avermectins	Abamectin B1, Emamectin benzoate	Chloride channel activators	Nerve post-synapse
Oxadiazins	Indoxacarb	Voltage-dependent sodium channel blocker	Axon of nerve
Spinosys	Spinosad	Nicotinic acetylcholine receptor agonists	Nerve post-synapse
Phenylpyrazoles	Fipronil	GABA-gated chloride channel antagonists	Nerve post-synapse

5. Insecticide resistance

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 a pesticide 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, by definition, 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 according to (Georghiou, 1980; Leeper *et al*., 1986; Plapp, 1986; Taylor, 1986; Croft, 1990; WHO, 1992) should be followed to avoid resistance of insecticides.

The resistance problems continued with the switch to newer insecticides such as the OP, carbamate, and pyrethroid insecticides. Three major enzyme groups are responsible for metabolically based resistance to organochlorines, OP, carbamate, and pyrethroid insecticides. The development of resistant populations is observed in the field as a progressive decrease in the control obtained by the dosage recommended based on 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 characteristics of insecticide resistance is inherited, and in most cases, it has proved to be due to unitary genetic factors (gene alleles) for resistance. When insecticide selection pressure is applied, the frequency of the resistant alleles increases. 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). 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 usually known as that they 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 *et al*., 1980).

Many studies have mapped and monitored resistance over the past two decades. There has been great dependence on studies *in vitro* using different methods with conflicting interpretations, and relatively limited knowledge of their implications for efficacy *in vivo* and clinical use under different epidemiological conditions. DDT dehydrochlorinase was first recognized as a glutathione S-transferase in *Anopheline* and *Aedes* mosquitoes (Grant *et al*., 1989; Prapanthadara *et al*., 1995). The esterase-based resistance mechanisms have been studied most extensively at the biochemical and molecular level in *Culex* mosquitoes (Vaughan *et al*., 1997). Elevated monooxygenase activity is associated with pyrethroid

resistance in *An. stephensi*, *An. subpictus*, *An. gambiae*, and *Cx. quinquefasciatus* (Hemingway *et al*., 1991; Vulule *et al*., 1994; Brogdon *et al*., 1997; Kasai *et al*., 1998). The organophosphorus, carbamate, organochlorines, and pyrethroid insecticides all target the nervous system. Compounds targeting the nicotinic acetylcholine receptor have recently made this transition from agriculture into public health. Alterations in AChE in organophosphate- and carbamate-resistant insects result in a decreased sensitivity to inhibition of the enzyme by these insecticides (Ayad *et al*., 1975). Studies show that cyclodiene-resistant insects are resistant to picrotoxin and phenylpyrazole insecticides, and that the effect of ivermectin on cultured neurons can be reversed by picrotoxin pretreatment. It suggests that these insecticides exert their effect by interacting with the chloride ionophore associated with the insect GABA receptor (Kadous *et al*., 1983). Also, in mosquitoes there have been many reports of suspected kdr-like resistance inferred from cross resistance between DDT and pyrethroids, which act on the same site within the sodium channel (Vatandoost *et al*., 1998).The widespread use of insecticides can lead to the development of insecticide resistance, making insecticide use ineffective and limiting the available options for disease control. Insecticide resistance status in culicine mosquitoes is summarized in Table 3.

Chemical pesticides 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 (Shim and Kim, 1981; Shim *et al*., 1995; Lee *et al*., 1997; Shin *et al*., 2003). Because of their increased usage, they demonstrated high to extremely high levels of SR values, while the less used

organophosphates demonstrated low to moderate SR values. 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 pesticides, all of which are major obstacles to cost-effective integrated mosquito management in the ROK (Kim *et al*., 2007). An effective insecticide resistance management strategy, based on the historical and current information on the distribution and potential for insecticide resistance in the ROK, is necessary (Kim *et al*., 2007; Kang *et al*., 2012).

Insecticide failures in the ROK 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). Continued or repeated use of these insecticides has often resulted in the development of resistance. In addition, factors such as increased costs of labor and pesticide application and safety issue have made mosquito difficult. 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). Pyrethroid insecticides have been used frequently, due to their strong insecticidal activity and relatively lower human toxicity compared to OP insecticides (Chang *et al*., 2009).

Table 3. Insecticide resistance in culicine mosquitoes

Species	Cases	Insecticides
Cx. pipiens pallens	117	DDT, chloropyriphos, diemthoate, dipterex, fenitrothion, fenthion, malathion, parathion, parathion-methyl, pirimifos-methyl, temephos, trichlorofon, deltamethrin,
Cx. pipiens molestus	15	Chlorpyrifos, chlorpyrifos-methyl, diazinon, dichlorvos, fenitrothion, fenthion, malathion, propetamphos, temephos, permethrin, phenothrin, etofenprox,
Cx. quinquefasciatus	295	DDT, dieldrin, chloropyriphos, deltamethrin, dichlorvos, diazinon, dichlorvos, fenitrothion, fenthion, malathion, permethrin, carbaryl, proxpur, temephos
Ae. aegypti	431	DDT, chlorphoxim, chlorpyrifos, dichlorvos, fenthion, malathion, temephos, tribufos, pirimiphos-methyl, propoxur, bioresmethrin, cyfluthrin, cyhalthrin-lambda, cybermethrin, permethrin, piperonyl butoxide,
Ae. albopictus	195	DDT, fenitrothion, fenthion, malathion, permethrin
Ae. nigromaculis	20	DDT, chlorpyrifos, chlorpyrifos-methyl, EPN, fenitrothion, fenthion, malathion, methidathion, parathion, parathion-methyl, phenthoate, temephos
An. sinensis	17	DDT, fenitrothion, fenthion, malathion,
An. gambiae	76	DDT, cyhalothrin-lambda

[Source] http://www.pesticideresistance.org/. Anonymous (2015)]

6. Plant-derived mosquito control agents

The appearance of mosquito resistance to conventional insecticides, 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, in recent years the use of environmentally friendly and easily biodegradable natural insecticides of plant origin has received renewed importance for malaria and other diseases control. Most plants contain compounds that they use in preventing attack from phytophagous insects. Interest in this field is based on the fact that these substances are least phytotoxic and do not lead to the accumulation of chemical residues in flora, fauna, soil and the entire environment in general.

Currently, numerous products of botanical origin, especially the secondary metabolites, have received considerable renewed attention as potentially bioactive agents used in insect vector management. 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 non-target 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, 2004).

Various secondary metabolites that may possess insecticidal, antimicrobial (Leeja and Thoppil, 2007), herbicidal and other biological activities (Tonk *et al*., 2006; Setia *et al*., 2007). At present, there are four major commercially available plant-based compounds (pyrethrum, rotenone, neem, and essential oils) and three limited use compounds (ryania, nicotine, and sabadilla) for insect control (Isman, 2006).

Due to the high degree of biodegradation, however, plant-derived bioproducts are currently attractive as replacements for synthetic insecticides or for use in integrated management programs to minimize human health hazards and reduce the accumulation of harmful residues in the environment. Furthermore, insect resistance to mosquitocidal botanical agents has not previously been documented (Shaalan *et al*., 2005). In recent years, the active insecticidal compounds isolated from plants have received much attention due to their pronounced larvicidal efficacy.

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 (Table 4). Due to the fact that application of adulticides may only temporarily diminish the adult population (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). 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, etc. depending on the polarity of the phytochemicals. Shallan *et al*. (2005) reviewed the current state of knowledge on larvicidal plant species, extraction processes, growth and reproduction inhibiting phytochemicals, botanical ovicides, synergistic, additive, and antagonistic joint action effects of mixtures, residual capacity, effects on non-target organisms, resistance and screening methodologies, and discussed some promising advances made in phytochemical research.

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), and tannins (Khanna, 2007) as mosquito larvicides. The plant-derived natural products as larvicides have the advantage of being harmless to beneficial non-target 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; Cavalcanti *et al*., 2004; Cheng *et al*., 2004) have received much attention as potentially useful bioactive compounds against insects (Kim *et al*., 2001). Among the

acetylenic compounds, falcarinol and falcarindiol isolated from *Cryptotaenia canadensis* display strong activity against *Cx. pipiens* larvae. The more lipophilic falcarinol with LC_{50} values of 3.5 and 2.9 mg L^{-1} in 24 hours and 48 hours, respectively exert strong toxicity than the more polar acetylene falcarindiol with LC_{50} values of 6.5 and 4.5 mg L^{-1} in 24 and 48 hours, respectively (Eckenbach *et al*., 1999). The lactones 30 and 31, isolated from *Hortonia floribunda*, *H. angustifolia*, exhibit potent larvicidal activity against the second instar larvae of *Ae. aegypti* with LC_{50} values of 0.41 and 0.47 mg L^{-1} (Ratnayake *et al*., 2001). The larvicidal activity of dioncophylline A, a naphthylisoquinoline alkaloid derived from the tropical vine *Triphyophyllum peltatum* (Dioncophyllaceae), was investigated against the malaria vector *Anopheles stephensi* with LC_{50} value below 1 mg L^{-1} (François *et al*., 1996). The stem bark of *Microcos paniculata* contained a new alkaloid, N-Methyl-6 beta-(deca-1', 3', 5'-trienyl)-3 beta-methoxy-2 beta-methylpiperidine, which showed good insecticidal activity against *Ae. aegypti* second instar larvae with LC_{50} value of 2.1 mg L^{-1} (Bandara *et al*., 2000). *Abutilon indicum* led to the separation and identification of a β-sitosterol as a potential new mosquito larvicidal compound with LC_{50} values of 11.49, 3.58, and 26.67 mg L^{-1} against *Ae. aegypti*, *An. stephensi* and *Cx. quinquefasciatus*, respectively (Sohel, 2015). The insecticidal constituent of *Tabebuia avellanedae* bark was isolated by chromatographic techniques and identified as 2-hydroxy-3-(3-methyl-2-butenyl)-1, 4-naphthalenedione with LC_{50} values of 8.30 and 8.30 mg L^{-1} against *Ae. aegypti* and *Cx. p. pallens* (Kim *et al*., 2013).

Table 4. Identification of various bioactive toxic principles from plant extract and their relative mosquitocidal efficacy

Active ingredient	Mosquito	Plants	LC_{50} values (mg L^{-1})	References
Falcarinol	*Cx. pipiens*	*Cryptotaenia canadensis*	3.5	Eckenbach *et al*., 1999
Falcarindiol	*Cx. pipiens*	*Cryptotaenia canadensis*	6.5	Eckenbach *et al*., 1999
Butenolides 1	*Ae. aegypti*	*Hortonia floribunda*	0.41	Ratnayake *et al*., 2001
Butenolide 2	*Ae. aegypti*	*Hortonia floribunda*	0.47	Ratnayake *et al*., 2001
Dioncophylline A	*An. stephensi*	*Triphyophyllum peltatum*	1.5	François *et al*., 1996
N-Methyl-6 beta-(deca-1',3',5'-trienyl)-3 beta-methoxy-2 beta-methylpiperidine	*Ae. aegypti*	*Microcos paniculata*	2.1	Bandara *et al*., 2000
β-sitosterol	*Ae. aegypti, An. stephensi* and *cx. quinquefasciatus*	*Abutilon indicum*	11.49, 3.58 and 26.67, respectively	Rahuman *et al*., 2008
2-hydroxy-3-(3-methyl-2-butenyl)-1,4-naphthalenedione	*Ae. Aegypti and Cx. p. pallens*	*Tabebuia avellanedae*	8.83 and 8.30, respectively	Kim *et al*., 2013

7. *Cnidium monnieri* and *Magnolia denudata*

Cnidium monnieri (L.) Cusson belonging to the Umbelliferae family is an important crude drug "Fructus Cnidii" used in traditional Chinese medicine for the treatment of impotence and frigidity (Ou, 1989). The dried fruits of *C. monnieri*, known in Chinese as "Shechuangzi" and Japanese name "Jashoshi", have been used as traditional remedies for the skin disease, gynecopathy, and stasis of the blood. Several investigations have reported that the fruits of *C. monnieri* exhibit various pharmacological effects including antidermatophytic effect (Honda *et al*., 1984), antipruritic action (Basnet *et al*., 2001), anti-allergic effect (Matsuda *et al*., 2002), antiosteoporosis (Matsuda *et al*., 2002), antiproliferation of vascular smooth muscle cells (Guh *et al*., 1996), vasorelaxation (Chiou *et al*., 2001), antifibrotic activity in hepatic cells (Shin *et al*., 2011), anti-adipogenic activity in 3T3-L1 cells (Kim *et al*., 2012), and inhibitory effect on pulmonary inflammation related with chronic obstructive pulmonary disease (Kwak *et al*., 2014). Coumarin derivatives such as osthole (Murayama *et al*., 1972), xanthotoxin (Basnet *et al*., 1993), isopimpinellin (Talapatra *et al*., 1973), bergapten (Gu *et al*., 1990), and imperatorin (Miyakado *et al*., 1978), have been reported as the main constituents from *C. monnieri* (Cai *et al*., 2000; Sagara *et al*., 1987).

Magnolia species are widely used in urban greening projects because it is highly ornamental. *Magnolia* species have a distribution in East and Southeast Asia used for centuries as a traditional medicine (Xu *et al*., 2008). Their dried flower buds of *Magnolia denudata* have been known as "Xin-Yi" and are used to clinical application for symptomatic management of allergic rhinitis, sinusitis and headache

(Jo *et al*., 2012). The chemical constituents *M. denudata* contain many phenyl propanoids, sesquiterpenes, alkaloids, terpenoids, lignans and neolignans (Kelm *et al*., 2000; Noshita *et al*., 2009). Plant materials constitute a rich source of active compounds that are eco-friendly and have been used to control mosquitoes in human communities for many centuries (Liu *et al*., 2012). They often act at multiple and novel target sites, thereby reducing the potential for resistance. The seeds of *M. denudata* have anti-allergy and anti-inflammation activities, and traditional and folk medicine uses of *Magnolia* species have been well reviewed (Li *et al*., 2013; Shaalan *et al*., 2005; Sukumar *et al*., 1991). However, no information is available concerning the potential of *M. denudate* seed-derived essential oil and their composition for mosquito larvicidal activity.

8. Perspectives

It is important to restate that herbal medicine practice that would be mainstreamed into healthcare should be backed by good science. The WHO has been pursuing a strategy toward institutionalizing procedures for the safety, efficacy, access, and rational use of herbal medicines under structured regulatory, legislative, and administrative frameworks since the year of 2002. The top priority in finding new insecticides is that, they must be plant origin and have no any ill effect on ecosystem.

In recent years, the emphasis to control the mosquito populations has shifted steadily from the use conventional chemicals toward more specific and environmentally friendly materials, which are generally of botanical origin. Modern synthetic chemicals could

provide immediate results for the control of insects/mosquitoes; on the contrary, they bring irreversible environmental hazard, severe side effects, and pernicious toxicity to human being and beneficial organisms. Therefore, use of eco-friendly and cost-free plant based products for the control of mosquitoes is inevitable.

CHAPTER I

Larvicidal Activity of *Cnidium monnieri* Fruit Furanocoumarins and Structurally Related Compounds against Insecticide-Susceptible and Insecticide-Resistant *Culex pipiens pallens* and *Aedes aegypti*

INTRODUCTION

Mosquitoes are cosmopolitan and common, and can transmit a variety of diseases, such as yellow fever, dengue hemorrhagic fever, malaria, several forms of encephalitis, and filariasis, as well as nuisance insect pests (Mullen and Durden, 2009; Pan *et al*., 2012). In 2009, it was estimated that 225 million people contract malaria each year, with at least 781,000 deaths annually, mostly among children (WHO, 2010). A recent estimate indicates 390 million dengue infections per year, of which 96 million manifest clinically (Bhatt *et al*., 2013). From 1999 to 2010, 37,088 cases of human West Nile virus disease (including 16,196 neuroinvasive disease cases) were reported in the United States (US), resulting in 1,549 deaths (USCDC, 2013). With global warming and increased international travel, a number of mosquitoes are distinctly increasing in incidence with a high occurrence of dengue fever all over the globe (Spielman and D'Antonio, 2001; Halstead, 2007).

Mosquito larval abatement has been achieved principally by the use of organophosphorus (OP) insecticides such as chlorpyrifos and temephos, insect growth regulators such as diflubenzuron and methoprene, and bacterial larvicides such as *Bacillus thuringiensis* H-14 and *Bacillus sphaericus* (Rozendaal, 1997), which continue to be effective larvicides. Continued and repeated use of these larvicides has disrupted natural biological control systems, led to resurgences of mosquitoes (Croft and Brown, 1975), and resulted in the widespread development of resistance (Mota-Sanchez *et al*., 2008; Vontas *et al*., 2012). Moreover, it has undesirable effects on aquatic non-target organisms (Brown, 1978; Rozendaal, 1997). Widespread insecticide resistance has been a major obstacle in the cost-effective integrated vector management program. In addition, the number of

approved insecticides may be reduced in the near future in the US (USEPA, 2015) and in the European Union (European Union, 2005) because of reregistration of conventional insecticides. The removal of conventional insecticide products from markets due to the increase in insecticide resistance or other concerns will have a serious impact on the mosquito proliferation. There is a pressing need for the development of selective alternatives for the control of mosquitoes, with novel target sites to establish a rational management strategy because vaccines have been proven that they have limited effectiveness in controlling malaria (Richie and Saul, 2002) or dengue (Mahoney, 2014).

Plants and their constituents have been suggested as potential alternative for arthropod control largely because they constitute a potential source of bioactive secondary substances 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; Shaalan *et al*., 2005). Secondary substances often act at multiple and novel target sites (Kostyukovsky *et al*., 2002; Priestley *et al*., 2003; Isman, 2008), thereby reducing the potential for resistance (Perumalsamy *et al*., 2010; Wang *et al*., 2012). Much effort has been focused on them as potential sources of commercial mosquito larvicides, in part, because certain plant preparations and their constituents meet the criteria of minimum risk pesticides (Isman, 2008). In the screening of plants for mosquito larvicidal activity, a methanol extract of the seed from *Cnidium monnieri* (L.) Cusson (Apiaceae) was shown to have good larvicidal activity against third instar larvae of northern house mosquito, *Culex pipiens pallens* (Coquillett), and yellow fever mosquito, *Aedes aegypti* (Linnaeus). No information is available concerning the potential of *C. monnieri* for managing mosquitoes for future commercialization, particularly insecticide-resistant mosquitoes, despite

excellent pharmacological actions of *C. monnieri* (Namba, 1993).

In this study, an assessment is made of the contact toxicity of two coumarins from the fruits of *C. monnieri* and 11 structurally related coumarins to third instar larvae from insecticide-susceptible *Cx. p. pallens* (KS-CP strain) and *Ae. aegypti* as well as a wild colony of *Cx. p. pallens* (YS-CP colony), which are identified by polymerase chain reaction (PCR) and resistant to various insecticides. The toxicities of these 13 coumarins were compared with those of the currently available organophosphorus larvicide temephos to assess their use as future commercial mosquito larvicides. Quantitative structure-activity relationship (QSAR) of test coumarins was also discussed.

MATERIALS AND METHODS

General instrumental methods

^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectra were recorded in CD_3OD on an AVANCE 600 spectrometer (Bruker, Karlsruhe, Germany) using tetramethylsilane as an internal standard, and chemical shifts are given in δ (ppm). Ultraviolet (UV) spectra were obtained in methanol on a Mecasys Optizen POP spectrophotometer (Daejeon, Republic of Korea (ROK)) and mass spectra on a JMS-DX 303 spectrometer (Jeol, Tokyo, Japan). Silica gel (0.063–0.2 mm) (Merck, Darmstadt, Germany) was used for column chromatography. Merck precoated silica gel plates (Kieselgel 60 F_{254}) were used for analytical thin-layer chromatography (TLC). An Agilent 1200 series high-performance liquid chromatography (HPLC) (Agilent, Santa Clara, CA, USA) was used for isolation of active principles.

Chemicals

The two coumarins isolated in this study and the 11 commercially available organic coumarins examined are listed in Table 5, along with their sources. For the QSAR analysis, values of molecular weight (MW), hydrophobic parameter (log *P*), and steric effects for the test coumarins were obtained from ChemDraw Ultra 10.0 (Cambridge Soft Corporation, Cambridge, MA), and these are recorded in Table 5. Molecular refraction (MR) was used as the parameter for describing steric effects. The five insecticides examined in this study were as follows: β-cyfluthrin (98.0% purity) and chlorfenapyr (99.0%) purchased from Sigma-Aldrich (St Louis, MO); deltamethrin (99.0%) and fenthion (98.4%) purchased from

Supelco (West Chester, PA); temephos (97.3%) purchased from Riedel (Seelze, Germany). Triton X-100 was supplied by Shinyo Pure Chemicals (Osaka, Japan). All of the other chemicals used in this study were of reagent-grade quality and available commercially.

Mosquitoes

The stock cultures of *Cx. p. pallens* (susceptible KS-CP strain) and *Ae. aegypti* have been maintained in the laboratory without exposure to any known insecticide (Yang *et al*., 2004). Larvae of wild *Cx. p. pallens* were field collected near rice paddy fields and cowsheds in Yusung (Daejeon) in September 2010 (Perumalsamy *et al*., 2010). The rice paddy fields and cowsheds had varying histories of insecticide use. They have been separately maintained in temperature-controlled insect rearing rooms (Seoul National University) to prevent cross-contamination. Larvae were reared in plastic trays (24 × 35 × 5 cm) containing 0.5 g of sterilized diet (40-mesh chick chow powder/yeast, 1/1 by weight). Adult mosquitoes were maintained on a 10% sucrose solution and blood fed on live mice. All stages were held at 27 ± 1°C and 65–75% relative humidity (RH) under a 12:12 hour light: dark cycle. Species identification based on PCR revealed that larvae from the field-collected colony (designated YS-CP) belonged to *Cx. p. pallens*. The wild mosquitoes were reared for four generations to ensure sufficient numbers for testing.

Table 5. Values of physical parameters of 13 coumarins derivatives examined in this study

Compound	MW[b]	Log *P*[c]	MR[d]	source
Linear FCs[a]				
Bergapten	216.19	1.31	59.03	S-A[e]
Imperatorin	270.28	2.55	78.63	CI[f]
Isoimperatorin	270.28	2.55	78.63	FL[g]
Isopimpinellin	246.22	1.18	66.28	FL
Psoralen	186.16	1.44	51.78	S-A
Xanthotoxin	216.19	1.31	59.03	FA[h]
Angular FC				
Angelicin	186.16	1.44	51.78	S-A
Simple coumarins				
Coumarin	146.14	1.82	42.09	S-A
7-Ethoxycoumarin	190.20	2.04	54.14	S-A
Hemiarin	176.17	1.70	49.34	S-A
Limettin	206.19	1.57	56.59	S-A
Osthole	244.29	3.29	74.64	CI
Umbelliferone	162.14	1.44	43.91	S-A

[a] Furanocoumarins.

[b] Molecular weight.

[c] Hydrophobic parameter expressed as the log of the octanol/water partition coefficient.

[d] Parameter for steric effects as described using molecular refraction.

[e] Purchased from Sigma-Aldrich (St Louis, MO).

[f] Compounds isolated in this study.

[g] Purchased from Futoro Laboratories (Ghatkopar, Mumbai, India).

[h] Purchased from Fluka (Bucks, Switzerland).

Plant material

Fruits of *C. monnieri* were purchased from a local medicinal herb shop, Chengdu (Sichuan, China). And the plant was identified by a certified botanical taxonomist. A voucher specimen (CM-01) was deposited in the Research Institute of Agriculture and Life Sciences, College of Agriculture and Life Sciences, Seoul National University.

Extraction and isolation

Air-dried fruits (3 kg) of *C. monnieri* were pulverized, extracted with methanol (3 × 10 L) at room temperature for 2 days, and filtered. The combined filtrate was concentrated under vacuum at 40°C to yield ~111 g of a dark greenish tar. The extract (20 g) was sequentially partitioned into hexane- (8.60 g), chloroform- (7.40 g), ethyl acetate- (0.93 g), butanol- (0.81 g), and water-soluble (2.26 g) portions for subsequent bioassay (Fig. 1). 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^{-1} of each *C. monnieri* fruit-derived material was tested in a direct-contact mortality bioassay as described previously (Perumalsamy *et al*., 2010).

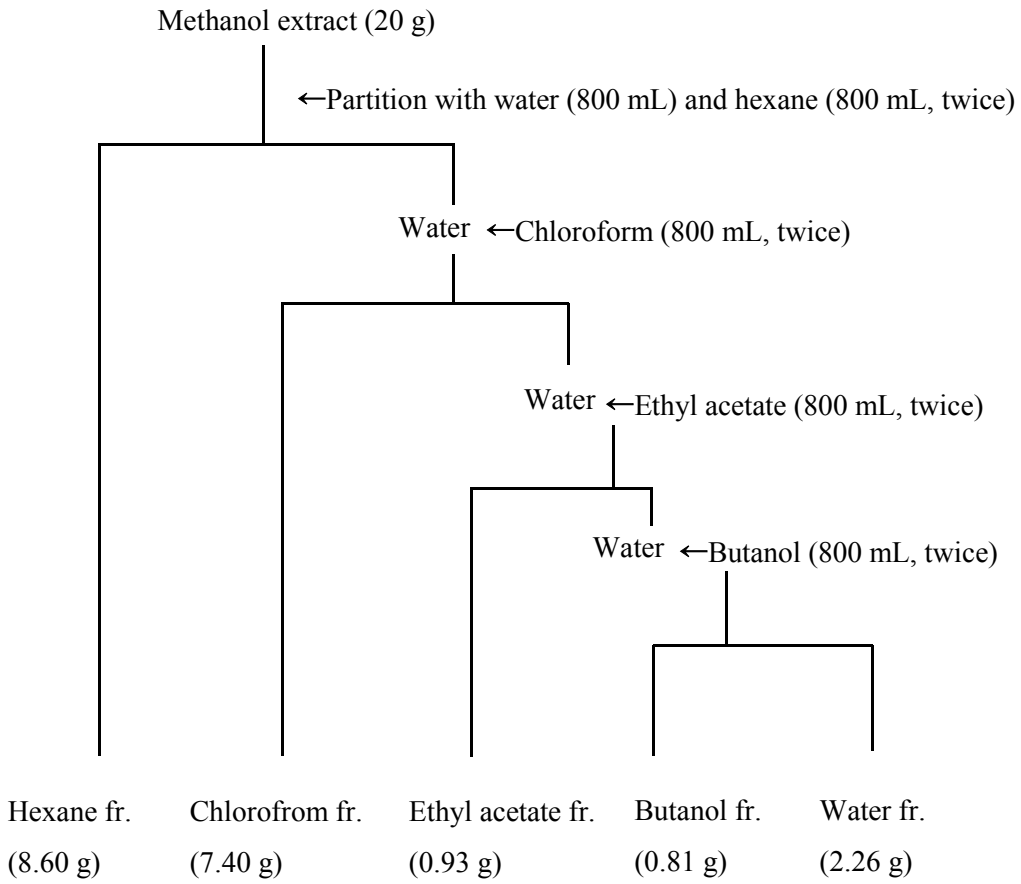


Fig. 1. Solvent fractionation procedures. The methanol extract from the fruits of *C. monnieri* was sequentially partitioned into hexane-, chloroform-, ethyl acetate-, butanol-, and water-soluble portions.

The hexane-soluble fraction (19.1 g) was most biologically active and was chromatographed on a 5.5 × 70 cm silica gel (580 g) column by elution with a gradient of hexane and ethyl acetate [95:5 (3 L), 90:10 (1 L), 80:20 (1 L), 75:25 (2 L), 70:30 (1 L), 65:35 (1 L) and 50:50 (1 L) by volume] and finally with methanol (1 L) to provide 44

fractions (each about 250 mL) (Fig. 2). Column fractions were monitored by TLC on silica gel plates developed with hexane and ethyl acetate (85:15 by volume) mobile phase. Fractions with similar R_f values on the TLC plates were pooled. Spots were detected by spraying with 10% H_2SO_4 and then heating on a hot plate. Active fractions 16 to 22 (3.47 g) were rechromatographed on a 4.5 × 77 cm silica gel (300 g) column by elution with a gradient of hexane and ethyl acetate [95:5 (1 L), 90:10 (1 L), 80:20 (2 L), 70:30 (2 L), 60:40 (1 L), 55:45 (1 L) and 20:80 (1 L) by volume] and finally with methanol (1 L) to provide 40 fractions (each about 250 mL). A preparative HPLC was used for separation of the constituents from the active fractions 12 to 14 (540 mg). The column was a 21.2 mm i.d. × 250 mm Phenomenex ODS (Prodigy, Torrance, CA) with a mobile phase of acetonitrile and water (9:1 by volume) at a flow rate of 1.0 mL min^{-1}. Chromatographic separations were monitored using a UV detector at 251 nm. Finally, an active principle **1** (520 mg) was isolated at a retention time of 13.2 min (Fig. 3).

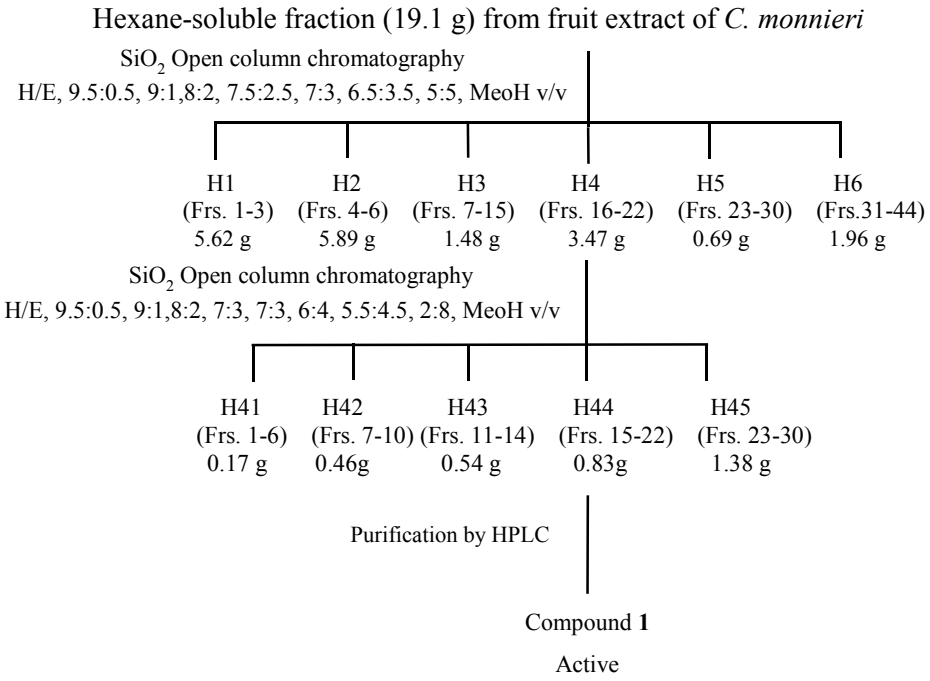


Fig. 2. Isolation procedures of larvicidal principles. The *C. monnieri* fruit methanol extract was sequentially partitioned into hexane-, chloroform-, ethyl acetate-, butanol-, and water-soluble portions. For isolation of active principles from the hexane-soluble fraction, 50 mg L^{-1} of each *C. monnieri* fruit-derived material was tested in a direct-contact mortality bioassay toward third instar larvae from *Cx. p. pallens*.

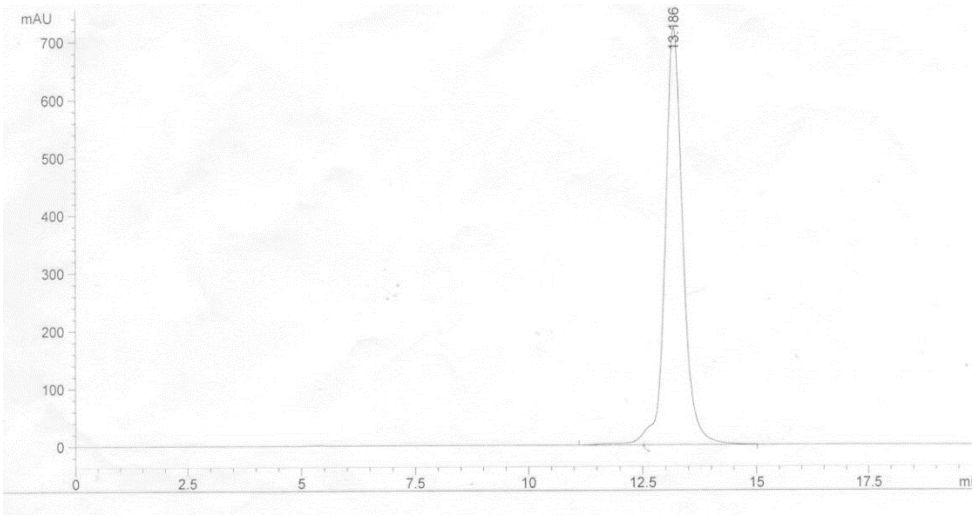


Fig. 3. High-performance liquid chromatogram of compound 1.

The other active chloroform-soluble fraction (5.1 g) was chromatographed on a 4.5 × 77 cm silica gel (300 g) column by elution with a gradient of chloroform and methanol [10:1 (6 L), 9:1 (1 L), 8:2 (1 L), 7:3 (1 L), 6:4 (1 L), and 0:10 (1 L) by volume] to provide 44 fractions (each about 250 mL) (Fig. 4). Column fractions were monitored by TLC on silica gel plates developed with chloroform and methanol (100:5 by volume) mobile phase. Fractions with similar R_f values on the TLC plates were pooled. The most active 1 to 12 (146 mg) was offered crystal particle which was recrystallized in acetone at -4°C to afford an active principle **2** (135 mg) (Fig. 5).

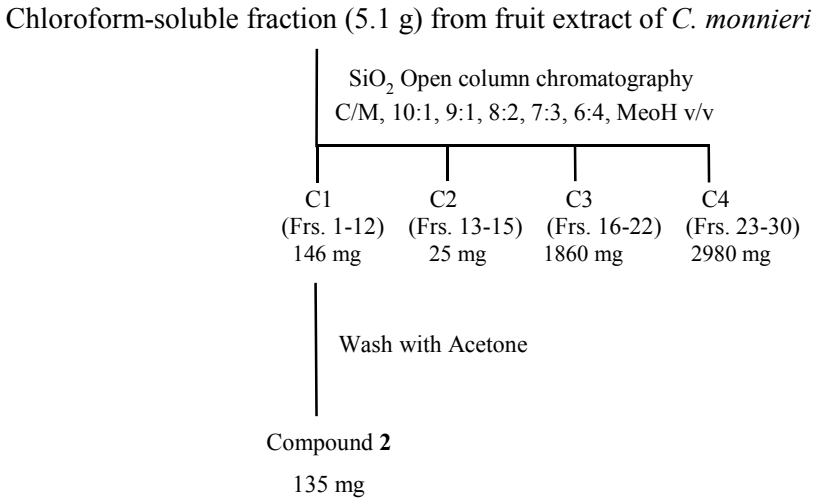


Fig. 4. Isolation procedures of larvicidal principles. For isolation of active principles from the chloroform-soluble fraction, 50 mg L^{-1} of each *C. monnieri* fruit-derived material was tested in a direct-contact mortality bioassay toward third instar larvae from *Cx. p. pallens*.

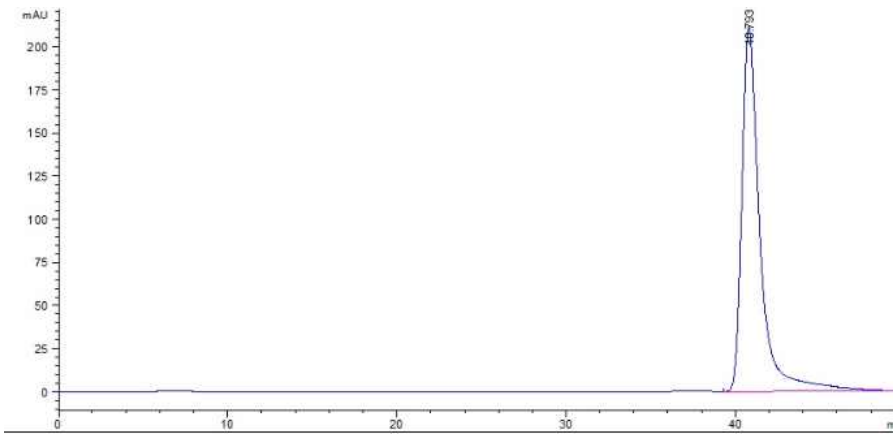


Fig. 5. High-performance liquid chromatogram of compound 2.

Bioassay

A direct-contact mortality bioassay was used to evaluate the toxicity of all compounds to third instar larvae from the susceptible and wild mosquito populations (Kim *et al*., 2007). Each compound in methanol was suspended in distilled water with Triton X-100 (20 μL L^{-1}). Groups of 20 mosquito larvae were separately put into paper cups (270 mL) containing each test compound solution (250 mL). Temephos served as a positive control and was similarly prepared. Negative controls consisted of the methanol-Triton X-100 carrier solution in distilled water. Based on the preliminary test results, the toxicity of each coumarin and insecticide examined was determined with four to six concentrations ranging from 0.1 to 400 mg L^{-1} and from 0.00001 to 10 mg L^{-1}, respectively.

Treated and control (methanol-Triton X-100 solution only) larvae were held under the same conditions as those used for colony maintenance. Mortalities were determined 24-hour post-treatment. A larva was considered dead if it did not move when prodded with a fine wooden dowel (Kim *et al*., 2007). All treatments were replicated three times using 20 larvae per replicate.

Data analysis

Data were corrected for control mortality using Abbott's (1925) formula. Concentration-mortality data were subjected to probit analysis (SAS Institute, 2004). The LC_{50} values for each species and their treatments were considered significantly different from one another when their 95% confidence limits did not overlap. A resistance ratio (RR) was calculated according to the formula RR = LC_{50} of larvae from YS-CP colony/LC_{50} of larvae of 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).

Correlation coefficient analysis of the toxicities of compounds to larvae of two mosquito species was done using their LC_{50} values and physical parameter (MW, MR, and log *P*) values for the test coumarins.

RESULTS

Insecticide resistance

The toxicity of the five insecticides to third instar larvae from the susceptible KS-CP strain and the wild YS-CP colony of *Cx. p. pallens* was evaluated by a direct-contact mortality bioassay (Table 6). YS-CP larvae exhibited extremely high levels of resistance to fenthion and deltamethrin, moderate levels of resistance to cyfluthrin and temephos and a low level of resistance to chlorfenapyr compared with KS-CP larvae. Mortality in the methanol-Triton X-100-water-treated controls for *Cx. p. pallens* larvae in this study was less than 2%.

Bioassay-guided fractionation and isolation

Fractions obtained from the solvent partitionings of the methanol extract of *C. monnieri* fruits were bioassayed against third-instar larvae from insecticide-susceptible *Cx. p. pallens* and *Ae. aegypti*, as stated above (Table 7). Significant differences in toxicity 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 hours of exposure, the hexane- and chloroform-soluble fractions were the most toxic materials. No toxicity was obtained from the ethyl acetate-, butanol-, and water-soluble fractions.

Table 6. Contact toxicity of the five insecticides to third instar larvae from the insecticide-susceptible KS-CP strain and wild YS-CP colony of *Cx. p. pallens* during a 24-h exposure

Compound	KS-CP larvae		YS-CP larvae		RR[b]
	Slope ± SE	LC_{50}, mg L^{-1} (95% CL[a])	Slope ± SE	LC_{50}, mg L^{-1} (95% CL[a])	
Deltamethrin	2.7 ± 0.33	0.00030 (0.00026–0.00035)	0.9 ± 0.31	0.04923 (0.02527–0.08509)	164.1
Cyfluthrin	2.2 ± 0.26	0.00043 (0.00035–0.00052)	3.8 ± 0.77	0.00618 (0.00520–0.00687)	14.4
Fenthion	3.2 ± 0.58	0.0028 (0.0025–0.0032)	2.8 ± 0.53	1.0930 (0.8879–1.3686)	390.4
Temephos	2.5 ± 0.43	0.011 (0.010–0.014)	4.1 ± 0.52	0.149 (0.132–0.167)	13.5
Chlorfenapyr	1.6 ± 0.26	0.008 (0.006–0.010)	3.4 ± 0.49	0.037 (0.032–0.044)	4.6

[a] CL denotes confidence limit.

[b] Resistance ratio.

Table 7. Contact toxicity of fractions obtained from solvent partitionings of the methanol extract of *C. monnieri* fruits to third instar larvae from two mosquito species during a 24-h exposure

Compound	*Culex pipiens pallens*		*Aedes aegypti*	
	Slope ± SE	LC_{50}, mg L^{-1} (95% CL[a])	Slope ± SE	LC_{50}, mg L^{-1} (95% CL[a])
Methanol extract	3.9 ± 0.56	25.80 (22.80–29.33)	3.5 ± 0.53	23.83 (20.99–27.26)
Hexane-soluble fr.[b]	2.3 ± 0.40	14.89 (11.98–18.17)	2.1 ± 0.40	16.47 (13.14–20.70)
Chloroform-soluble fr.	4.6 ± 0.54	16.91 (14.92–17.81)	4.1 ± 0.58	17.89 (15.72–20.09)
Ethyl acetate-soluble fr.		>100		>100
Butanol-soluble fr.		>100		>100
Water-soluble fr.		>100		>100

[a] CL denotes confidence limit.

[b] Fraction.

The larvicidal activity of each subfraction derived from the hexane-soluble and chloroform-soluble fractions is given in Tables 8 and 9, respectively.

Table 8. Larvicidal activity of each subfraction from hexane-soluble fraction derived from *C. monnieri* fruits against third instars of insecticide-susceptible KS-CP strain of *Cx. p. pallens*

Fraction	Mortality, % (± SE)	
	10 mg L^{-1}	50 mg L^{-1}
H1	–	10 ± 0.0
H2	–	12 ± 1.7
H3	20 ± 0.0	33 ± 1.7
H4	43 ± 3.3	97 ± 1.7
H5	15 ± 0.0	49 ± 0.8
H6	–	32 ± 1.7
Fraction	50 mg L^{-1}	
H41	–	
H42	8 ± 1.6	
H43	21 ± 1.9	
H44	100 ± 0.0	
H45	5 ± 0.0	

Table 9. Larvicidal activity of each subfraction from chloroform-soluble fraction derived from *C. monnieri* fruits against third instars of insecticide-susceptible KS-CP strain of *Cx. p. pallens*

Fraction	Mortality, % (± SE)	
	10 mg L^{-1}	50 mg L^{-1}
C1	97 ± 1.7	100
C2	3 ± 1.7	23 ± 1.7
C3	0	18 ± 1.7
C4	0	17 ± 1.7

The direct-contact mortality assay-guided fractionation of the methanol extract from *C. monnieri* fruits afforded two active principles (compound **1** and **2**). Compound **1** was obtained as a white powder and identified by spectroscopic analysis, including electron ionized mass spectrometry (EI-MS) (Fig. 6), ^{1}H NMR (Fig. 7), and ^{13}C NMR (Fig. 8). EI-MS revealed a molecular ion at *m/z* 270 $[M]^{+}$ and its ^{13}C NMR spectra showed 16 carbons in the molecule, suggesting the molecular formula $C_{16}H_{14}O_4$. The ^{1}H NMR spectra of compound **1** contained resonance signals due to H-4, relatively upfield at δ 8.04, indicating that these compounds were substituted at C-8; the chemical shift of H-5 was found at δ 7.87. The furanoprotons appeared as doublets with J = 2.2 Hz. The position of the signals of the gem-dimethyl grouping δ 1.69 and δ 1.65 compared with the spectrum showing the methyl proton signal on the second carbon atom of the side chain. The protons of the -OCH_2 group as a doublet at δ 4.96 are also readily visible. This compound was thus identified as imperatorin (**1**) (9-(3-methylbut-2-enoxy)-7-furo [3, 2-g] chromenone) (CAS no. 482-44-0) (Fig. 9). The interpretations of proton and carbon signals were largely

consistent with those of Yun and Yoichiro (2006). Imperatorin (**1**) was identified on the basis of the following evidence: white powder. UV (MeOH): λ_{max}nm = 222, 251, 310. (EI-MS) (70 eV), *m/z* (% relative intensity): 270 $[M]^+$ (3), 202 (100), 174 (50), 146 (9), 118 (6), 89 (14), 67 (30), 53 (16). ^{1}H NMR and ^{13}C NMR spectral data of imperatorin is given in Table 10.

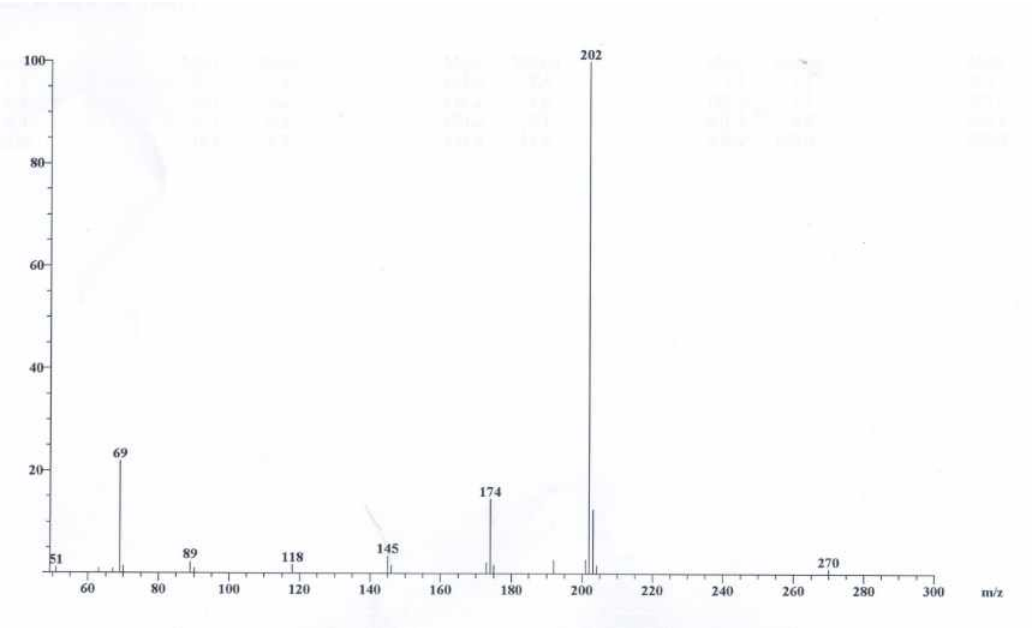


Fig. 6. EI-MS spectrum of compound 1.

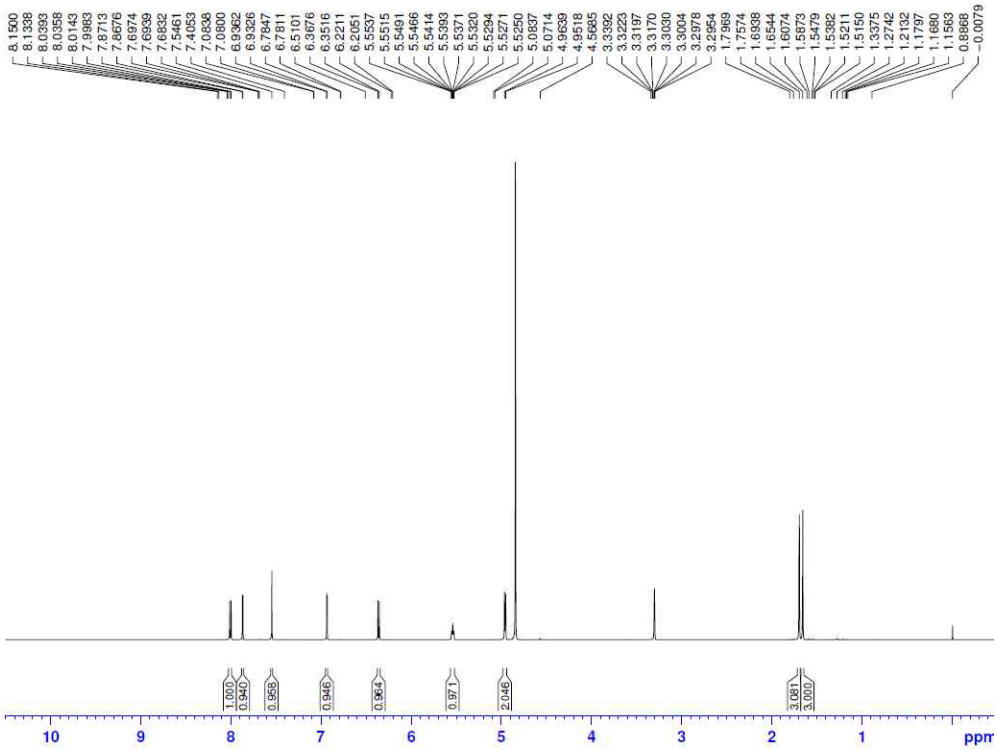


Fig. 7. ^{1}H NMR spectrum of compound 1.

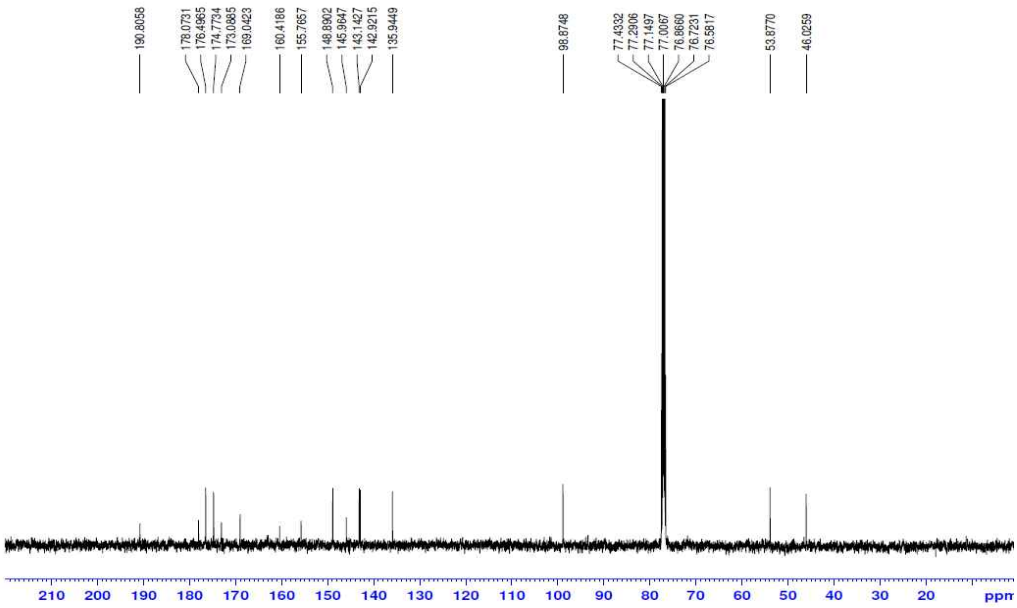


Fig. 8. ^{13}C NMR spectrum of compound 1.

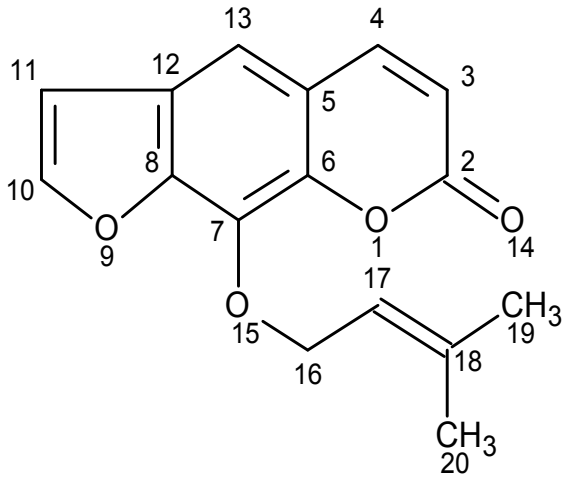


Fig. 9. Structure of imperatorin (1). The chemical formula of this compound is $C_{16}H_{14}O_4$; the molar mass is 270.28 g/mol.

Table 10. ^{1}H-NMR (600 MHz) and ^{13}C-NMR (150 MHz) spectral data for compound 1

Position	Partial Structure	δ_C, ppm (MeOD, 150 MHz)	δ_H, ppm (MeOD, 600 MHz)	δ_C (ppm) (Yun and Yoichiro, 2006)	δ_H (ppm) (Yun and Yoichiro, 2006)
2	C	190.8		159.8	
3	CH	143.1	6.36 d (J = 9.6 Hz)	114.2	6.29
4	CH	174.8	8.04 d (J = 2.1 Hz)	145.3	7.73
5	C	146.0		116.4	
6	C	173.1		143.2	
7	C	160.4		130.5	
8	C	178.1		147.8	
10	CH	176.5	7.87 d (J = 2.2 Hz)	146.4	7.67
11	CH	135.9	6.93 d (J = 2.2 Hz)	107.1	6.78
12	C	155.8		125.7	
13	CH	142.9	7.55 s	114.1	7.29
16	CH_2	98.9	4.96 d (J = 2.2 Hz)	69.4	4.98
17	CH	148.9	5.54 m	119.7	5.56
18	C	169.0		139.1	
19	CH_3	53.9	1.69 m	17.8	1.79
20	CH_3	46.0	1.65 s	25.5	1.79

Compound **2** was obtained as a colorless needle and identified by spectroscopic analysis, including electron ionized mass spectrometry (EI-MS) (Fig. 10), ^{1}H NMR (Fig. 11), and ^{13}C NMR (Fig. 12). It showed a molecular ion at *m/z* 244 $[M]^{+}$ in EI-MS spectrum suggesting the molecular weight 244 Da. The structure of the side chain at position 8 was determined by ^{1}H NMR spectra. It indicated the presence of olefinic proton (H-2') at 5.17 ppm (m) with the same coupling constant (J = 7.3 Hz) as that of protons (H-1'). In the aromatic proton region, 2 pairs of doublets at δ 6.18 (J = 9.4 Hz) and 7.78 (J = 14.9 Hz) and at δ 6.96 (J = 8.6 Hz) and δ 7.36 (J = 8.6 Hz) were attributed to the protons at C3, C4, C10 and C9, respectively. The ^{1}H NMR spectrum of **2** also showed a pair doublets (J = 8.6 Hz) cis vinylic signal at δ 7.62, and δ 6.24 corresponding to the coumarin with substitutent at C-7 and C-8. The existence of isoprenyl chain of compound **2** showed the presence of two methyl groups (δ 1.80, and δ 1.62), one methylene group (δ 4.41), and one vinyl group (δ 5.17). The methoxyl group showed singlet proton signal at δ 3.48. And its ^{13}C NMR spectra showed 15 carbons in the molecule. This compound was characterized as osthole (**2**) (7-methoxy-8-(3-methylbut-2-enyl)-2-chromenone) (CAS no. 484-12-8). The interpretations of proton and carbon signals were largely consistent with those of Riviere *et al.* (2011). Osthole (**2**) was identified on the basis of the following evidence: colorless needle. UV (MeOH): λ_{max} nm = 208, 322. EI-MS (70 eV), *m/z* (% relative intensity): 244 $[M]^{+}$ (100), 229 (73), 214 (5), 201 (35), 189 (49), 131 (15). ^{1}H NMR and ^{13}C NMR spectral data of osthole is given in Table 11.

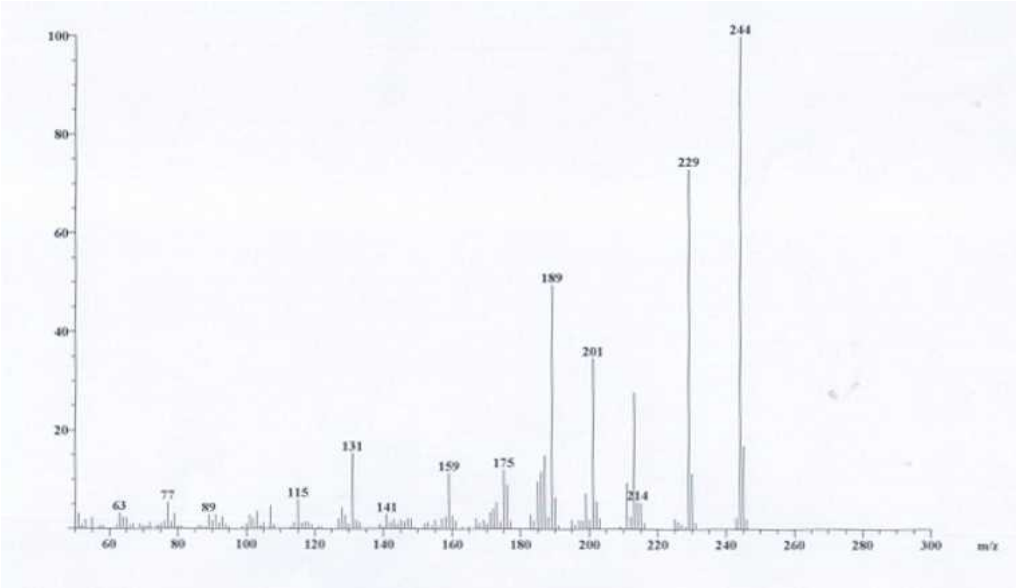


Fig. 10. EI-MS spectrum of compound 2.

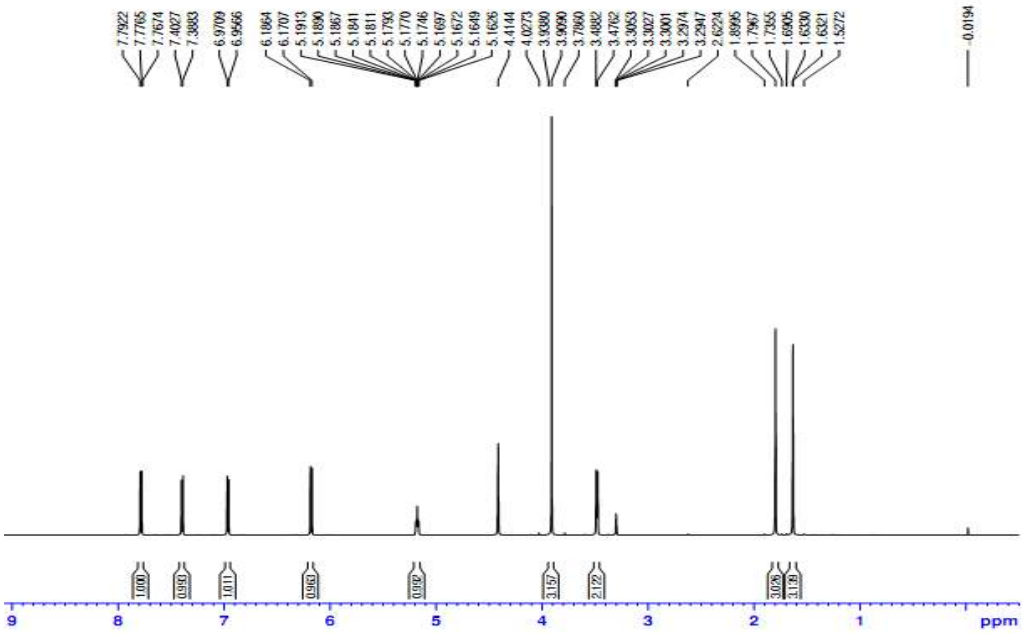


Fig. 11. ^{1}HNMR spectrum of compound 2.

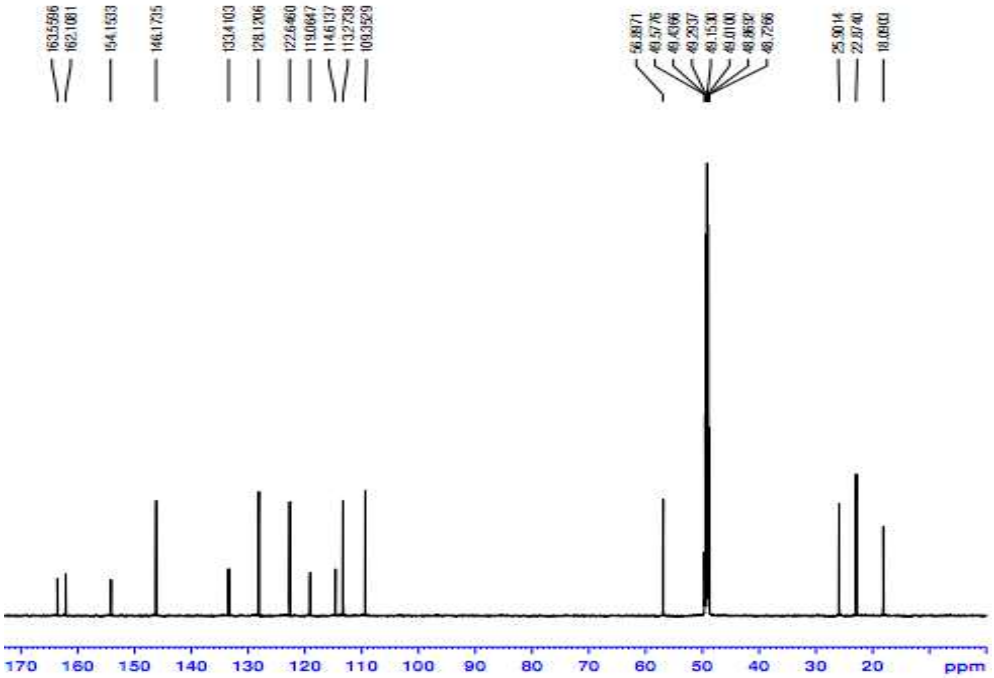


Fig. 12. ^{13}CNMR spectrum of compound 2.

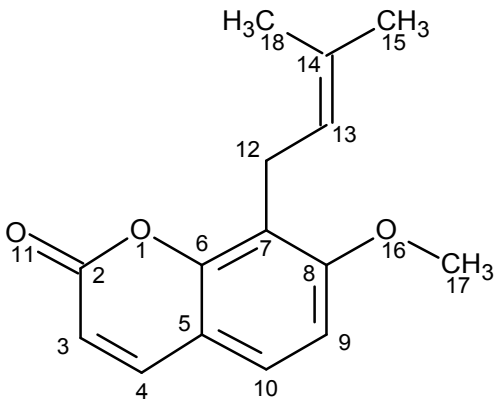


Fig. 13. Structure of osthole (2). The chemical formula of this compounds is $C_{15}H_{16}O_3$; the molar mass is 244.28 g/mol.

Table 11. ^{1}H-NMR (600 MHz) and ^{13}C-NMR (150 MHz) spectral data for compound 2

Position	Partial Structure	δ_C, ppm (MeOD, 150 MHz)	δ_H, ppm (MeOD, 600 MHz)	δ_C (ppm) (Riveere *et al*, 2006)	δ_H (ppm) (Riveere *et al*, 2006))
2	C	163.6		161.2	
3	CH	114.6	6.18 d (J = 9.4 Hz)	112.8	6.22 d (J = 9.5 Hz)
4	CH	146.2	7.78 d (J = 14.9 Hz)	143.6	7.60 d (J = 9.5 Hz)
5	C	113.3		113.2	
6	C	154.2		152.7	
7	C	119.1		117.8	
8	C	162.1		160.1	
9	CH	109.4	6.96 d (J = 8.6 Hz)	107.3	6.82 d (J = 8.6 Hz)
10	CH	128.1	7.40 d (J = 8.6 Hz)	126.1	7.28 d (J = 8.6 Hz)
12	CH_2	22.9	3.48 d (J = 7.2 Hz)	21.8	3.53 d (J = 7.3 Hz)
13	CH	122.6	5.17 m	121.1	5.22 t (J = 7.3 Hz)
14	C	133.4		132.4	
15	CH_3	25.9	1.80 s	25.6	1.64 s
17	CH_3	56.9	4.41 s	55.9	3.96 s
18	CH_3	18.1	1.62 s	17.8	1.83 s

Larvicidal activity of test coumarins

The toxicity of the two coumarins from the fruits of *C. monnieri* and 11 structurally related coumarins to third instar larvae from the KS-CP strain of *Cx. p. pallens* was investigated by direct-contact application (Table 12). Based on 24-hour LC_{50} values, imperatorin (**1**) (3.14 mg L^{-1}) was 1.9-, 3.7- and 4.2-fold more toxic than isopimpinellin, isoimperatorin, and osthole (**2**) against KS-CP larvae, respectively. Moderate toxicity was produced by angelicin, psoralen, 7-ethoxycoumarin, herniarin, and xanthotoxin (LC_{50} = 22.84–39.35 mg L^{-1}). Low toxicity was obtained from limettin, bergapten, and coumarin (LC_{50} = 57.03–73.95 mg L^{-1}). The toxicity of umbelliferone was the lowest of any of the coumarins examined. Overall, all of the compounds were at least 300-fold less toxic than temephos (LC_{50} = 0.011 mg L^{-1}) (Table 12).

The toxic effects of 13 test coumarins on YS-CP larvae were likewise compared (Table 12). Interestingly, all of the compounds were of equal toxicity against both KS-CP and YS-CP larvae, indicating a lack of cross-resistance in the YS-CP.

Against third instar larvae from *Ae. aegypti*, imperatorin (**1**) (2.88 mg L^{-1}) was 2.4, 4.5 and 4.6 times more toxic than isopimpinellin, isoimperatorin, and osthole (**2**), respectively, as judged by 24-hour LC_{50} values (Table 13). These compounds were less toxic than temephos (LC_{50} = 0.019 mg L^{-1}). Moderate toxicity was obtained from angelicin, psoralen, 7-ethoxycoumarin, herniarin, and xanthotoxin (LC_{50} = 26.40–45.11 mg L^{-1}). Low toxicity was observed with limettin, bergapten, and coumarin (LC_{50} = 60.50–76.35 mg L^{-1}). The toxicity of umbelliferone was the lowest of any of the coumarins.

Table 12. Contact toxicity of the 13 different coumarins to third instar larvae from the insecticide-susceptible KS-CP strain and wild YS-CP colony of *Cx. p. pallens* during a 24-h exposure

Compound	KS-CP larvae		YS-CP larvae	
	Slope ± SE	LC_{50}, mg L^{-1} (95% CL)	Slope ± SE	LC_{50}, mg L^{-1} (95% CL[a])
Linear FCs[b]				
Imperatorin	3.4 ± 0.51	3.14 (2.78–3.54)	2.3 ± 0.31	4.60 (3.22–5.63)
Isopimpinellin	2.7 ± 0.68	5.89 (4.51–6.80)	2.8 ± 0.35	6.85 (5.82–7.98)
Isoimperatorin	2.5 ± 0.35	11.70 (10.07–14.29)	2.3 ± 0.34	11.67 (9.48–14.81)
Psoralen	3.7 ± 0.69	26.64 (22.56–30.35)	3.7 ± 0.50	29.23 (25.73–33.26)
Xanthotoxin	4.6 ± 0.54	39.35 (36.24–42.90)	5.2 ± 0.96	43.13 (37.10–46.02)
Bergapten	5.3 ± 0.86	72.42 (67.03–78.97)	5.0 ± 0.79	70.64 (65.32–77.52)
Angular FC				
Angelicin	4.7 ± 0.71	22.84 (19.57–26.11)	3.2 ± 0.66	30.73 (24.21–39.92)
Simple coumarins				
Osthole	3.2 ± 0.44	13.11 (11.77–14.80)	2.4 ± 0.40	15.26 (12.39–18.56)
7-Ethoxycoumarin	3.9 ± 0.85	31.73 (27.13–37.00)	3.9 ± 0.87	34.82 (30.21–42.83)
Hemiarin	4.5 ± 0.71	39.27 (35.15–44.46)	4.7 ± 0.65	45.06 (41.01–51.17)
Limettin	4.9 ± 0.87	57.03 (51.48–62.71)	4.9 ± 0.71	53.82 (49.26–58.77)
Coumarin	3.9 ± 0.59	73.95 (65.98–83.66)	3.7 ± 0.55	83.20 (73.64–95.35)
Umbelliferone	3.9 ± 0.70	106.02 (93.38–122.61)	4.9 ± 0.94	124.18 (109.17–137.04)

[a] CL denotes confidence limit.

[b] Furanocoumarins.

Table 13. Toxicity of the 13 different coumarins and temephos to third-instar larvae from insecticide-susceptible *Ae. aegypti* during a 24-h exposure

Compound	Slope ± SE	LC_{50}, mg L^{-1}(95% CL[a])
Linear FCs[b]		
Imperatorin	1.8 ± 0.16	2.88 (2.40–3.33)
Isopimpinellin	2.6 ± 0.14	6.82 (6.32–7.34)
Isoimperatorin	2.3 ± 0.45	12.97 (10.45–17.94)
Psoralen	2.4 ± 0.48	34.22 (26.94–46.42)
Xanthotoxin	5.1 ± 0.81	45.11 (40.39–49.38)
Bergapten	5.6 ± 0.96	72.49 (65.43–81.55)
Angular furanocoumarin		
Angelicin	3.5 ± 0.53	26.40 (22.99–30.41)
Simple coumarins		
Osthole	2.3 ± 0.36	13.14 (11.11–16.70)
7-Ethoxycoumarin	3.4 ± 0.97	40.54 (34.39–51.26)
Hemiarin	2.6 ± 0.85	44.39 (34.86–59.58)
Limettin	4.7 ± 0.73	60.50 (55.37–67.23)
Coumarin	5.1 ± 0.70	76.35 (69.90–82.51)
Umbelliferone	5.0 ± 0.76	132.65 (121.36–145.90)
Larvicide		
Temephos	3.2 ± 0.40	0.019 (0.016–0.022)

[a] CL denotes confidence limit.

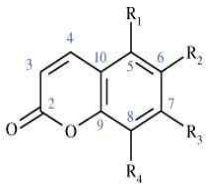
[b] Furanocoumarins.

Quantitative structure-activity relationship

Comparisons were made to determine toxicity differences involving the chemical structures and functional groups of the coumarins (Fig. 14), using the toxicity data obtained from Tables 12 and 13. Generally, furanocoumarins were more pronounced in toxicity than simple coumarins. Furanocoumarins having C8 alkoxy substituents such as imperatorin and isopimpinellin (except for xanthotoxin) were significantly more toxic than furanocoumarins lacking C8 alkoxy substituents but having C5-alkoxy substituents such as bergapten and isoimperatorin. Furthermore, the degree of toxicity appeared to be affected by the length of the C8 alkoxyl side chain. For example, imperatorin with an isoprenyl side chain at the C8 position was significantly more toxic than xanthotoxin and isopimpinellin, both of which have a methoxy substituent. A methoxy substitution at position C5 (bergapten) or C8 (xanthotoxin) caused a considerable reduction in toxicity relative to psoralen. Of the simple coumarin series examined, osthole having a C8 prenyl (dimethylallyl) side chain was significantly more toxic than coumarins with a simple benzopyrone ring moiety such as coumarin and umbelliferone. An alkoxy substitution in the C7 position (7-ethoxycoumarin, herniarinm, and limettin) of the coumarin nucleus enhanced the toxicity, whereas a hydroxy substitution in that position (umbelliferone) reduced the toxicity.

Correlation coefficient (r) analysis showed that MW, MR, and log *P* may be negatively correlated with LC_{50} (MW, r = -0.707 for *Cx. p. pallens* and r = -0.716 for *Ae. aegypti*; MR, r = -0.724 for *Cx. p. pallens* and r = -0.734 for *Ae. aegypti*; log *P*, r = -0.435 for *Cx. p. pallens* and r = -0.437 for *Ae. aegypti*).

Simple coumarins



Coumarin $R_1, R_2, R_3, R_4 = H$

Umbelliferone $R_1, R_2, R_4 = H; R_3 = OH$

Herniarin $R_1, R_2, R_4 = H; R_3 = OCH_3$

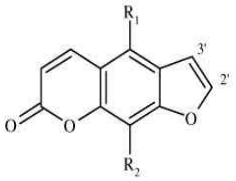
7-Ethoxycoumarin $R_1, R_2, R_4 = H; R_3 = OC_2H_5$

Limettin $R_1, R_3 = OCH_3; R_2, R_4 = H$

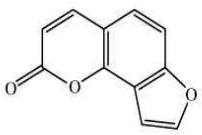
Osthole (**2**) $R_1, R_2 = H; R_3 = OCH_3$

$R_4 = CH_2CH=C(CH_3)_2$

Linear furanocoumarins



	R_1	R_2
Psoralen	H	H
Xanthotoxin	H	OCH_3
Bergapten	OCH_3	H
Isopimpinellin	OCH_3	OCH_3
Imperatorin (**1**)	H	$O\text{-}CH_2CH=C(CH_3)_2$
Isoimperatorin	$O\text{-}CH_2CH=C(CH_3)_2$	H



Angular furanocoumarin

Angelicin

Fig.14. Structures of the 13 different coumarins examined.

DISCUSSION

It is well recognized that certain plant preparations can be developed into products suitable for integrated mosquito management because they can be selective, biodegrade to nontoxic products and have few harmful effects on non-target organisms and are environmentally nonpersistent (Sukumar *et al*., 1991; Kurt *et al*., 1997; Shaalan *et al*., 2005; Isman, 2006).They also can be used in conjunction with biological control (Isman, 2006). They can be applied to mosquito breeding places in the same manner as conventional insecticides. Certain plant-derived materials and their constituents manifest larvicidal activity against different mosquito species (Sukumar *et al*., 1991; Park *et al*., 2002; Shaalan *et al*., 2005; Perumalsamy *et al*., 2010) and have been proposed as alternatives to conventional larvicides. For example, the isobutylamide alkaloids pellitorine, guineensine, pipercide, and retrofractamide A possess potent toxicity against larvae of *Cx. p. pallens*, *Ae. aegypti*, and *O. togoi* (Park *et al*., 2002). In the present study, a direct-contact mortality bioassay was used to identify the larvicidal constituents of *C. monnieri* fruit. The larvicidal principles were identified as the linear furanocoumarin imperatorin (**1**) and the simple coumarin osthole (**2**). These coumarins were highly effective against larvae of *Cx. p. pallens* and *Ae. aegypti*. In addition, they were also effective against *Cx. p. pallens* larvae resistant to various insecticides. This original finding indicates that the *C. monnieri* fruit-derived preparations containing imperatorin and osthole may hold promise for the development of novel and effective mosquito larvicides even against currently insecticide-resistant mosquito populations, although imperatorin (LC_{50} = 3.14 mg L^{-1} for KS-CP and 2.88 mg L^{-1} for *Ae. aegypti*) and osthole (13.11 and 13.14 mg L^{-1}) fail to meet the stage 3

criteria (LC_{50} < 1 mg L^{-1}) set by Shaalan *et al*. (2005). 4-Methyl-7-hydroxy coumarin is considered to be a lead molecule as a biopesticide, and its monobromo and tribromo derivatives were highly effective against larvae of *Ae. aegypti* and *Cx. quinquefasciatus* (Deshmukh *et al*., 2008). Coumarins are naturally occurring plant metabolites whose multiple bioactivities have been well documented by Hoult *et al*. (1996) and Santana *et al*. (2004).

The QSAR

s of phytochemicals in many insect pests have been well studied. For example, Tsao *et al*. (1995) found that the derivatization of the hydroxyl group in selected monoterpenoids and phenols enhanced biological activity. This was suggested to result from an increase in vapor pressure, which increased the fumigation activity and or increased the lipophilicity, which led to better penetration of the insect cuticle and availability in the insect's body. Park *et al* (2002) studied the toxicity of isobutylamide alkaloids (pellitorine, guineensine, pipercide, and retrofractamide A) and indicated that the *N*-isobutylamine moiety in the alkaloids might play a crucial role in the larvicidal activity. From the limited number of compounds examined in this study, the chemical characteristics, such as chemical structure (simple coumarin versus furanocoumarin), methoxy substitution at the C8 position of furanocoumarin (imperatorin and isopimpinellin versus bergapten and isoimperatorin), length of the C8 alkoxyl side chain (imperatorin versus xanthotoxin and isopimpinellin; osthole versus coumarin and umbelliferone) and methoxy substitution in the C7 position (7-ethoxycoumarin, herniarin, and limettin), of the coumarin nucleus appear to play a role in determining the coumarin toxicities to two mosquito species. The MW, hydrophobic parameter (log *P*), and MR were negatively related to the observed coumarin toxicities.

Calcagno *et al*. (2002) studied the feeding deterrence of five furanocoumarins on larvae of *Spodoptera littoralis* Boisduval, 1833 and suggested that a methoxy group on position C5 (bergapten) or C8 (xanthotoxin) enhanced the activity relative to psoralen or imperatorin. The difference between the present and the previous study might be attributable to the difference in the behavioral (feeding deterrence) and physiological (acute toxicity) characteristics as described by Isman (2006).

Investigations of the modes of action and resistance mechanisms of plant-based biocides are of practical importance for mosquito control because they may give useful information on the most appropriate formulations and delivery means to be adopted for their future commercialization and for future resistance management (Perumalsamy *et al*., 2010). Major mechanisms of resistance to insecticides currently available to control mosquitoes are target-site insensitivity, which reduces sodium channel sensitivity to pyrethroid insecticides or AChE sensitivity to OP and carbamate insecticides, as well as enhanced metabolism of various groups of insecticides (Hemingway *et al*., 2004; Li *et al*., 2007). Many of the insecticides currently used in the ROK have failed to control *Cx. p. pallens*, most probably because of the development of resistance (Kim *et al*., 2007; Perumalsamy *et al*., 2010). Alternative mosquito control agents with novel modes of action, low mammalian toxicity, low toxicity to aquatic non-target organisms and little aquatic environmental impact are urgently needed. In addition, certain plant constituents were found to be highly effective against insecticide-resistant mosquitoes, and they are likely to be useful in resistance management strategies. For example, the phenylpropanoids methyleugenol and α-asarone, the saturated hydrocarbon pentadecane, the lignan (–)-asarinin and the isobutylamide alkaloid pellitorine are effective against larvae from wild

Cx. p. pallens with extremely high to high levels of resistance to chlorpyrifos, fenitrothion, fenthion, α-cypermethrin, deltamethrin, and chlorfenapyr (Perumalsamy *et al*., 2010). The present findings that seven furanocoumarins and six simple coumarins examined are virtually equal in toxicity to both insecticide-susceptible and insecticide-resistant larvae of *Cx. p. pallens* suggest that these coumarins and the pyrethroid and OP insecticides do not share a common mode of action or elicit cross-resistance. Detailed tests are needed fully to understand the modes of action of the coumarins, although the octopaminergic and γ-aminobutyric acid receptors have been suggested as novel target sites for some monoterpenoid essential oil constituents by Kostyukovsky *et al*. (2002) and Priestley *et al*. (2003), respectively.

In conclusion, *C. monnieri* fruit-derived preparations containing imperatorin and osthole could be useful as larvicides in the control of mosquito populations, particularly in the light of their activity against insecticide-resistant mosquito larvae. However, these compounds can be cytotoxic (Hitotsuyanagi *et al*., 1996; Luszczki *et al*., 2009). For practical use of these materials as novel mosquito larvicides to proceed, further research is needed to establish safety to humans, although historically *C. monnieri* fruit has been used as a herbal anti-itch lotion, as a remedy for various skin ailments and as a reproductive and aphrodisiac (Namba, 1993). In addition, their effects on non-target organisms 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 II

Larvicidal Activity of *Magnolia denudata* Seed Constituents against Insecticide-Susceptible *Culex pipiens pallens* and *Aedes aegypti* as well as Wild *Aedes albopictus* and *Anopheles sinensis*

INTRODUCTION

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 the ROK have failed to control the mosquito species in the field, most probably because of the development of resistance (Kim *et al*., 2007; Chang *et al*., 2009; Shin *et al*., 2012). Increasing public concern for the environmental effects of insecticides including groundwater contamination, human health effects, and undesirable effects on non-target organisms intensifies when repeated applications of conventional insecticides become necessary (Romeis *et al*., 2008). These problems substantiate the need for the development of new improved alternatives, with novel target sites to establish a rational resistance management strategy and tactics based on all available information on the extent and nature of resistance in mosquito.

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. Complex mixtures exerting synergistic or potentiating actions are considered to have higher and longer-lasting effects through various mechanisms than pure compound alone (Berenbaum 1985, Hummelbrunner and Isman 2001). Previous studies have shown that a hydrodistillation from the seeds of yulan magnolia (lily tree), *Magnolia denudata* (Magnoliaceae), possessed

good larvicidal activity toward *Culex pipiens pallens* and *Aedes aegypti*. No information is available concerning the potential of binary mixtures of the constituents from *M. denudata* seed for managing mosquitoes, although joint toxic effects of insecticides with plant extracts on different mosquito species have been well-noted (Shaalan *et al*., 2005). *Magnolia* species have a distribution in East and Southeast Asia (Xu *et al*., 2008). *M. denudata* contains many phenylpropanoids, terpenoids, alkaloids, lignans and neolignans (Kelm *et al*., 2000; Noshita *et al*., 2009). Traditional and folk medicine uses of *Magnolia* species have been well documented by Kelm and Nair (2000) and Li *et al*. (2013).

The aim of the study was to assess the contact toxicity of a lignin honokiol and two fatty acids (linoleic acid and palmitic acid) from *M. pinnata* seed extract and 17 constituents from *M. denudata* seed hydrodistillation (MD-SHD) to third instar larvae from insecticide-susceptible *Cx. p. pallens* and *Ae. aegypti*, as well as wild *Aedes albopictus* and *Anopheles sinensis*. Results were compared with those of the four conventional insecticides fenitrothion, fenthion, temephos, and deltamethrin. The efficacy of four liquid formulations (10, 20, 30, and 50 mg L^{-1} liquids) containing the seed hydrodstillate was compared with that of the commercial larvicide temephos 200 g L^{-1} emulsifiable concentrate (EC) because the larvicide has low toxicity to mammals and aquatic organisms and is less persistent in the environment (Opong-Mensah, 1984). Quantitative structure-activity relationships (QSAR) of the most toxic constituent 2, 4-di-*tert*-butylphenol (DTBP) and its 13 analogues were also discussed. Lastly, the potential of honokiol alone or in combination with linoleic acid and palmitic acid at five tested ratios (1:1, 1:2, 1:3, 2:1, and 3:1) was evaluated toward third instar larvae from insecticide-susceptible and -resistant *Ae. albopictus* and *An. sinensis*

MATERIALS AND METHODS

Instrumental analysis

^{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 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 TLC. An Isolera One medium-pressure liquid chromatography (Biotage, Uppsala, Sweden) and an Agilent 1200 high-performance liquid chromatography were used for isolation of active principles.

Chemicals

The 28 commercially-available organic pure compounds used in this study are listed in Table 6, along with their sources and purity. For the QSAR analysis, values of molecular weight (MW), hydrophobic parameter (log *P*), and steric effects for 2,4-DTBP and its 13 analogues examined were obtained from ACD/ChemSketch (ACD/LAB 12.0 for Microsoft Windows; Advanced Chemistry Development, Inc., Montreal, Canada) and are recorded in Table 14. Molecular refraction (MR) was used as the parameter for describing steric effects. The insecticides used in this study were as follows: deltamethrin (99.0% purity) purchased from Supelco (West Chester, PA); fenitrothion (98.5%), fenthion (95.5%), and temephos

(97.3%) purchased from Sigma-Aldrich (St. Louis, MO, USA). A commercially-available mosquito larvicides examined in this study was temephos 200 g L^{-1} EC (Dongsung Pharmaceuticals, Asan, Chungnam, ROK). Triton X-100 was obtained from Shinyo Pure Chemicals (Osaka, Japan). Polyoxyethylene + polyoxypropylene (9:1) styrenated phenyl ether, a surfactant, was a gift from Hannong Chemical (Anyang, Gyeonggi, ROK). All of the other chemicals used in this study were of reagent-grade quality and available commercially.

Plant material

Fresh mature fruits of *M. denudata* were collected in August 2012 from Huazhong Agriculture University garden (Wuhan, Hubei, China), and were identified by a certified botanical taxonomist. A voucher specimen (MD-SD-01) was deposited in the Research Institute of Agriculture and Life Sciences, College of Agriculture and Life Sciences, Seoul National University.

Hydrodistillation

Seeds (200 g) of *M. denudata* were pulverized and subjected to hydrodistillation at 100°C for 2 hours 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 hydrodistillation from the seeds was 0.12 ± 0.002% based on dried weight of the plant.

Table 14. The 30 organic pure compounds examined in this study

Compound	Source[a]	Purity (%)	Compound	Source[a]	Purity (%)
Behenic acid[b]	TCI	>95	*n*-Hexadecane[b]	S-A	99
2-*sec*-Butylphenol[c]	S-A	98	α-Humulene[b]	S-A	≥96
4-*sec*-Butylphenol[c]	TCI	96	4-Isopropylphenol[c]	TCI	>99
2-*tert*-Butylphenol[c]	S-A	99	2-Isopropyl-5-MP[c]	WK	98
3-*tert*-Butylphenol[c]	TCI	>98	5-Isopropyl-2-MP[c]	WK	95
4-*tert*-Butylphenol[c]	TCI	>98	(±)-Limonene[b]	TCI	>95
2,4-Di-*tert*-butylphenol[b]	S-A	99	Linoleic acid[b]	TCI	90
2,6-Di-*tert*-butylphenol[c]	S-A	99	Methyl linolelaidate[b]	TCI	98
2,4,6-Tri-*tert*-butylphenol[c]	S-A	96	Nerolidol[b]	S-A	98
4-*sec*-Butyl-2,6-di-*tert*-BP[c]	S-A	>98	Palmitic acid[b]	S-A	99
2-*tert*-Butyl-4-methylphenol[c]	S-A	99	Phenol[c]	S-A	99
β-Caryophyllene[b]	TCI	>90	(±)-Terpinen-4-ol[b]	TCI	95
p-Cymene[b]	S-A	>99	α-Terpinene[b]	S-A	85
Ethyl palmitate[b]	S-A	≥99	γ-Terpinene[b]	S-A	97
Geranic acid[b]	S-A	85	α-Terpineol[b]	S-A	90

[a] S-A, Sigma-Aldrich (St. Louis, MO); TCI, Tokyo Chemical Industry (Tokyo, Japan); WK, Wako (Osaka, Japan).

[b] Constituents identified in this study.

[c] Compounds examined for QSAR.

Mosquitoes

The stock cultures of *Cx. p. pallens* and *Ae. aegypti* have been maintained as described in Chapter 1. Engorged *Anopheles* and *Ae. albopictus* females were collected from rice paddy fields and cowsheds in Osong (Chungbuk, ROK) and bamboo forest near a village in Jeonju (Jeonbuk, ROK), respectively, from late July to late August 2011 using a D-CELL collecting aspirator (Gemplers, Janesville, WI, USA) and black light FL-6w traps (Shinyoung, Seoul, ROK), respectively. The rice paddy fields and cowsheds had varying histories of insecticide use, while no insecticide had been applied to bamboo forest. They have been separately maintained in temperature-controlled insect rearing rooms (Seoul National University) to prevent cross-contamination. Females were placed individually in paper cups (270 mL) lined with filter paper and filled with 150 mL distilled water. Larvae were separately reared in plastic trays (24 × 35 × 5 cm) containing 0.5 g of sterilized diet (40-mesh chick chow powder/yeast, 1/1 by weight) from Sewhapet (Inchon, ROK). Adults were maintained on a 10% sucrose solution and were allowed to blood fed on mice. All stages were maintained at 27 ± 1°C and 65–75% RH under a 16:8 hour light:dark cycle. The wild mosquitoes were reared for 4-5 generations to ensure sufficient numbers for testing.

Polymerase chain reaction (PCR) identification of *Anopheles* species

Primer pairs described in Table 15 were purchased from Bioneer Corporation (Daejeon, Republic of Korea). The PreMIx (Bioneer AccuPower PCR PreMix) was performed using the product performed for 3 min at 94°C condition to maintain the first one conducted, 94°C for 30 s, 55°C for 30 s, 72°C for 2 min then 30 cycles of conditions the final 7 min

to maintain the reaction at 72°C will be terminated. Mixture obtained on a 1.5% agarose (Agarose) gel electrophoresis performed in the reactant (DNA) confirmed the size of the. Mosquito wing smear China (*An. sinensis*), the band size 500 bp (Fig. 15) appears as a gray stain wing mosquitoes (*An. pullus*), the band will appear as the size of 240 bp. Species identification for *Anopheles* females confirmed that females from the wild collections were *An. sinensis*.

Table 15. Primer sequence used for polymerase chain reaction in this study

Species	Primer	Dignostic band (bp)
Universal		
Forward	TGT GAA CTG CAG GAC ACA TGA A	
Species-specific reverse		
An. sienesis	ATT GTT GTC CAG CCC GCT AAC	500
An. pullus	ATA TCA TGG CTT AAC ACC GCG T	240
An. kleini	GCG TCC ATA CTG TCT CAA CGA	400
An. lesteri	TGC CTA GAA CTT CCG CCA ATC	300
An. belenrae	CAT TTT TCA CGA CTG CGA CGG	190

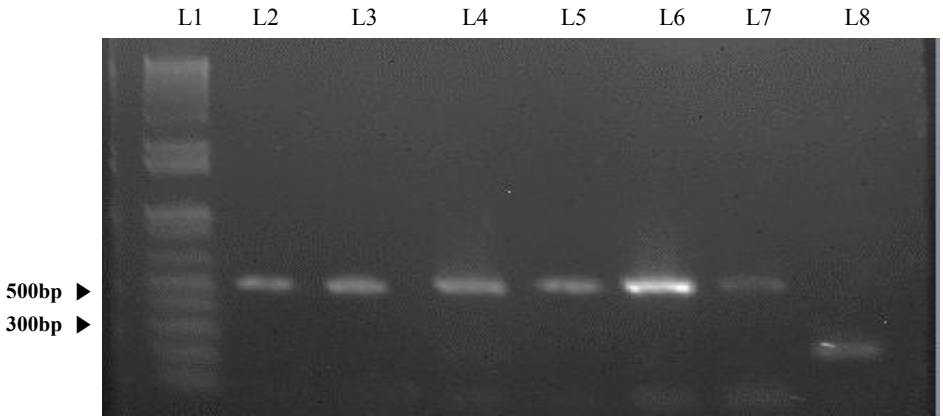


Fig. 15. DNA electrophoresis of *Anopheles* species collected from Osong (Chungbuk, ROK). Lane 1-1 kb plus ladder. Lane 2-7 were *Anopheles* species collected. PCR-Based Identification of *An. sinensis* at 500 bp (Lane 2-7) and *An. pullus* at 240 bp (Lane 8).

Experimental liquid formulations

Four experimental liquid formulations containing MD-SHD were formulated to determine the effective mosquito larvicide products. The 1% (10 mg L^{-1}), 2% (20 mg L^{-1}), 3% (30 mg L^{-1}) and 5% (50 mg L^{-1}) liquid products containing the hydrodistillation were, respectively, composed of 1, 2, 3, or 5% of the hydrodistillation; 2% surfactant (polyoxyethylene + polyoxypropylene (9:1) styrenated phenyl ether); 5% ethanol; and sterile distilled water at 92.5, 92, 91, and 90%.

Chromatographic analysis

An Agilent 7890A gas chromatography (GC) system (Agilent, Palo Alto, CA, USA)

equipped with a split injector and a flame ionization detection (FID) system, was used to separate and detect the constituents of MD-SHD. Constituents were separated with an Agilent 30 m × 0.32 mm i.d. (d_f = 0.25 μm) HP-5 capillary column (J&W Scientific, Folsom, CA). The oven temperature was kept at 50°C (5 min isothermal) and programmed to 280°C at a rate of 5°C min^{-1}, then isothermal at 280°C for 10 min. The linear velocity of the nitrogen carrier gas was 19.15 cm s^{-1} (at 50°C) at a split ratio of 5:1. The injector temperature was 280°C. Chemical constituents were identified by coelution of authenticated samples following coinjection.

Gas chromatography-mass spectrometry (GC-MS) analysis was performed using a Clarus 680/680 T gas chromatograph-mass spectrometer (PerkinElmer, Fort Belvoir, VA, USA). An Agilent 30 m × 0.25 mm i.d. (d_f = 0.5 μm) DB-5MS capillary column was employed. The oven temperature was kept at 60 °C (5 min isothermal) and programmed to 260°C at a rate of 2°C min^{-1}. Flow rate of the helium carrier gas was 1.0 mL min^{-1}. The ion source temperature was 250°C, and mass spectra were obtained in EI-scan mode at 70 eV electron energy. The sector mass analyzer was set to scan from 35 to 550 amu every 0.2 s. Chemical constituents were identified by comparison of mass spectra of each peak with those of authentic samples in a mass spectrum library (Anonymous, 2008).

Isolation of active constituents from *Magnolia denudata*

The air-dried seed (1.5 kg) of *M. denudata* was pulverized, extracted with methanol (5 × 3 L) at room temperature for 2 days, and filtered through Whatman no.2 filter paper (Whatman, Maidstone, UK). The combined filtrate was concentrated to dryness by rotary

evaporation at 40°C to yield approximately ~266.4g of a dark olive brown tar. The extract (20 g) was sequentially partitioned into hexane- (3.11 g), chloroform- (5.10 g), ethyl acetate- (6.09 g), butanol- (1.53 g) and water-soluble (4.17 g) portions for subsequent bioassay (Fig. 13). 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^{-1} of each *M. denudata* seed-derived material was applied to a direct-contact mortality bioassay as described previously (Kim *et. al*., 2008).

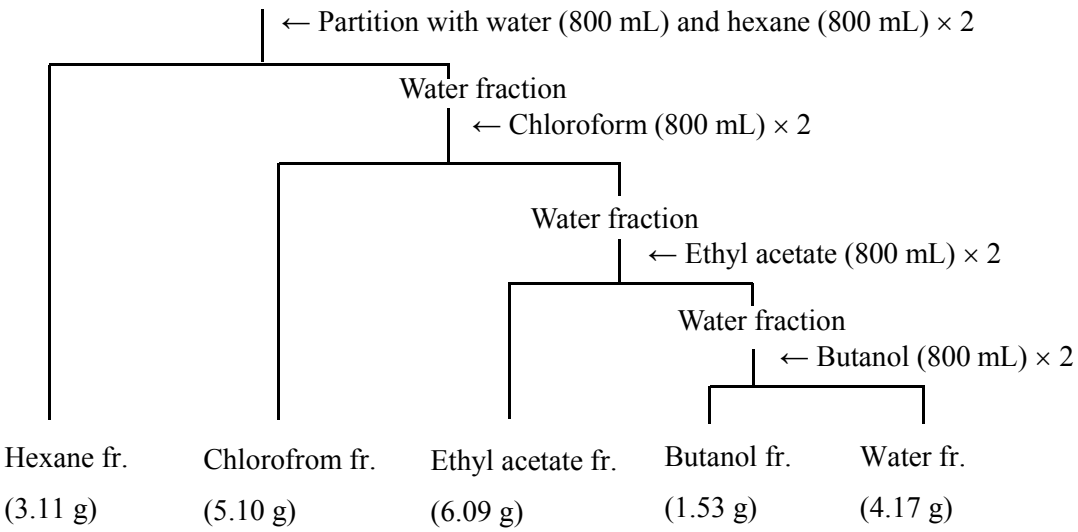


Fig. 16. Solvent fractionation procedures of methanol extract from the seed of *M. denudata*.

The hexane-soluble fraction (32.4 g) was most biologically active and medium-pressure liquid chromatography (MPLC) was performed using an Isolera apparatus equipped with a UV detector at 254 and 365 nm and a column cartridge SNAP (100 g silica gel) with column

volume of 132 mL. Separation was achieved with a gradient of hexane and ethyl acetate (100:0, 98:2, 90:10, 80:20, 70:30, 60:40, 50:50, 60:40, and 0:100 by volume) and finally with methanol (1 L) at a flow rate of 30 mL min^{-1} to provide 42 fractions (each about 100 mL) (Fig. 17). Column fractions were monitored by TLC on silica gel developed with chloroform and methanol (8:2 by volume) mobile phase. Fractions with similar R_f values on the TLC plates were pooled. Spots were detected by spraying with 2% sulfuric acid and the heating on a hot plate. The active fractions 3 (H2, 10.5 g) and 14–18 (H6, 2.2 g) were obtained. Fraction H2 was separated by MPLC with a UV detector at 254 and 360 nm and column cartridge (100 g silica gel) by elution with a gradient of hexane and ethyl acetate (98:2, 97:3, 96:4, 95:5, 90:10, 87:13, 83:17, 80:20, 73:17, 65:35, 60:40, and 50:50 by volume) and finally with methanol (1 L) at a flow rate of 50 mL min^{-1} to provide 233 fractions (each about 22 mL). Column fractions were monitored by TLC on silica gel plates, as stated previously. Active fractions 103–125 (H22, 1 g) was obtained. Fraction H22 was separated by MPLC with a UV detector at 254 and 280 nm and a column cartridge (25 g silica gel) by elution with a gradient of chloroform and methanol (100:0, 99:1, 98:2, 97:3 and 80:20 by volume) and finally with methanol (1 L) at a flow rate of 15 mL min^{-1} to provide 70 fractions (each about 20mL). The active fractions 8 to 45 (H222, 926 mg) was obtained. Preparative TLC (chloroform:methanol (98:2) by volume) of fraction H222 to provide two fractions. Finally, a potent active principle **1** (21.4 mg) was obtained. As shown in Fig. 18, **1** is obtained with a radiochemical purity greater than 97.5%

Fraction H6 was separated by MPLC with a UV detector at 254 and 360 nm and column cartridge (100 g silica gel) by elution with a gradient of chloroform and methanol (100:0, 99:1, 98:2, 97:3, 96:4, 93:7, 90:10, 89:11 and 60:40 by volume) and finally with methanol

(0.5 L) at a flow rate of 25 mL min^{-1} to provide 204 fractions (each ~22 mL). Column fractions were monitored by TLC on silica gel plates, with Hexane and Ethyl acetate (9:1 by volume) mobile phase. The active fractions 4–21 (H62, 488 mg) and 45–51 (H64, 1318 mg) were obtained. Fraction H62 was separated by MPLC with a UV detector at 254 and 280 nm and column cartridge (100 g silica gel) by elution with a gradient of hexane and ethyl acetate (96:4, 94:6, 90:10, 85:15, 80:20, 77:23, 73:27, 60:40, and 40:60 by volume) and finally with methanol (350 mL) at a flow rate of 40 mL min^{-1} to provide 110 fractions (each about 22 mL). Column fractions were monitored by TLC on silica gel plates, with Hexane and Ethyl acetate (8:2 by volume) mobile phase. The active fractions 57–77 (H623) (203 mg) was obtained. Fraction H623 was separated by MPLC with a UV detector at 254 and 365 nm and column cartridge (25 g silica gel) by elution with a gradient of hexane and ethyl acetate (100:0, 96:4, 90:10, 85:15, 82:18, 81:19, 75:25, 70:30, 57:43, and 20:80 by volume) and finally with methanol (80 mL) at a flow rate of 25 mL min^{-1} to provide 68 fractions (each about 22 mL). The active fractions 8–16 (H6232, 86 mg) was obtained. A preparative high-performance liquid chromatography (HPLC) was used for further separation of the constituents from the fraction H6232. The column was a 7.8 mm i.d. × 300 mm μBondapak C18 (Waters, Milford, MA, USA) with a mobile phase of acetonitrile and water (93:7 by volume) at a flow rate of 1 mL min^{-1}. Chromatographic separations were monitored using a UV detector at 210 nm. Finally, an active principle **2** (28 mg) was isolated at a retention time of 19. 81 min (Fig. 19).

Fraction H64 was separated by MPLC with a UV detector at 254 and 280 nm and column cartridge (100 g silica gel) by elution with a gradient of hexane and ethyl acetate (96:4, 94:6, 90:10, 85:15, 80:20, 77:23, 73:27, 60:40, and 40:60 by volume) and finally with

methanol (350 mL) at a flow rate of 40 mL min^{-1} to provide 110 fractions (each about 22 mL). Column fractions were monitored by TLC on silica gel plates, with Hexane and Ethyl acetate (8:2 by volume) mobile phase. The active fractions 15–25 (H642, 260 mg) was obtained. Fraction H643 was separated by MPLC with a UV detector at 254 and 365 nm and column cartridge (100 g silica gel) by elution with a gradient of chloroform and ethyl acetate [100:0, 99:1, 98:2, 97:3, and 96:4 by volume] and finally with methanol (300 mL) at a flow rate of 40 mL min^{-1} to provide 147 fractions (each about 22 mL). The active fractions 60–72 (H6433, 295.13 mg) was obtained. Preparative TLC [chloroform: ethyl acetate (80:20) by volume] of fraction H64333 to provide four fractions. A preparative HPLC was used for further separation of the constituents from the fraction H64333, as stated previously. Finally, a potent active principle **3** (43.25 mg) were isolated at a retention time of 13.57 min (Fig. 20).

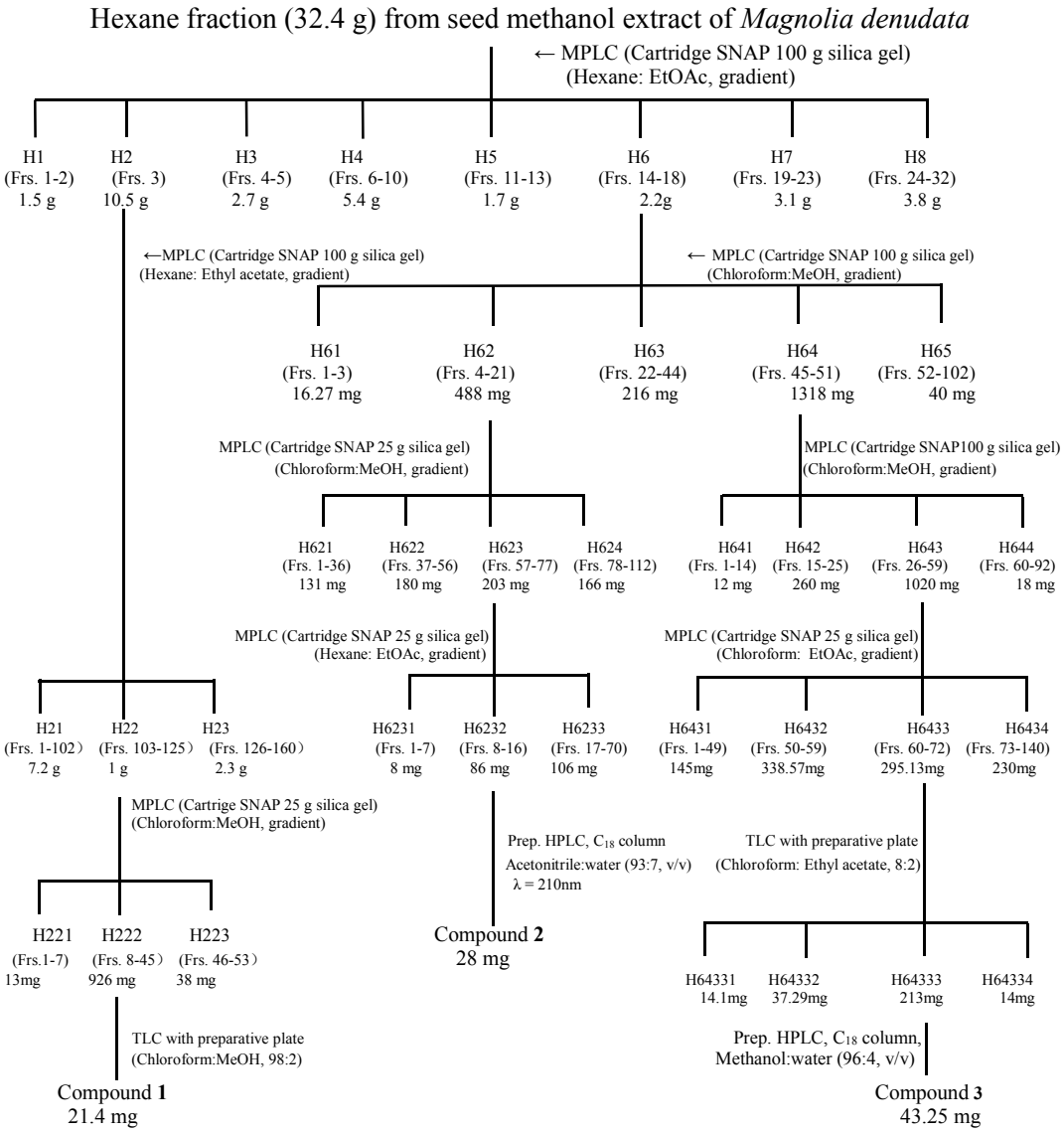


Fig. 17. Isolation procedures of larvicidal principles. The *M. denudata* seed methanol extract was sequentially partitioned into hexane-, chloroform-, ethyl acetate-, butanol-, and water-soluble portions. For isolation of active principles from the hexane-soluble fraction, 50 mg L^{-1} of each *M. denudata* seed-derived material was tested in a direct-contact mortality bioassay toward third instar larvae from *Cx. p. pallens*.

Bioassay

A direct-contact mortality bioassay was used to evaluate the toxicity of all compounds to third instar larvae from the insecticide-susceptible and wild mosquito populations (Kim *et al*., 2007). Each test compound in acetone (for deltamethrin) or methanol (for the other materials) was suspended in distilled water with Triton X-100 (20 μL L^{-1}). Groups of 20 mosquito larvae were separately put into paper cups (270 mL) containing each test compound solution (250 mL). The OPs fenitrothion, fenthion, and temephos served as positive controls and were similarly formulated. Negative controls consisted of the acetone- or methanol-Triton X-100 solution in distilled water. Based on the preliminary test results, the toxicity of each compound and insecticide was determined with four to six concentrations ranging from 0.1 to 400 mg L^{-1} and 0.0001 to 0.1 mg L^{-1}, respectively. Treated and control (acetone- or methanol-Triton X-100 solution only) larvae were held under the same conditions as those used for colony maintenance for 24 hours. A larva was considered dead if it did not move when prodded with a fine wooden dowel (Park *et al*., 2002). 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 (Robertson *et al*., 2007). Freshly prepared compound solutions were used for each block of bioassays. All treatments were replicated three times using 20 larvae per replicate.

The toxicity of honokiol alone or in combination with linoleic acid or palmitic acid at five tested ratios (1:1, 1:2, 1:3, 2:1, and 3:1) to third instar larvae from insecticide-susceptible and -resistant *Ae. albopictus* and *An. sinensis* was likewise elucidated. Each compound and binary mixture in methanol was suspended in distilled water with Triton X-

100 (20 μL L^{-1}). The toxicity of each tested material was determined with four to six concentrations ranging from 0.1 to 200 mg L^{-1}. Controls received methanol-Triton X-100 solution in distilled water. Mortalities were recorded as stated previously. All treatments were replicated three times using 20 larvae per replicate.

In separate experiments, the efficacy of the four experimental liquid formulations was evaluated as stated previously. Groups of 20 mosquito larvae were separately put into paper cups containing each test solution. Temephos 200 g L^{-1} EC served as a positive control. Negative controls consisted of the polyoxyethylene + polyoxypropylene (9:1) styrenated phenyl ether solution in distilled water or water. Mortalities were recorded as stated previously.

Data analysis

Data were corrected for control mortality using Abbott's (1925) formula. Mortality percentages were transformed to arcsine square root values for analysis of variance (ANOVA). The Bonferroni multiple-comparison method was used to test for significant differences among the treatments (SAS Institute, 2004). Means ± SE of untransformed data are reported. Concentration-mortality data were subjected to probit analysis (SAS Institute, 2004). The LC_{50} values for each mosquito species and their treatments were considered to be significantly different from one another when 95% confidence limits did not overlap. A compound having LC_{50} >100 mg L^{-1} was considered to be ineffective as described previously by Kirin *et al*. (2006). The relative susceptibility ratio (RSR) was defined as the ratio of LC_{50} of third instar larvae from wild *Ae. albopictus* or *An. sinensis* to LC_{50} of third

instar larvae from *Ae. aegypti* (Chang *et al*., 2014). RSR values of <10, 10–40, 40–160, and >160 were classified as low, moderate, high, and extremely high, respectively (Chang *et al*., 2014). The co-toxicity coefficient (CC) and synergistic factor (SF) were calculated according to the methods of Sun and Johnson (1960), Kalyanasundaram and Das (1985), respectively. Values of SF <1 indicate synergism and SF >1 indicate antagonism.

RESULTS

Comparative toxicity of test insecticides

The toxicity of four insecticides examined to third instar larvae of insecticide-susceptible *Cx. p. pallens* and *Ae. aegypti* was evaluated using a direct-contact mortality bioassay (Table 16). Deltamethrin (24-h LC_{50}, 0.0028 and 0.0034 mg L^{-1}) was the most toxic insecticide, followed by temephos, fenthion, and fenitrothion. Mortality in the ethanol-Triton X-100-water-treated controls was less than 2%.

The toxic effects of all insecticides on third instar larvae of wild *Ae. albopictus* and *An. sinensis* were likewise compared (Table 16). *An. sinensis* larvae were less susceptible than either *Ae. aegypti* or *Ae. albopictus* larvae. The RSR varied according to the insecticides. RSR of the four insecticides was between 1.0 and 2.0 for *Ae. albopictus* larvae. For *An. sinensis* larvae, extremely high RSR (611) was observed with deltamethrin while moderate RSR was obtained from fenthion and temephos. Low RSR was produced by fenitrothion.

Table 16. Toxicity of four test insecticides to third instar larvae from insecticide-susceptible *Cx. p. pallens* and *Ae. aepypti* and from wild *Ae. albopictus* and *An. sinensis* during a 24-h exposure

	$LC_{50,}$ mg L^{-1} (95% CL)					
Insecticides	*Cx. p. pallens* larvae	*Ae. aepypti* larvae	*Ae. albopictus* larvae	RSR	*An. sinensis* larvae	RSR
Temephos	0.0116 (0.0107–0.0125)	0.0121 (0.0105–0.0139)	0.0135 (0.0121–0.0151)	1.1	0.3554 (0.2992–0.4258)	29.4
Fenthion	0.0234 (0.0221–0.0248)	0.0290 (0.0273–0.0307)	0.0311 (0.0287–0.0334)	1.1	0.3220 (0.2748–0.3839)	11.1
Fenitrothion	0.0261 (0.0215–0.0312)	0.0399 (0.0373–0.0425)	0.0417 (0.0389–0.0444)	1.0	0.0783 (0.0718–0.0860)	2.0
Deltamethrin	0.0028 (0.0024–0.0033)	0.0034 (0.0027–0.0036)	0.0068 (0.0060–0.0078)	2.0	2.0780 (1.6784–2.5732)	611.2

Composition of *Magnolia denudata* seed hydrodistillation

MD-SHD was composed of nine major (≥3%) and 18 minor constituents by comparison of mass spectral data and co-elution of authenticated samples following co-injections (Table 17). The nine major constituents were β-caryophyllene (peak no. 7), unknown (peak no. 21), unknown (peak no. 20), methyl linolelaidate (peak no. 25), palmitic acid (peak no. 26), behenic acid (peak no. 24), α-humulene (peak no. 8), unknown (peak no. 13) and unknown (peak no. 9), and comprised 18.89, 11.35, 9.53, 9.25, 7.76, 5.27, 5.15, 4.13 and 3.13% of the hydrodistillation, respectively (Fig. 21).

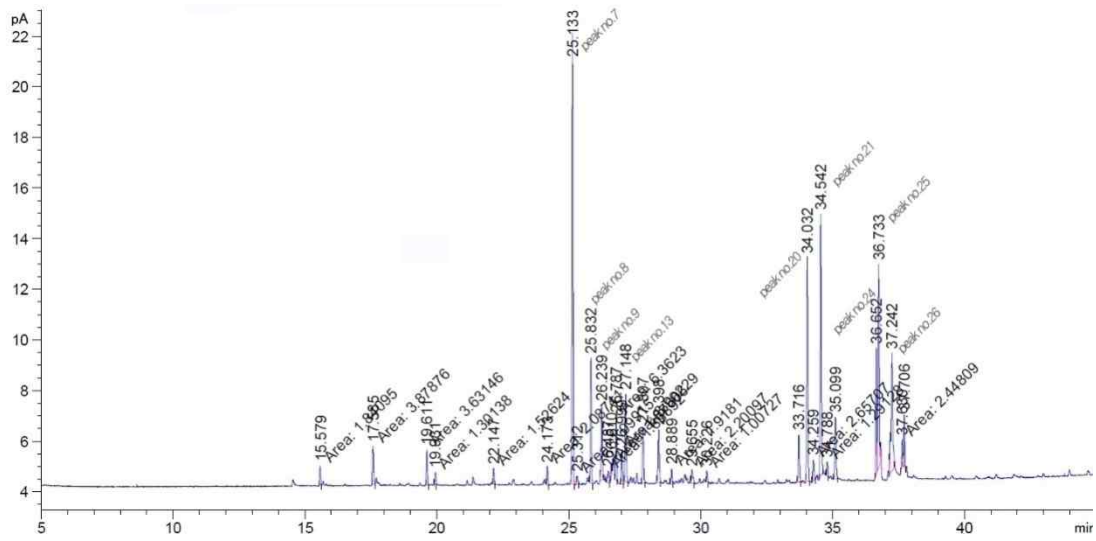


Fig. 21. Gas chromatogram of steam distillate from *Magnolia denudata* seed.

Toxicity of *Magnolia denudata* seed hydrodistillation constituents

The toxicity of MD-SHD and 17 compounds examined to third instar *Cx. p. pallens* larvae was evaluated (Table 9). Responses varied according to compound tested. As judged by 24-h LC_{50} values, MD-SHD was highly toxic (19.60 mg L^{-1}). 2,4-DTBP (LC_{50}, 1.98 mg L^{-1}) was the most toxic compound and was 171, 85 and 76 times less toxic than temephos, fenthion and fenitrothion respectively (Table 10). High toxicity was also obtained from linoleic acid, nerolidol and (±)-limonene (LC_{50}, 7.28–14.05 mg L^{-1}). LC_{50} of α-terpinene, γ-terpinene, *p*-cymene, palmitic acid, α-terpineol and methyl linolelaidate was between 21.30 and 35.51 mg L^{-1}. LC_{50} of β-caryophyllene, geranic acid, (±)-terpinen-4-ol and ethyl palmitate was between 44.99 and 56.39 mg L^{-1}. Low toxicity were produced by α-humulene, behenic acid and *n*-hexadecane.

Table 17. Chemical composition of *M. denudata* seed hydrodistillate

Peak number	Compound	RT[a] (min)	% area in GC[b]-FID	Identification	
				CI[c]	MS[d]
1	*p*-Cymene	15.58	1.03	O	O
2	Limonene	17.59	1.49	O	O
3	Terpine-4-ol	19.61	1.50	O	O
4	α-Terpinene	19.93	0.55	O	O
5	α-Terpineol	22.15	0.65	O	O
6	γ -Terpinene	24.18	0.86	O	O
7	β-Caryophyllene*	25.13	18.89	O	O
8	α-Humulene*	25.83	5.15	O	O
9	Unknown*	26.24	3.13		
10	Unknown	26.49	0.64		
11	Geranic acid	26.70	1.71	O	X
12	2,4-Di-*tert*-butylphenol	26.79	2.43	O	O
13	Unknown*	27.15	4.13		
14	Unknown	27.81	2.38		
15	Unknown	28.40	2.49		
16	Unknown	28.89	0.91		
17	Unknown	29.65	0.80		
18	Unknown	30.23	0.60		
19	Nerolidol	33.72	2.18	O	X
20	Unknown*	34.03	9.53		
21	Unknown*	34.54	11.35		
22	*n*-Hexadecane	34.79	0.60	O	O
23	Ethyl palmitate	35.10	2.79	O	X
24	Behenic acid*	36.65	5.27	O	X
25	Methyl linolelaidate*	36.73	9.25	O	X
26	Palmitic acid*	37.24	7.76	O	X
27	Linoleic acid	37.71	1.93	O	X

[a] Retention time of GC-FID

[b] Gas-liquid chromatography with flame ionisation detection.

[c] Co-injection with authentic samples.

[d] Mass spectrometry.

* Major constituent (>3%).

Toward third instar *Ae*. *aegypti* larvae (Table 10), 2, 4-DTBP (24-h LC_{50}, 3.28 mg L^{-1}) was the most toxic compound and was 271, 113 and 82 times less toxic than temephos, fenthion and fenitrothion respectively (Table 10). High toxicity was also obtained from linoleic acid, nerolidol, (±)-limonene and MD-SHD (LC_{50}, 7.19–19.30 mg L^{-1}). LC_{50} of α-terpinene, γ-terpinene, α-terpineol, palmitic acid, and *p*-cymene was between 23.49 and 33.93 mg L^{-1}. LC_{50} of methyl linolelaidate, (±)-terpinen-4-ol, geranic acid, ethyl palmitate and β-caryophyllene was between 41.93 and 54.95 mg L^{-1}. Behenic acid, α-humulene and *n*-hexadecane were ineffective.

The toxic effects of the test materials on third instar larvae from wild *Ae. albopictus* and *An*. *sinensis* were likewise compared (Table 11). 2,4-DTBP (LC_{50}, 1.98 mg L^{-1}) was the most toxic compound and was 249, 108 and 81 times less toxic than temephos, fenthion and fenitrothion towards *Ae. albopictus* larvae respectively (Table 11). High toxicity was also produced by linoleic acid, (±)-limonene and nerolidol (LC_{50}, 8.79–16.34 mg L^{-1}). LC_{50} of α-terpinene, MD-SHD, γ-terpinene, *p*-cymene, α-terpineol, methyl linolelaidate and palmitic acid was between 21.26 and 40.96 mg L^{-1}. LC_{50} of geranic acid, ethyl palmitate, (±)-terpinen-4-ol and β-caryophyllene was between 49.50 and 53.14 mg L^{-1}. α-Humulene, behenic acid and *n*-hexadecane were ineffective. Interestingly the toxicity of all materials was virtually identical towards *Ae. albopictus* and *An*. *sinensis* larvae.

Table 18. Toxicity of *M. denudata* seed hydrodistillate and 17 organic compounds to third instar larvae from insecticide-susceptible *Cx. p. pallens* and *Ae. aepypti* during a 24-h exposure

Material	*Cx. p. pallens* larvae		*Ae. aegypti* larvae	
	Slope ± SE	LC_{50}, mg L^{-1} (95% CL[a])	Slope ± SE	LC_{50}, mg L^{-1} (95% CL[a])
MD-SHD[b]	2.4 ± 0.28	19.60 (16.40–23.65)	3.1 ± 0.37	19.30 (16.86–22.29)
2,4-Di-*tert*-butylphenol	2.0 ± 0.25	1.98 (1.55–2.44)	2.3 ± 0.28	3.28 (2.65–3.94)
Linoleic acid	4.5 ± 0.59	7.28 (6.53–8.00)	4.2 ± 0.57	7.19 (6.38–7.95)
Nerolidol	2.0 ± 0.24	9.84 (7.78–12.11)	2.3 ± 0.28	13.85 (11.42–16.79)
(±)-Limonene	3.3 ± 0.33	14.05 (12.14–16.17)	2.8 ± 0.30	17.04 (14.42–19.91)
α-Terpinene	3.2 ± 0.37	21.30 (18.66–24.46)	2.7 ± 0.33	23.49 (18.96–27.40)
γ-Terpinene	3.3 ± 0.38	24.70 (21.65–28.11)	3.0 ± 0.34	27.53 (23.85–31.78)
p-Cymene	2.5 ± 0.31	29.34 (24.58–34.48)	2.6 ± 0.33	33.93 (28.78–39.71)
Palmitic aicd	3.1 ± 0.38	32.56 (28.22–37.19)	3.1 ± 0.38	33.54 (29.06–38.38)
α-Terpineol	2.7 ± 0.35	35.08 (29.99–40.90)	2.8 ± 0.34	31.92 (27.21–37.09)
Methyl linolelaidate	4.1 ± 0.47	35.51 (31.87–39.48)	5.6 ± 0.65	41.93 (38.80–45.20)
β-Caryophyllene	4.9 ± 0.63	44.99 (41.39–49.41)	4.6 ± 0.67	54.95 (50.24–61.55)
Geranic acid	2.1 ± 0.30	45.03 (37.19–56.91)	2.3 ± 0.31	49.38 (40.95–62.54)
(±)-Terpinen-4-ol	3.6 ± 0.42	45.80 (40.23–51.42)	2.9 ± 0.36	42.45 (36.30–48.80)
Ethyl palmitate	4.8 ± 0.57	56.39 (51.61–62.30)	5.0 ± 0.58	53.43 (49.10–58.56)
α-Humulene	6.0 ± 0.75	96.35 (89.8–104.23)	6.4 ± 0.82	108.06 (101.26–116.37)
Behenic acid	5.8 ± 0.71	103.32 (96.91–110.01)	5.5 ± 0.62	104.79 (97.96–112.28)
n-Hexadecane	3.4 ± 0.62	103.71 (90.66–122.72)	5.0 ± 0.77	113.03 (102.89–125.70)

[a] CL denotes confidence limit.

[b] *Magnolia denudata* seed hydrodistillate.

Table 19. Toxicity of *M. denudata* seed hydrodistillate and 17 organic compounds to third instar larvae from wild *Ae. albopictus* and *An. sinensis* during a 24-h exposure

Material	*Ae. albopictus* larvae		*An. sinensis* larvae	
	Slope ± SE	LC_{50}, mg/L (95% CL[a])	Slope ± SE	LC_{50}, mg/L (95% CL[a])
MD-SHD[b]	2.2 ± 0.30	21.40 (17.83–25.84)	3.9 ± 0.57	23.30 (10.85–24.89)
2,4-Di-*tert*-BP[c]	2.3 ± 0.28	3.36 (2.73–4.03)	2.2 ± 0.29	3.90 (3.07–4.73)
Linoleic acid	3.0 ± 0.41	8.79 (7.42–10.40)	2.5 ± 0.36	10.49 (8.61–12.79)
(±)-Limonene	2.7 ± 0.32	15.63 (13.30–18.56)	2.2 ± 0.26	18.91 (15.51–22.94)
Nerolidol	2.5 ± 0.30	16.34 (13.80–19.28)	2.9 ± 0.35	20.84 (18.10–24.02)
α-Terpinene	2.9 ± 0.36	21.26 (18.26–24.53)	2.8 ± 0.35	27.16 (23.43–31.71)
γ-Terpinene	3.2 ± 0.37	30.03 (26.31–34.28)	2.9 ± 0.34	36.42 (31.36–42.01)
p-Cymene	3.4 ± 0.44	35.10 (30.33–39.62)	2.8 ± 0.36	38.07 (32.74–44.40)
α-Terpineol	3.3 ± 0.43	36.70 (34.93–45.21)	2.8 ± 0.37	39.98 (34.45–46.47)
Methyl linolelaidate	4.7 ± 0.50	38.96 (35.40–42.93)	5.2 ± 0.61	45.16 (41.62–49.08)
Palimite aicd	3.5 ± 0.81	40.96 (34.70–46.40)	2.5 ± 0.70	49.88 (40.22–61.27)
Geranic acid	2.1 ± 0.38	49.50 (39.72–64.86)	2.4 ± 0.44	54.35 (45.14–68.26)
Ethyl palmitate	4.4 ± 0.55	52.40 (47.62–57.50)	5.1 ± 0.60	62.09 (57.37–67.75)
(±)-Terpinen-4-ol	3.7 ± 0.43	52.61 (46.26–58.98)	3.5 ± 0.42	52.84 (46.30–59.52)
β-Caryophyllene	4.1 ± 0.59	53.14 (48.12–60.08)	4.3 ± 0.56	60.17 (54.77–66.97)
α-Humulene	5.8 ± 0.75	106.25 (98.99–114.82)	5.8 ± 0.75	107.35 (101.88–113.40)
Behenic acid	5.3 ± 0.64	110.87 (102.50–120.78)	5.5 ± 0.61	114.29 (106.83–122.50)
n-Hexadecane	2.2 ± 0.42	124.55 (100.37–173.29)	1.8 ± 0.37	132.88 (101.88–206.27)

[a] CL denotes confidence limit.

[b] *Magnolia denudata* seed hydrodistillate.

[c] Butylphenol.

Structure-activity relationship

Because of the potent larvicidal activity of 2, 4-DTBP, comparisons were made to determine toxicity differences involving the chemical structures and functional groups of its analogues (Fig. 22) using the toxicity data obtained (Table 21). Toward third instar *Cx. p. pipiens* larvae, the toxicity of phenol, a basic structure, was the lowest of any of the phenolic compounds. 2,4-DTBP was the most toxic compound, followed by 2,6-DTBP. High toxicity was produced by 2-*tert*-butyl-4-methylphenol (MP), 2-TBP and 2,4,6-TTBP (LC_{50}, 7.25–8.23 mg L^{-1}). LC_{50} of 2-SBP and 4-isopropylphenol was 10.30 and 11.19 mg L^{-1} respectively. LC_{50} of 3-TBP, 5-isopropyl-2-MP, 4-SBP and 2-isopropyl-5-MP was between 31.35 and 42.83 mg L^{-1}. Low toxicity was observed with 4-SBP and 4-*sec*-butyl-2,6-DTBP. Similar results were likewise observed with third instar *Ae. aegypti* larvae.

Multiple regression analysis of the contact toxicities of compounds to third instar larvae from *Cx. p. pallens* and *Ae. aegypti* was examined using their LC_{50} values and the values of the physical parameters (MW, MR and VP) for the 14 compounds ($R^2 = 0.197$ for *Cx. p. pallens* larvae; $R^2 = 0.199$ for *Ae. aegypti* larvae). Correlation coefficient (r) analysis showed that MW, log P and MR may be negatively loosely correlated with LC_{50} (MW, r = –0.285 for *Cx. p. pallens* and r = –0.31 for *Ae. aegypti*; log P, r = –0.271 for *Cx. p. pallens* and r = –0.296 for *Ae. aegypti*; MR, r = –0.283 for *Cx. p. pallens* and r = –0.307 for *Ae. aegypti*).

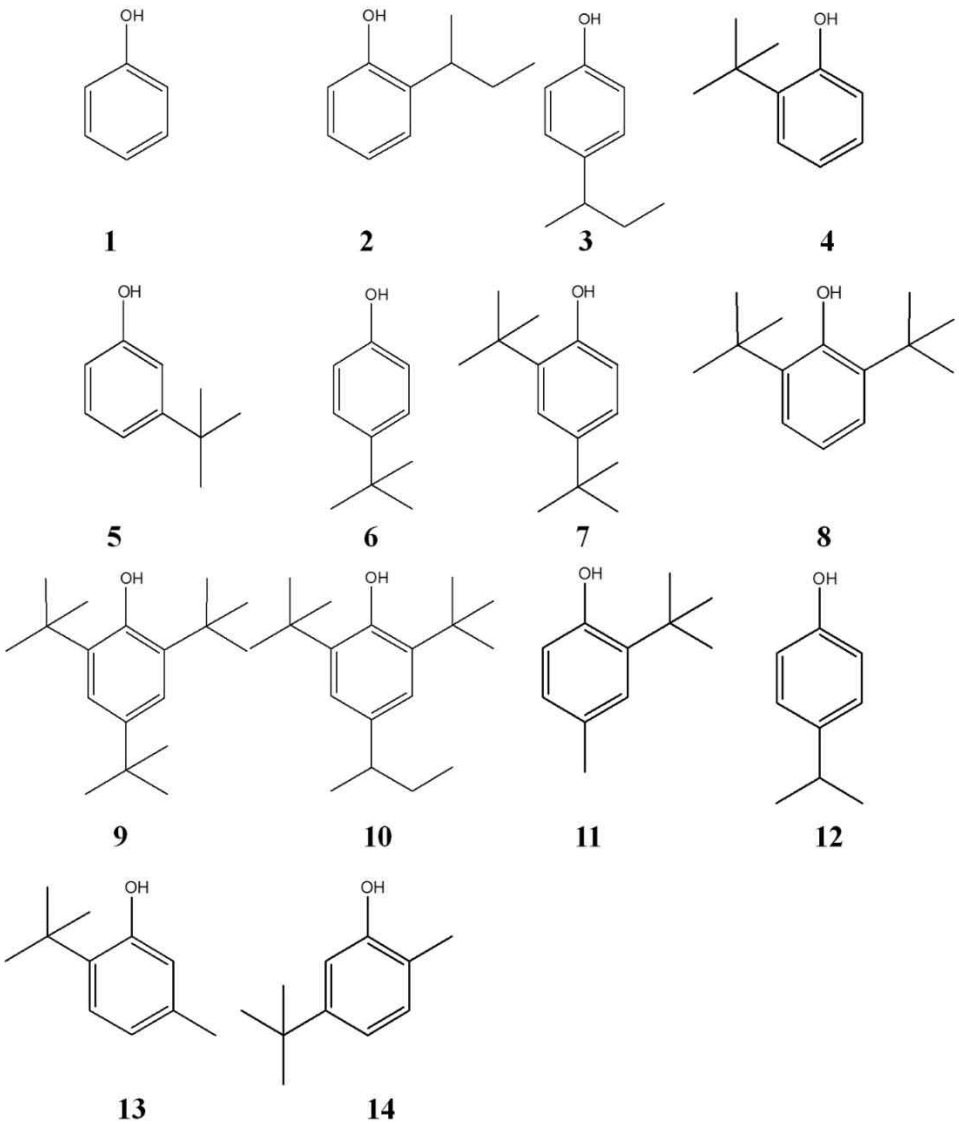


Fig. 22. Structures of 14 phenol compounds.

Table 20. Toxicity of 14 organic pure phenolic compounds to third instar larvae from insecticide-susceptible *Culex pipiens pallens* and *Aedes aepypti* during a 24-h exposure

Compound	*Cx. p. pallens* larvae		*Ae. aepypti* larvae	
	Slope ± SE	LC_{50}, mg L^{-1} (95% CL[a])	Slope ± SE	LC_{50}, mg L^{-1} (95% CL[a])
Phenol (**1**)	4.9 ± 0.62	66.90 (60.72–72.69)	5.1 ± 0.62	73.92 (67.35–80.18)
2-*sec*-Butylphenol (**2**)	3.4 ± 0.44	10.30 (8.74–11.76)	3.7 ± 0.40	12.95 (11.44–14.58)
4-*sec*-Butylphenol (**3**)	5.3 ± 0.65	41.90 (38.62–45.31)	5.5 ± 0.64	43.14 (39.85–46.60)
2-*tert*-Butylphenol (**4**)	3.5 ± 0.45	8.04 (7.14–9.05)	2.7 ± 0.31	9.06 (7.70–10.79)
3-*tert*-Butylphenol (**5**)	2.5 ± 0.41	31.35 (26.17–36.63)	5.4 ± 0.70	37.60 (34.12–41.27)
4-*tert*-Butylphenol (**6**)	5.8 ± 0.64	53.56 (49.44–57.79)	4.4 ± 0.60	54.00 (48.28–61.77)
2,4-Di-*tert*-butylphenol (**7**)	2.3 ± 0.27	2.92 (2.31–3.54)	2.1 ± 0.27	3.36 (3.11–4.72)
2,6-Di-*tert*-butylphenol (**8**)	4.1 ± 0.42	3.98 (3.52–4.46)	2.8 ± 0.27	4.73 (4.08–5.45)
2,4,6-Tri-*tert*-butylphenol (**9**)	2.1 ± 0.22	8.23 (6.83–9.83)	2.3 ± 0.24	9.55 (8.02–11.21)
4-*sec*-Butyl-2,6-di-*tert*-BP[b] (**10**)	4.8 ± 0.65	55.06 (50.53–60.04)	5.8 ± 0.73	58.77 (54.72–63.30)
2-*tert*-Butyl-4-MP[c] (**11**)	3.3 ± 0.43	7.25 (6.26–8.23)	3.8 ± 0.48	9.03 (8.11–10.12)
4-Isopropylphenol (**12**)	3.0 ± 0.36	11.19 (9.76–12.94)	3.4 ± 0.39	14.17 (12.49–16.24)
2-Isopropyl-5-MP (**13**)	4.2 ± 0.47	42.83 (38.17–47.51)	5.6 ± 0.66	47.77 (44.11–51.50)
5-Isopropyl-2-MP (**14**)	4.5 ± 0.58	35.17 (31.22–38.62)	5.5 ± 0.69	42.09 (38.47–45.40)

[a] CL denotes confidence limit.

[b] BP, butylphenol.

[c] MP, methylphenol.

Efficacy of experimental liquid formulations

The control efficacy of four liquid formulations significantly differed towards *Cx. p. pallens* ($F = 498.28$; df = 3, 8; $P < 0.0001$), *Ae. aegypti* ($F = 669.73$; df = 3, 8; $P < 0.0001$), *Ae. albopictus* ($F = 232.23$; df = 3, 8; $P < 0.0001$) and *An. sinensis* ($F = 543.44$; df = 3, 8; $P < 0.0001$) (Table 22). MD-SHD applied as 50 mg L^{-1} liquid provided 100, 100, 98 and 92% mortality towards *Cx. p. pallens*, *Ae. aegypti*, *Ae. albopictus* and *An. sinensis* larvae respectively. The lethalities of the MD-SHD 30 mg L^{-1} liquid and MD-SHD 30 mg L^{-1} liquid were 92, 87, 95 and 74% and 67, 62, 61 and 49% towards *Cx. p. pallens*, *Ae. aegypti*, *Ae. albopictus* and *An. sinensis* larvae respectively. A commercial temephos 200 g L^{-1} treatment resulted in 100% mortality towards *Cx. p. pallens*, *Ae. aegypti* and *Ae. albopictus* larvae and 30% mortality towards wild *An. sinensis* larvae.

Bioassay-guided fractionation and isolation

Fractions obtained from the solvent partitionings of the methanol extract of *M. denudata* seed were bioassayed against third instar larvae from insecticide-susceptible *Cx. p. pallens* and *Ae. aegypti*, as stated above (Table 23). Significant differences in toxicity 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 hours of exposure, the hexane- and chloroform-soluble fractions were the most toxic materials. No toxicity was obtained from the ethyl acetate-, butanol-, and water-soluble fractions.

Table 21. Effectiveness of four experimental spray formulations containing *M. denudata* seed hydrodistillate and commercial larvicide temephos towards four mosquito species larvae during a 24-h exposure

Treatment	Larval mortality[a] (%) (± SE)			
	Cx. p. pallens	*Ae. aepypti*	*Ae. albopictus*	*An. sinensis*
MD-SHD[b] 10 mg L^{-1}[c]	22 ± 1.7 d	21 ± 1.6 d	19 ± 2.8 d	9 ± 0.0 a
MD-SHD 20 mg L^{-1}	67 ± 1.7 c	62 ± 1.5 c	61 ± 0.6 c	49 ± 0.8 c
MD-SHD 30 mg L^{-1}	92 ± 1.7 b	87 ± 1.6 b	95 ± 1.5 b	74 ± 1.1 d
MD-SHD 50 mg L^{-1}	100 a	100 a	98 ± 1.7 a	92 ± 1.7 e
Temephos 200 g L^{-1} EC[d]	100 a	100 a	100 a	30 ± 1.6 b

[a] Means within a column followed by the same letter are not significantly different at $P = 0.05$ (Bonferroni test).

[b] *Magnolia denudata* seed hydrodistillate.

[c] Liquid.

[d] Emulsifiable concentrate.

Table 22.Contact toxicity of fractions obtained from solvent partitionings of the methanol extract of *M. denudata* seed to third-instar larvae from two mosquito species during a 24-h exposure

Compound	*Culex pipiens pallens*		*Aedes aegypti*	
	Slope ± SE	LC_{50}, mg L^{-1} (95% CL[a])	Slope ± SE	LC_{50}, mg L^{-1} (95% CL[a])
Methanol extract	4.5 ± 0.63	43.30 (29.44–66.60)	2.7 ± 0.33	58.70 (56.29–61.30)
Hexane-soluble fr.[b]	3.3 ± 0.63	21.21 (16.52–26.26)	3.1 ± 0.41	26.10 (19.57–33.95)
Chloroform-soluble fr.		>100		>100
Ethyl acetate-soluble fr.		>100		>100
Butanol-soluble fr.		>100		>100
Water-soluble fr.		>100		>100

[a] CL denotes confidence limit.

[b] Fraction.

The larvicidal activity of hexane-soluble fraction derived from *M. denudata* seed is given in Tables 23.

Table 23. The larvicidal activity of hexane-soluble fraction derived from *M. denudata* seed against third instars of insecticide-susceptible KS-CP strain of *Cx. p. pallens*

	Mortality, % (± SE)	
Fraction	10 mg L^{-1}	50 mg L^{-1}
H1	0	68 ± 1.4
H2	33 ± 2.6	80 ± 2.6
H3	15 ± 2.6	73 ± 3.3
H4	17 ± 1.7	55 ± 0.9
H5	7 ± 1.7	44 ± 0.9
H6	65 ± 2.9	99 ± 1.5
H7	15 ± 2.9	35 ± 1.5
H8	0	0
Fraction	50 mg L^{-1}	
H21	2 ± 1.7	
H22	85 ± 0	
H23	16 ± 3.6	
H61	32 ± 1.7	
H62	32 ± 1.7	
H63	80 ± 0	
H64	23 ± 1.7	
H65	30 ± 0	
H221	16 ± 3.6	
H222	88 ± 1.7	
H223	20 ± 0	
H621	27 ± 1.7	
H622	25 ± 2.8	
H623	85 ± 0	
H624	42 ± 4.4	
H641	8 ± 1.7	
H642	8 ± 1.7	
H643	80 ± 0	
H644	16 ± 3.6	
H6231	42 ± 0	
H6232	87 ± 1.7	
H6233	20 ± 0	
H6431	32 ± 1.7	
H6432	38 ± 1.7	
H6433	90 ± 1.7	
H6434	42 ± 1.7	
H64331	33 ± 3.3	
H64332	25 ± 1.7	
H64333	100	
H64334	55 ± 2.9	

The direct-contact mortality assay-guided fractionation of the methanol extract from *M. denudata* seed afforded three active principles. The compound **1** was obtained as white crystal and identified as palmitic acid by spectroscopic analysis, including EI-MS (Fig. 23), ^{1}H NMR (Fig. 24), ^{13}C NMR (Fig. 25). The EI-MS revealed a molecular ion at *m/z* 256 $[M]^{+}$ and the ^{1}H NMR spectra of compound **1** at δ H = 1.29 indicating that these compounds compared with the spectrum showing a long chain methylene signal. A peak showing up at a particular shift between 11 and 12 ppm (δ H = 11.6) indicates which peak belongs to carboxylic acid. Moreover, its ^{13}C NMR spectra showed 16 long carbonated chain including a hydroxyl group in the molecule suggesting the molecular formula $C_{16}H_{32}O_{2}$. Compound **1:** white crystal; UV (EtOH): λ_{max} = 254; EI-MS (70 eV), *m/z* (rel. int.): 256 $[M]^{+}$ (base peak), 241, 213, 185, 129, 97, 73, 57**;** The interpretations of proton and carbon signals of compounds **1** was largely consistent with those of Ragona *et al*., (2000).(Fig. 26) ^{1}H NMR and ^{13}C NMR spectral data of palmitic acid is given in Table 24.

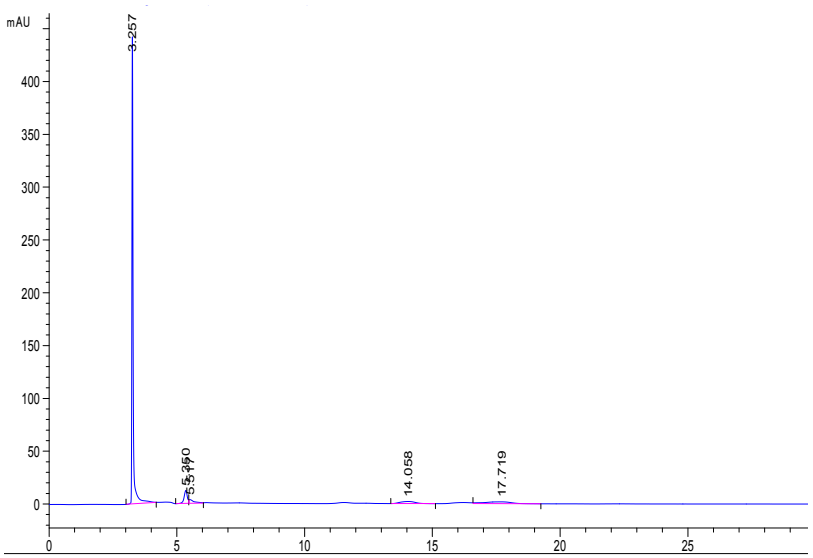


Fig. 18. High-performance liquid chromatogram of compound 1.

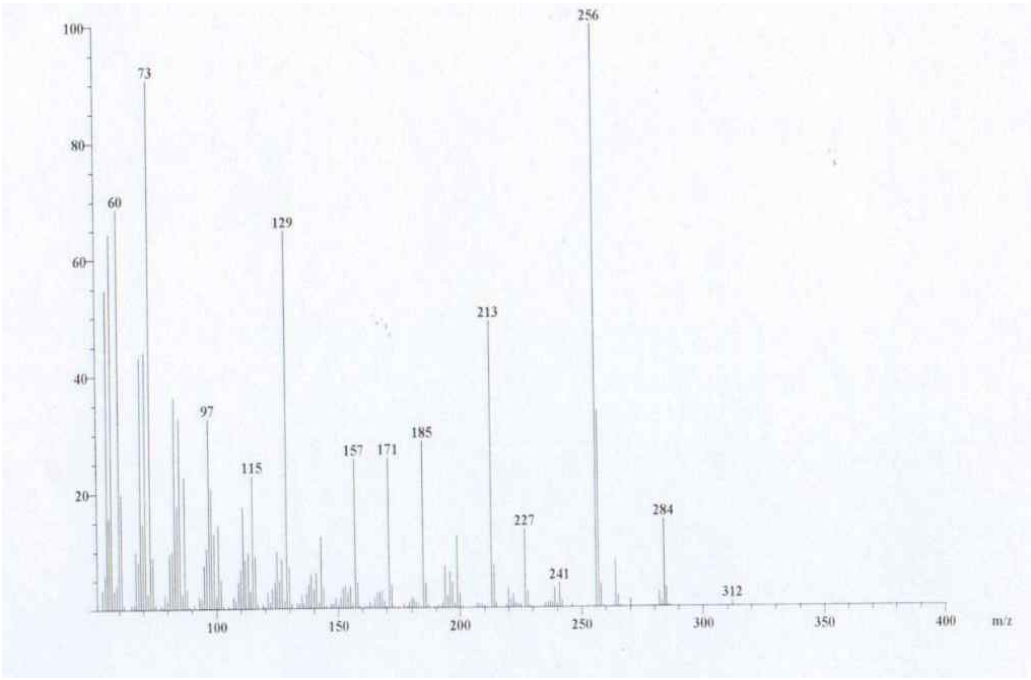


Fig. 23. EI-MS spectrum of compound 1.

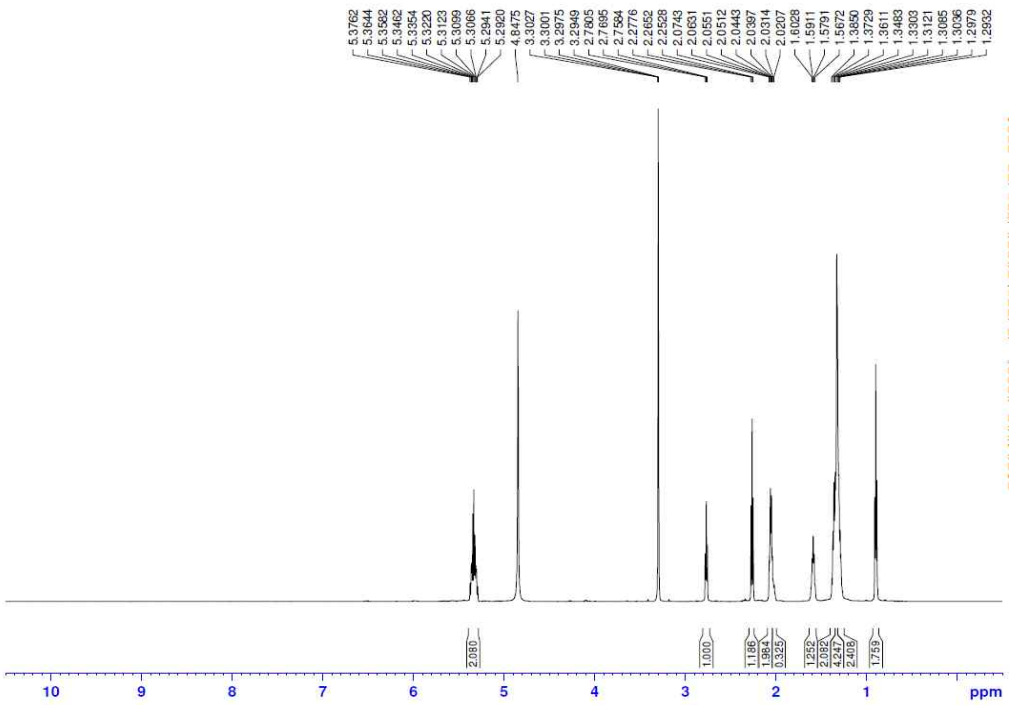


Fig. 24. ^{1}H NMR spectrum of compound 1.

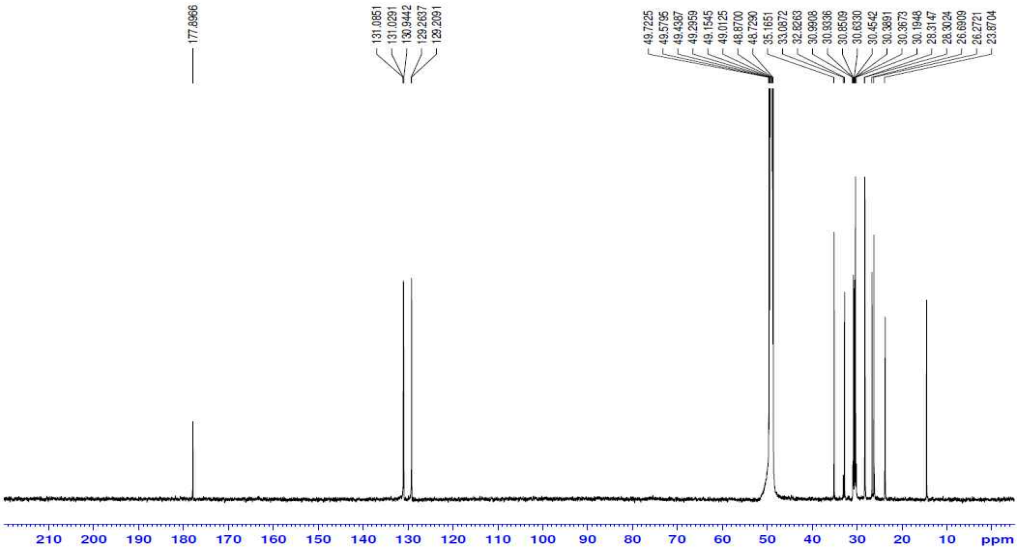


Fig. 25. ^{13}C NMR spectrum of compound 1.

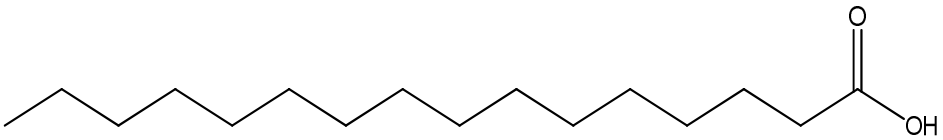


Fig. 26. Structure of palmitic acid (1). The chemical formula of this compound is $C_{16}H_{32}O_2$; the molar mass is 256.43 g/mol.

Table 24. ^{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) (Ragona *et al*, 2000)	δ_H (ppm) (Ragona *et al*, 2000)
1	C	177.9			180.1
2	CH_2	35.2	2.05, s	2.12	34.7
3	CH_2	27.3	2.03, m	2.01	26.4
4	CH_2	27.3	2.05, m	2.02	16.5
5	CH_2	26.7	1.64, m	1.50	14.9
6	CH_2	30.9	1.29, m	1.24	29.2
7	CH_2	30.9	1.29, m	1.24	29.2
8	CH_2	30.1	1.29, m	1.24	29.2
9	CH_2	28.3	1.30, s	1.50	28.9
10	CH_2	28.3	1.29, m	1.24	28.9
11	CH_2	28.3	1.29, m	1.24	28.9
12	CH_2	30.6	1.29, m	1.24	29.5
13	CH_2	30.4	1.29, m	1.24	29.3
14	CH_2	35.1	1.29, m	1.24	32.4
15	CH_2	26.4	1.33, s	1.50	25.3
16	CH_3	15.1	0.88, s	0.54	16.5
17	OH		11.6		11.4

The compound **2** was obtained as white crystal and identified as linoleic acid by spectroscopic analysis, including EI-MS (Fig. 27), 1H NMR (Fig. 28), 13C NMR (Fig. 29). DEPT spectrum **((**Fig. 30**).** The EI-MS revealed a molecular ion at m/z 280 [M]+ and its 13C NMR spectra showed 18 long carbonated chain including a hydroxyl group in the molecule suggesting the molecular formula $C_{18}H_{32}O_2$. According to the ^{1}H NMR method, terminal methyl protons are useful to calculate linoleic acid, The terminal methyl group gave a chemical shift between 0.7 and 1.0ppm regions. The signals at 0.87 ppm referred to the methylene group (–CH3) . There are 18 carbon atoms in the molecule, 2 double bonds (=), and the last double bond is located 6 carbon atoms down from the omega or methyl end of the fatty acid. Similarly, the interpretations of proton and carbon signals of compound **2** was largely consistent with those of Kim *et al.* linoleic acid (Fig 31) was identified on the basis of the following evidence: colorless oil. UV (MeOH): λ_{max}nm = 290. EI-MS (70 eV), *m/z* (% relative intensity): 280 $[M]^+$ (65), 236 (3), 210 (4), 182 (7), 168 (6), 137 (15), 123 (22), 108 (11), 95 (70), 81 (100), 67 (91), 55 (54). ^{1}H NMR and ^{13}C NMR spectral data of linoleic acid is given in Table 25.

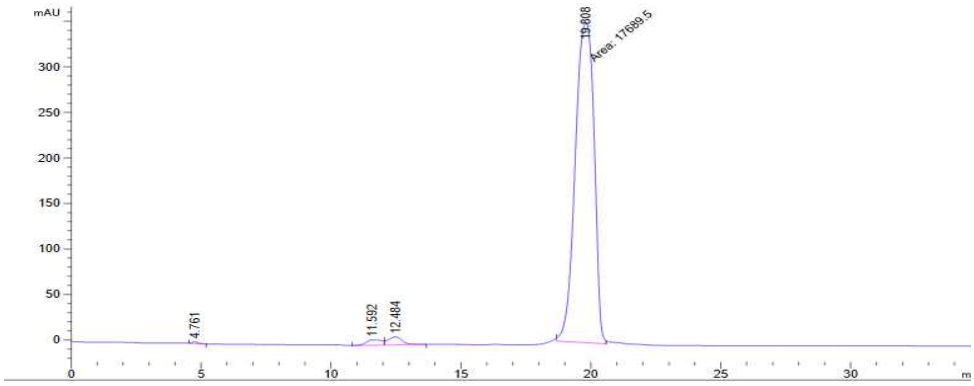


Fig. 19. High-performance liquid chromatogram of compound 2.

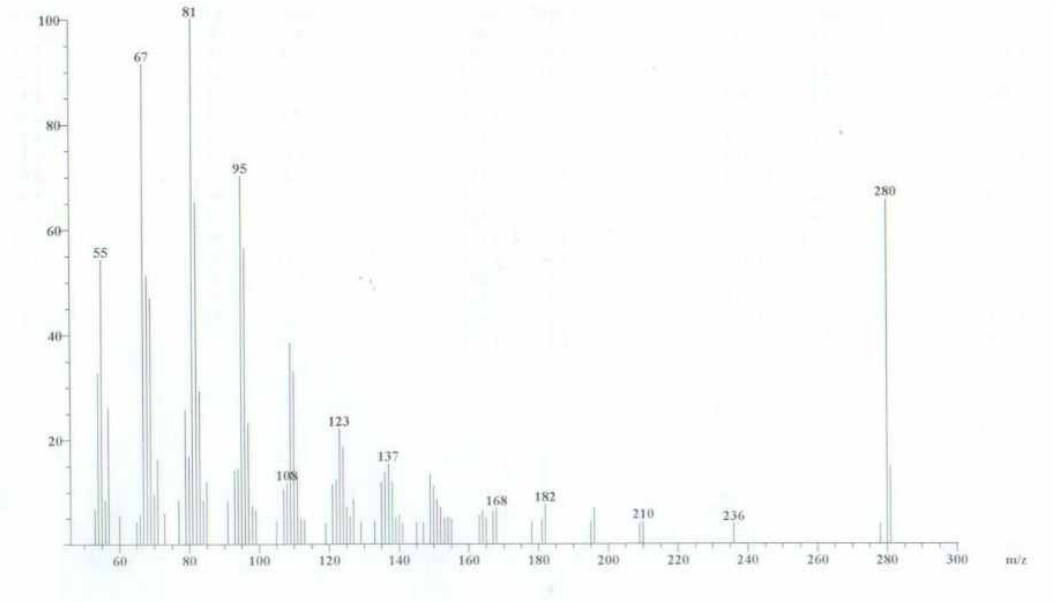


Fig. 27. EI-MS spectrum of compound 2.

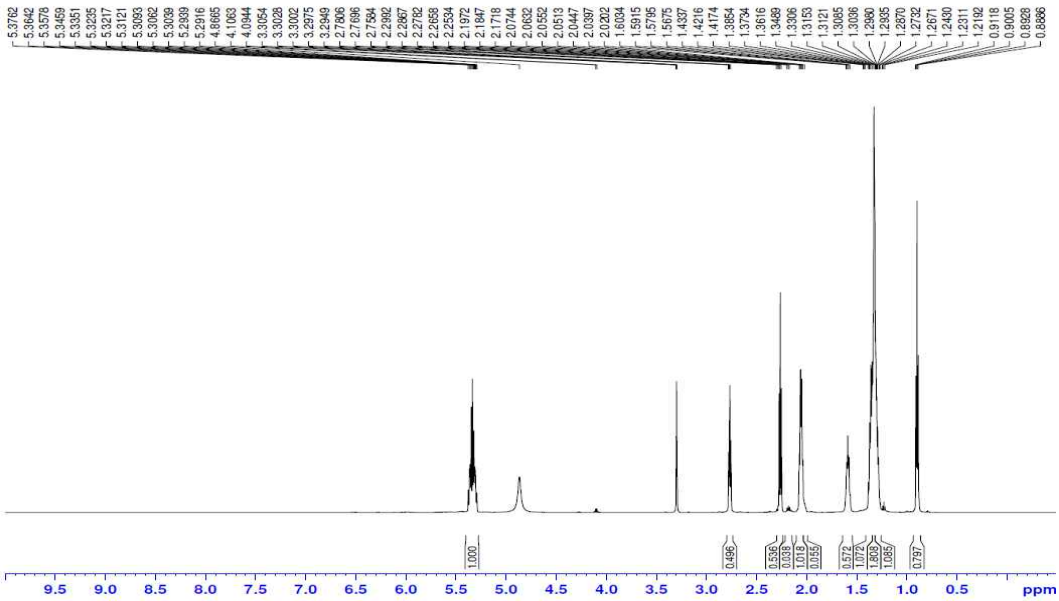


Fig. 28. ^{1}H NMR spectrum of compound 2.

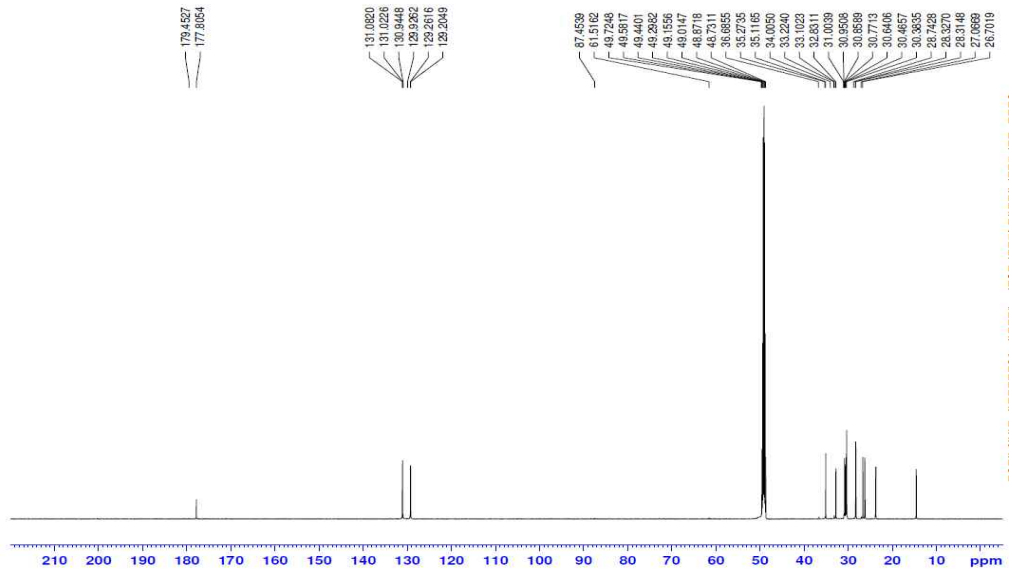


Fig. 29. ^{13}C NMR spectrum of compound 2

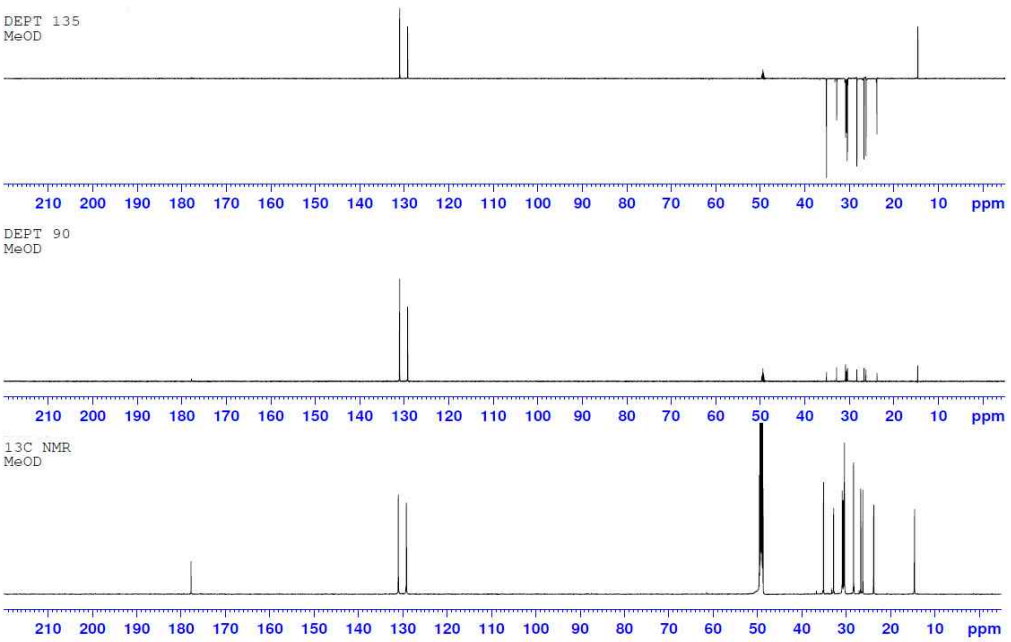


Fig. 30. DEPT spectrum of compound 2.

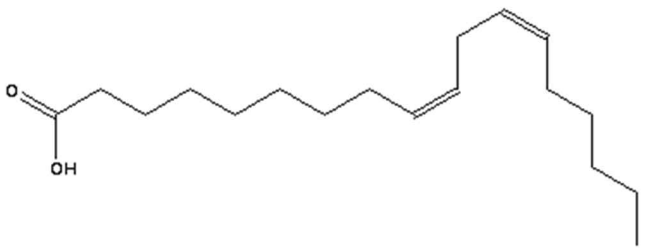


Fig. 31. Structure of linoleic acid (2). The chemical formula of this compound is $C_{18}H_{32}O_2$; the molar mass is 280.44 g/mol.

Table 25. ^{1}H NMR (600 MHz) and ^{13}C NMR (150 MHz) spectral data of compound 2

Position	Partial structure	δ_H (ppm)	δ_C (ppm)	δ_H (ppm) Kim *et al*., 2010	δ_C (ppm) Kim *et al*., 2010
1	C		180.13		173.9
2	CH_2	2.34 t (J=7.5Hz)	34.03	2.04	27.2
3	CH_2	1.62 m	24.63	1.96	24.9
4	CH_2	1.31 m	29.00	1.29	29.7
5	CH_2	1.31 m	29.05	1.29	29.7
6	CH_2	1.31 m	29.33	1.29	29.7
7	CH_2	1.31 m	29.56	1.28	29.5
8	CH_2	2.05 d (J=6.6Hz)	27.21	2.02	27.2
9	CH	5.35 m	130.00	5.27	129.8
10	CH	5.35 m	128.04	5.34	129.8
11	CH_2	2.78 t (J=5.9Hz)	25.60	2.50	34.3
12	CH	1.31 m	27.88	1.28	29.5
13	CH	1.31 m	30.20	1.28	29.5
14	CH_2	2.05 d (J=13.3Hz),	27.25	2.04	27.2
15	CH_2	1.31 m	29.12	1.28	29.3
16	CH_2	1.31 m	31.51	1.28	29.6
17	CH_2	1.31 m	22.56	1.34	22.4
18	CH_3	0.87 s	14.05	0.90	14.2
	OH	11.3		11.4	

The compound **3** was obtained as white crystal and identified as honokiol by sp ectroscopic analysis, including EI-MS (Fig. 32), ^{1}H NMR (Fig. 33), ^{13}C NMR (Fi g. 34). DEPT spectrum (Fig. 35) The EI-MS revealed a molecular ion at m/z 266 [M]+ and the 1^{3}C NMR and DEPT spectra showed 18 carbon signals attributed to two methylene (δ 35.5), one oxymethylene (δ 64.5), and 14 sp2 carbons. The ^{1}H spectra revealed the presence of two methylene (δ 3.30, d, 6 Hz), six aromatic protons (δ 6.77－7. 22). The structure consists of *para*-allyl-phenol and an *ortho*-allyl-phenol which link together through *ortho, para*-C-C-couplng suggesting the molecular formula $C_{18}H_{18}O_2$. The interpretations of proton and carbon signals were largely consistent with those of Lo *et al*., honokiol (compound **3**) was identified on the basis of the following evidence: colorless solid. UV (MeOH): λ_{max}nm = 293. (EI-MS) (70 eV), *m/z* (% relative intensity): 266 (100), 237(18), 224 (8), , 197 (8), 184 (8), 152 (3),133 (4), 105 (2), 77 (1).). ^{1}H NMR and ^{13}C NMR spectral data of honokiol is given in Table 26.

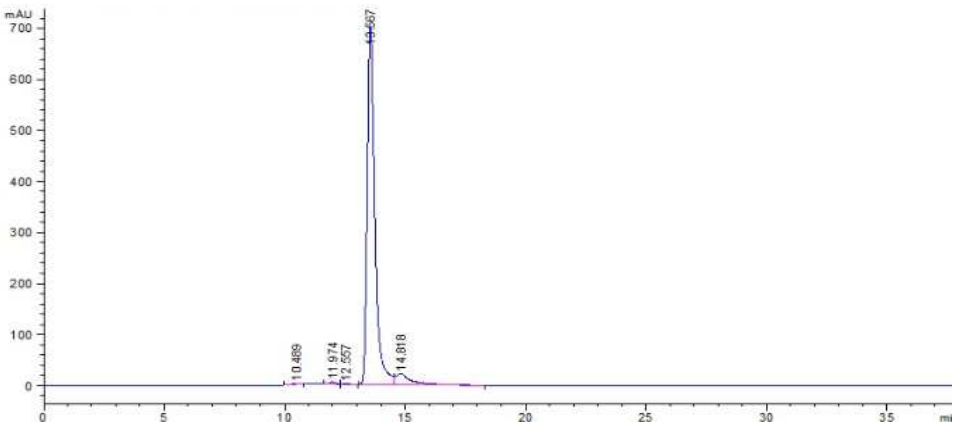


Fig. 20. High-performance liquid chromatogram of compound 3

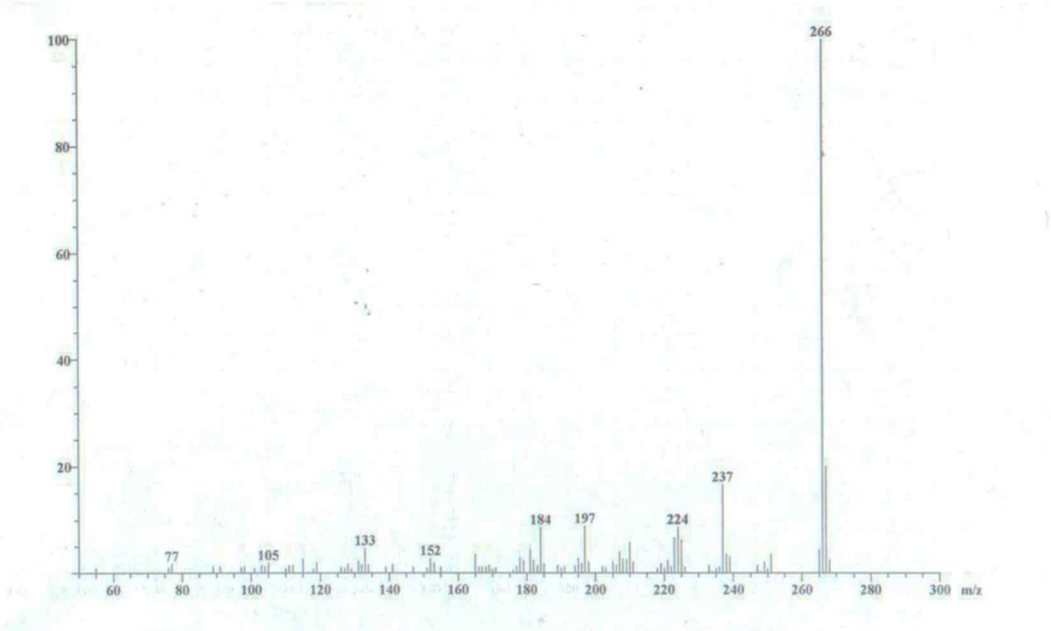


Fig. 32. EI-MS spectrum of compound 3.

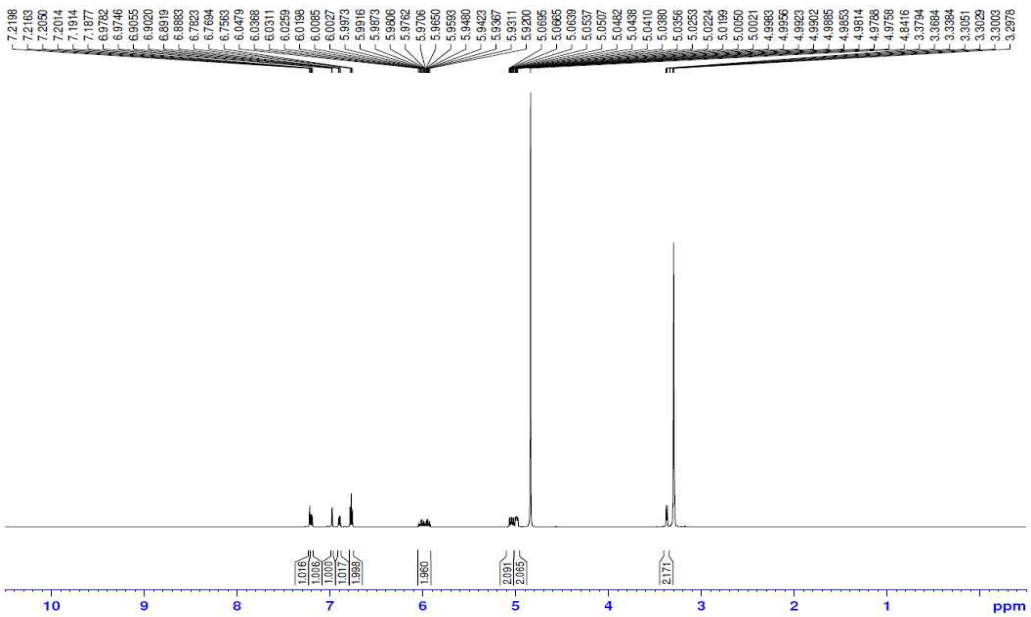


Fig. 33. ^{1}H NMR spectrum of compound 3.

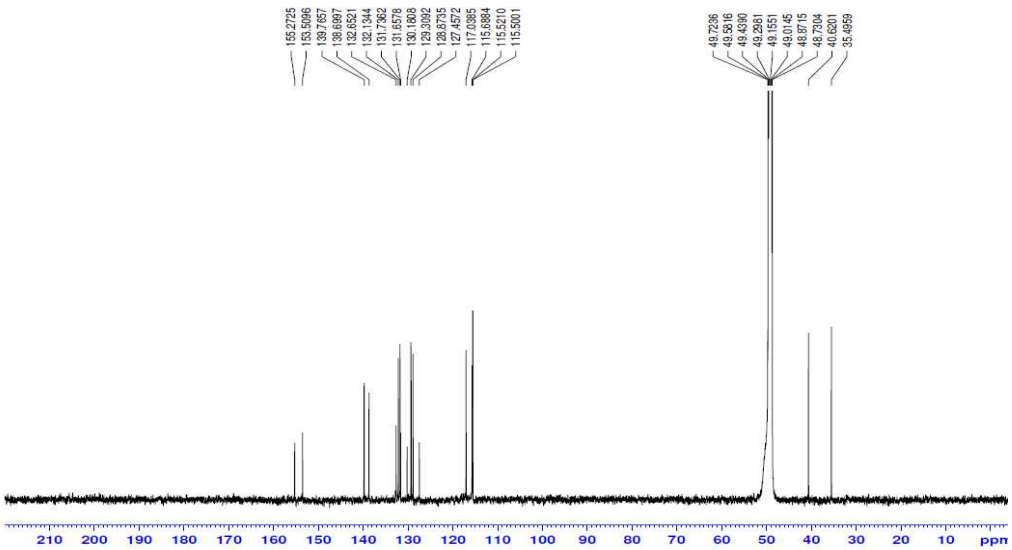


Fig. 34. ^{13}C NMR spectrum of compound 3.

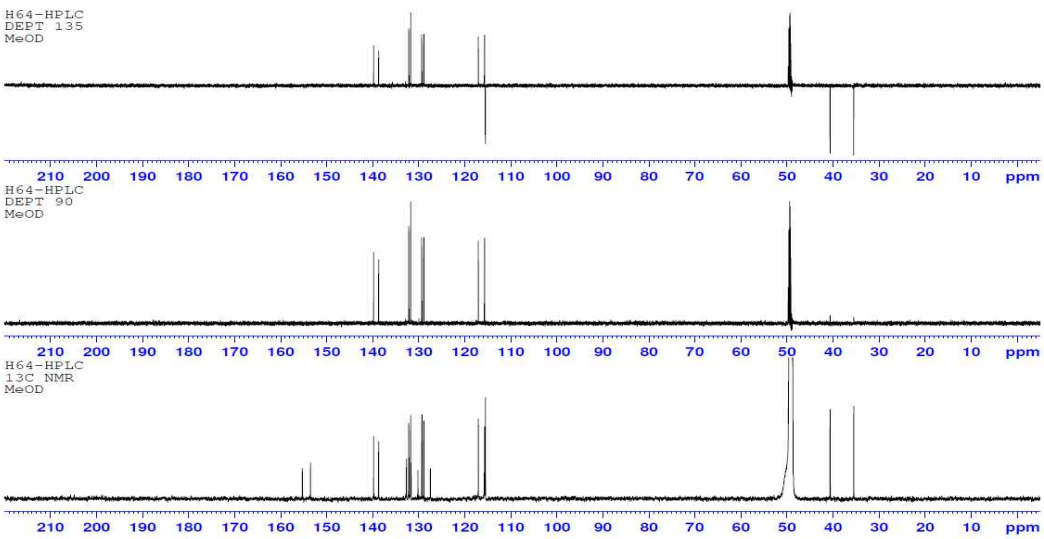


Fig. 35. DEPT spectrum of compound 3.

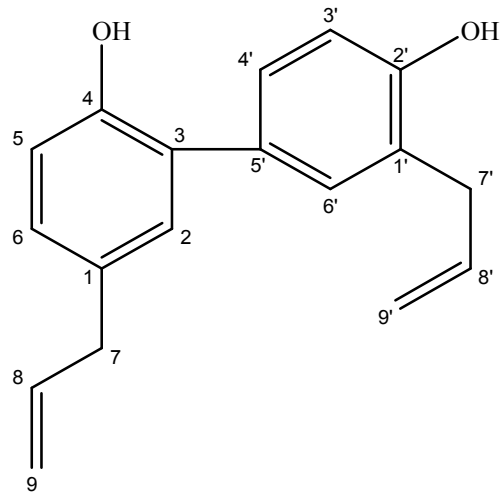


Fig. 36. Structure of honokiol (3). The chemical formula of this compound is $C_{18}H_{18}O_2$; the molar mass is 266.334 g/mol.

Table 26. ^{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)	δc (ppm) Agrawal *et. al.,* 1985	δ_H (ppm) Agrawal *et. al.,* 1985
1	C	155.3	—	132.2	—
2	CH	132.7	7.19 s	131.0	7.22 (br,s)
3	C	138.7	—	132.2	—
4	C	127.5	—	127.7	—
5	CH	153.5	7.20 *m*	153.5	6.92 d (*J*=8.4)
6	CH	115.5	6.77 d (*J*=12)	115.5	7.07 dd (*J*=8.0, 2.0)
7	CH_2	35.5	3.30 d (*J*=6)	35.0	3.36 d (*J*=6.8)
8	CH	132.1	5.98 m	137.7	6.00 (m)
9	CH_2	115..8	6.89 m	115.2	5.22 (m)
1’	C	128.8	—	129.5	—
2’	C	115.7	—	153.8	—
3’	CH	153.5	6.77 d (*J*=12)	116.4	6.90 d (*J*=8.4)
4’	CH	127.5	7.22 s	128.4	7.21 dd (*J*=8.4, 2.0)
5’	C	128.8		126.4	—
6’	CH	139.2	5.98 m	130.2	7.05 dd (*J*=2.4)
7’	CH_2	35.5	3.30 d (*J*=6)	39.3	3.46 d (*J*=6.4)
8’	CH	139.2	5.98 m	135.9	6.07 (m)
9’	CH_2	115.7	5.05 m	115.5	5.32 (m)

Larvicidal activity of test compounds

The toxicity of the three isolated compounds and four structurally related compounds of honokiol to third-instar larvae from the KS-CP strain of *Cx. p. pallens* and *Ae. aegypti* was investigated by direct-contact application. (Table 27.) Based on 24-hour LC_{50} values, honokiol was ($LC_{50,}$ 6.32 and 6.51 mg L^{-1}) the most toxic and followed by linoleic acid ($LC_{50,}$ 6.97 and 7.19 mg L^{-1}), palmitic acid ($LC_{50,}$ 32.56 and 33.54 mg L^{-1}). Magnolol exists with its structural isomer honokiol, which differs from honokiol only by the position of one hydroxyl group also show the good larvicidal activity ($LC_{50,}$ 25.70 and 26.00 mg L^{-1}), followed by eugenol ($LC_{50,}$ 74.35 and 80.80 mg L^{-1}), isoeugenol ($LC_{50,}$ 79.72 and 89.90 mg L^{-1}) and caffeic acid ($LC_{50,}$ 119.31 and 143.00 mg L^{-1}).

Table 27. Toxicity of *M. denudata* seed-derived materials to third instar larvae from insecticide-susceptible KS-CP strain of *Cx. p. pallens* and *Ae. Aegypti* using direct-contact mortality bioassay during a 24-h exposure

	KS-CP *Culex pipiens pallens*		*Aedes Aegypti*	
Material	Slope ± SE	LC_{50}, mg L^{-1} (95% CL[a])	Slope ± SE	LC_{50}, mg L^{-1} (95% CL[a])
Natural HK[b]	2.4 ± 0.30	6.32 (5.07–7.60)	2.3 ± 0.30	6.51 (5.19–7.89)
Pure HK	2.1 ± 0.30	6.38 (5.01–7.78)	2.1 ± 0.30	6.48 (5.11–7.90)
Magnolol	2.5 ± 0.34	26.00 (22.17–30.75)	4.5 ± 0.53	25.70 (21.0–30.5)
Eugenol	2.9 ± 0.39	74.35 (64.14–85.27)	2.4 ± 0.30	80.80 (74.4–87.9)
Isoeugenol	2.7 ± 0.39	79.72 (68.33–92.65)	2.3 ± 0.42	89.90 (82.4–98.1)
Caffeic acid	4.2 ± 0.61	119.31 (108.50–131.90)	3.4 ± 0.44	143.00 (132.7–153.9)
LA[c]	4.4 ± 0.59	6.97 (6.21–7.68)	4.2 ± 0.57	7.19 (6.38–7.95)
PA[d]	3.1 ± 0.38	32.56 (28.22–37.19)	3.1 ± 0.38	33.54 (29.06–38.38)
Fenthion	1.3 ± 0.17	0.0234 (0.0221–0.0248)	1.5 ± 0.18	0.0290 (0.0273–0.0307)
Temephos	1.2 ± 0.17	0.0116 (0.0107–0.0125)	1.7 ± 0.21	0.0121 (0.0105–0.0139)

[a] CL denotes confidence limit.

[b] Honokiol.

[c] Linoleic acid.

[d] Palmitic acid.

Toxicity of binary mixtures

The toxicity of honokiol alone or in combination with linoleic acid or palmitic acid to the insecticide-susceptible and -resistant *Ae. albopictus* and *An. sinensis* was evaluated using the direct-contact mortality bioassay (Table 28, 29). Binary mixtures of honokiol and linoleic acid were significantly more toxic than either honokiol or linoleic acid alone. Based on the SF values, the five binary mixtures of honokiol and linoleic acid operated in a synergy pattern (SF, 2.0–3.5). Binary mixtures of palmitic acid and linoleic acid were significantly more toxic than palmitic acid alone. Based on the SF values, the five binary mixtures of honokiol and linoleic acid operated in a synergy pattern (SF, 1.1–2.3).The toxicity of the other binary mixtures of honokiol and palmitic acid were significantly less toxic than either honokiol or linoleic acid alone. Based on the SF values, the five binary mixtures of honokiol and linoleic acid operated in a synergy pattern (SF, 0.3–0.5). Mortality in the methanol-Triton X-100-water-treated controls was <2%.

Table 28. Toxicity of honokiol alone or in combination with linoleic acid or palmitic acid to third instar larvae from wild *Ae. albopictus* during a 24-h exposure

Treatment	Slope ± SE	$LC_{50,}$ mg L^{-1} (95% CL[a])	CC[b]	SF[c]
Honokiol (HK) only	2.9 ± 0.52	6.13 (5.19–7.38)		
Palmitic acid (PA) only	3.7 ± 0.81	36.91 (31.30–41.64)		
HK + PA (1:1)	3.3 ± 0.32	12.11 (10.52–13.74)	87	0.5
HK + PA (2:1)	2.8 ± 0.31	11.89 (10.40–13.61)	72	0.5
HK + PA (3:1)	3.0 ± 0.35	14.10 (12.19–16.31)	55	0.4
HK + PA (1:2)	2.8 ± 0.29	16.63 (14.38–19.10)	81	0.4
HK + PA (1:3)	2.2 ± 0.28	23.70 (20.04–28.70)	69	0.3
Linoleic acid (LA) only	4.5 ± 0.59	7.28 (6.53–8.00)		
HK + LA (1:1)	3.7 ± 0.48	2.90 (2.53–3.25)	229	2.5
HK + LA (2:1)	4.8 ± 0.51	2.11 (1.90–2.31)	306	3.5
HK + LA (3:1)	2.7 ± 0.30	2.24 (1.90–2.59)	290	3.3
HK + LA (1:2)	3.3 ± 0.40	2.54 (2.23–2.90)	274	2.9
HK + LA (1:3)	2.9 ± 0.38	3.68 (3.17–4.25)	189	2.0
PA+ LA (1:1)	2.7 ± 0.42	16.86 (13.78–19.64)	72	2.2
PA+ LA (2:1)	5.0 ± 0.65	31.32 (28.61–34.01)	50	1.2
PA+ LA (3:1)	4.9 ± 0.66	34.92 (32.09–38.07)	52	1.1
PA+ LA (1:2)	5.1 ± 0.60	16.38 (15.03–17.78)	60	2.3
PA+ LA (1:3)	2.2 ± 0.33	15.79 (13.23–19.16)	58	2.3

[a] CL denotes confidence limit.

[b] Co-toxicity coefficient.

[c] Synergistic factor, LC_{50} of compound alone /LC_{50} of compound in mixture.

Table 29. Toxicity of honokiol alone or in combination with linoleic acid or palmitic acid to third instar larvae from wild *An sinensis* during a 24-h exposure

Treatment	Slope ± SE	LC_{50}, mg L^{-1} (95% CL[a])	CC[b]	SF[c]
Honokiol (HK) only	1.9 ± 0.34	7.37 (5.64–9.78)		
Palmitic acid (PA) only	4.0 ± 0.81	49.74 (44.44–58.97)		
HK + PA (1:1)	2.7 ± 0.40	12.06 (10.16–13.97)	94	0.6
HK + PA (2:1)	2.6 ± 0.33	12.67 (10.74–14.80)	81	0.6
HK + PA (3:1)	2.8 ± 0.34	15.44 (13.30–18.05)	60	0.5
HK + PA (1:2)	2.5 ± 0.29	18.97 (15.94–22.68)	90	0.4
HK + PA (1:3)	2.4 ± 0.31	25.79 (24.42–36.04)	82	0.3
Linoleic acid (LA) only	3.9 ± 0.56	7.50 (6.65–8.32)		
HK + LA (1:1)	3.6 ± 0.47	3.03 (2.64–3.39)	245	2.5
HK + LA (2:1)	4.3 ± 0.48	2.19 (1.97–2.42)	338	3.4
HK + LA (3:1)	2.4 ± 0.29	2.49 (2.04–2.96)	300	3.0
HK + LA (1:2)	3.0 ± 0.42	2.90 (2.46–3.44)	256	2.6
HK + LA (1:3)	2.7 ± 0.37	3.95 (3.38–4.59)	189	1.9
PA+ LA (1:1)	2.5 ± 0.42	20.92 (17.36–25.43)	62	2.4
PA+ LA (2:1)	4.7 ± 0.65	33.61 (30.72–36.69)	51	1.5
PA+ LA (3:1)	4.9 ± 0.66	34.92 (32.09–38.07)	59	1.4
PA+ LA (1:2)	4.8 ± 0.58	17.16 (15.71–18.73)	55	2.8
PA+ LA (1:3)	4.1 ± 0.47	14.43 (12.46–16.22)	81	3.4

[a] CL denotes confidence limit.

[b] Co-toxicity coefficient.

[c] Synergistic factor, LC_{50} of compound alone /LC_{50} of compound in mixture.

DISCUSSION

Certain essential oil preparations can be developed into mosquito larvicide products suitable for integrated vector management because they can be target-specific, biodegrade to non-toxic products, have few harmful effects on non-target organisms and are environmentally nonpersistent (Sukumar *et al*., 1991; Shaalan *et al*., 2005; Isman, 2006; Dias *et al*., 2014). Komalamisra *et al*., (2005) considered products showing LC_{50} <50 mg L^{-1} active, 50 mg L^{-1} < LC_{50} <100 mg L^{-1} moderately active, 100 mg L^{-1} < LC_{50} <750 mg L^{-1} effective, and LC_{50} >750 mg L^{-1} inactive. Kiran *et al.* (2006) considered compounds with LC_{50} <100 mg L^{-1} as exhibiting a significant larvicidal effect. Dias and Moraes (2014) has pointed out that the majority of the active oils (LC_{50}, <100 mg L^{-1}) of 361 essential oils from 269 plant species toward *Ae. aegypti* larvae are plants in the families Lamiaceae, Myrtaceae and Rutaceae. The efficacy of the essential oils towards *Ae. aegypti* larvae (LC_{50}, 0.69–100 mg L^{-1}) has been well documented by Dias and Moraes , although the activity can vary significantly depending on plant species, plant tissue, age of plant, chemotypes, geographic conditions and mosquito species (Dias and Moraes, 2014). In the current study, *M. denudata* seed hydrodistillation exhibited potent larvicidal activity against *Ae*. *aegypti*, *Cx*. *p. pallens*, wild *Ae. albopictus* and *An. sienesis* (LC_{50}, 19.30–23.30 mg L^{-1}).

Many plant preparations manifest toxicity to different mosquito species larvae and have been proposed as potential alternatives to the conventional larvicides (Sukumar *et al*., 1991; Shaalan *et al*., 2005). Active larvicidal constituents (LC_{50}, <100 mg L^{-1}) derived from essential oils toward *Ae. aegypti* larvae include phenylpropanoids (e.g., *trans*-anethole and eugenol (Cheng *et al*., 2004), LC_{50} 42 and 33 mg L^{-1}; methyleugenol and β-asarone

(Perumalsamy *et al*., 2010), 57.65 and 26.99 mg L^{-1}; (*E*)-cinnamaldehyde, ethyl cinnamate, and ethyl *p*-methoxycinnamate (Kim *et al*., 2007), 51.3, 12.3, and 20.7 mg L^{-1}; estragole, myristicin, and safrole (Perumalsamy *et al*., 2009), 46.4, 72.98, and 9.88 mg L^{-1}), monoterpene hydrocarbons (e.g., δ-3-carene, fenchone, (+)-limonene, β-myrcene, α-phellandrene, and terpinolene (Perumalsamy *et al*., 2009), 19.2, 69.28, 24.47, 66.42, 23.08, and 15.32 mg L^{-1}; *p*-cymene (Santos *et al*., 2010), 51 mg L^{-1}), oxygenated monoterpenes (e.g., ascaridole (Torres *et al*., 2008), 9.6 mg L^{-1}; borneol, camphene, linalool, terpene-4-ol, thymol, and verbenone (Perumalsamy *et al*., 2009), 94.9, 67.02, 96.6, 64.76, 64.05, and 93.16 mg L^{-1}; carvacrol and 1,8-cineole (Lima *et al*., 2011), 58.9 and 47.9 mg L^{-1}), sesquiterpene hydrocarbons (e.g., germacrene D, geijerene, and pregeijerene (Kiran *et al*., 2006), 63.6, 43.4, and 28.3 mg L^{-1}; β-caryophyllene (Perumalsamy *et al*., 2009), 88.3 mg L^{-1}), oxygenated sesquiterpenes (e.g., β-bisabolol (Rajkumar *et al*., 2010), 33.2 mg L^{-1}; (*E*),(*E*)-farnesol and (*E*)-nerolidol (Simas *et al*., 2004), 13 and 17.0 mg L^{-1}), diterpenes (e.g., 16-kaurene (Cheng *et al*., 2009), 57 mg L^{-1}), and others (e.g., hexyl butyrate (Özek *et al*., 2012) and undecan-2-one (Tabanca *et al*., 2012), 74.9 and 14.37 mg L^{-1}).

In the current study, the larvicidal principles of MD-SHD were determined to be the alkylphenol 2,4-di-*tert*-butylphenol, the monoterpene hydrocarbons *p*-cymene, (±)-limonene, α-terpinene and γ-terpinene, the oxygenated monoterpenes (±)-terpinen-4-ol and α-terpineol, the sesquiterpene hydrocarbon β-caryophyllene, the oxygenated sesquiterpene nerolidol, the fatty acids geranic acid, linoleic acid and palmitic acid and the fatty acid esters methyl linolelaidate and ethyl palmitate. LC_{50} of these constituents was between 1.98 and 56.39 mg L^{-1} for *Cx. p. pallens* larvae, between 3.28 and 53.43 mg L^{-1} for *Ae. aegypti* larvae, between 3.36 and 53.14 mg L^{-1} for *Ae. albopictus* larvae and between 4.49 and

63.50 mg L^{-1} for *An. sinensis* larvae. The larvicidal principles of *M. denudata* seed extract were determined to be the lignin honokiol, linoleic acid, and palmitic acid. LC_{50} of honokiol was between 6.32 and 32.56 mg L^{-1} for *Cx. p. pallens* larvae, between 6.51 and 33.54 mg L^{-1} for *Ae. aegypti* larvae, between 6.13 and 36.91 mg L^{-1} for *Ae. albopictus* larvae, and between 7.37 and 49.74 mg L^{-1} for *An. sinensis* larvae. However, LC_{50} of the natural compounds stated previously is between 9.6 and 100 mg L^{-1}. These constituents were highly effective toward larvae of *Cx. p. pallens*, *Ae. aegypti*, and *Ae. albopictus*. In addition, they were also effective towards *An. sinensis* larvae resistant to temephos and deltamethrin. Furthermore, the MD-SHD 50 mg L^{-1} liquid resulted in good control towards four mosquito species larvae while commercial temephos 200 g L^{-1} EC was almost ineffective toward *An. sinensis* larvae. This susceptibility difference might be attributed to the development of insecticide resistance in wild *An. sinensis* collected near rice paddy fields and cowsheds with varying histories of insecticide use. 2,4-DTBP possesses antioxidant and neuronal protective, cytotoxic, antihyperalgesic, antimalarial, and antifungal actions (Malek *et al*., 2009; Kusch *et al*., 2011; Choi *et al*., 2013; Tibbs *et al*., 2013; Dharni *et al*., 2014). However, this compound can induce hepatic and renal toxicity to newborn and young animals. It produces moderate acute toxicity to animals (1500 mg kg body $weight^{-1}$) and skin irritation (Hasegawa *et al*., 2007). Honokiol possesses various biological activities, such as antimicrobial (Clark *et al*., 1981), anticancer (Franck *et al*., 2007), antioxidative (Masahiro *et al*., 1997; Li *et al*., 2003), antianxiety (Irie *et al*., 2001), anti-inflammatory (Jongsung *et al*., 2005), antiperoxidative (Hiroyuki *et al*., 1997), neurotropic (Yoshiyasu *et al*., 2002; Yi-Ruu *et al*., 2006), and antiangiogenic (Levi *et al*., 2009).

QSAR analysis of phytochemicals toward mosquito larvae has been well noted (Park *et*

al., 2002; Wang *et al*., 2012; Dias *et al*., 2014). However, limited information is available on larvicidal activity of phenolic compounds. The QSAR information can not only contribute to the search for additional compounds with higher activity but promote the understanding of the mode of action of the larvicidal constituents as described previously by Dias and Moraes (2014).The presence of lipophilic groups in aromatic rings or in hydroxyls results in increased toxicity while the presence of hydroxyls in aromatic or aliphatic rings results in decreased toxicity (Scotti *et al*., 2014). For example, phenol (LC_{50} 194 mg L^{-1}) exhibited larvicidal activity lower than compounds that have lipophilic groups like CH chains outside a phenyl ring (e.g., carvacrol and thymol, LC_{50} 69 and 81 mg L^{-1}) towards *Ae. aegypti* larvae. Wang *et al*. (2012) studied the toxicity of six simple coumarins and seven furanocoumarins to *Cx. p. pallens* and *Ae. aegypti* larvae. They reported that chemical structure and alkoxy substitution and length of the alkoxy side chain at the C8 position of the coumarin nucleus are essential for imparting toxicity to two mosquito species and MW, hydrophobicity, and MR parameters are negatively related to the observed coumarin toxicity.

In the current study, introduction of functional group, such as butyl or propyl, in phenol significantly increased the toxicity to *Cx. p. pallens* and *Ae. aegypti* larvae. 2,4- and 2,6-DTBPs were the most toxic compounds and were significantly more toxic than 2-TBP as basic structure as well as 2,4,6-TTBP and 2-*tert*-butyl-4-MP, although the latter three compounds did not differ significantly in toxicity from each other. 2-TBP was more pronounced in toxicity than either 3-TBP or 4-TBP. Similar results were observed with 2- and 4-SBPs. The toxicity of 2-TBP was higher than 2-SBP. Similar results were observed with 4-TBP and 4-SBP. Substitution of butyl group in 2, 4-DTBP to methyl group (2-*tert*-

butyl-4-MP) significantly reduced the toxicity. 2, 4, 6-TTBP was significantly more toxic than 4-*sec*-butyl-2,6-DTBP. Isopropylphenol was significantly more toxic than butylphenols, except for 2-TBP. This current finding indicate that structural characteristics, such as types of functional groups and the number of carbon atoms which are attached to the butyl-bearing carbon, appear to play a role in determining the toxicity to two mosquito species. MW, hydrophobicity and MR parameters appear to be norelated to the observed phenol toxicity.

Investigations on the modes of action and resistance mechanisms of plant-based biolarvicides are of practical importance for mosquito control because they may give useful information on the most appropriate formulations and delivery means to be adopted for their future commercialization and for future resistance management (Perumalsamy *et al*., 2010). Major mechanisms of resistance to insecticides currently available to control mosquitoes are target site insensitivity that reduces sensitivity of sodium channels of the nervous system to pyrethroid insecticides or sensitivity of a key enzyme in the nervous system AChE to OP and carbamate insecticides and enhanced metabolic detoxification of various groups of insecticides (Hemingway *et al*., 2004; Liu, 2015). Many of the insecticides currently used in the ROK have failed to control culine and *Anopheline* mosquitoes, most probably because of the development of resistance (Chang *et al*., 2009; Shin *et al*., 2012). Alternative mosquito larval control agents with novel modes of action, low mammalian toxicity, low toxicity to aquatic non-target organisms and little aquatic environmental impact are urgently needed. In addition, certain plant constituents were found to be highly effective against insecticide-resistant mosquitoes (Perumalsamy *et al*., 2010; Wang *et al*., 2012), and they are likely to be useful in resistance management

strategies and tactics. For example, methyleugenol, α-asarone, pentadecane, (–)-asarinin and pellitorine are effective against larvae from wild *Cx. p. pallens* with high levels of resistance to chlorpyrifos, fenitrothion, fenthion, α-cypermethrin, deltamethrin and chlorfenapyr (Perumalsamy *et al*., 2010). The current findings that one alkylphenol, eight terpenes, three fatty acids and two fatty acid esters examined are virtually equal in toxicity to both insecticide-susceptible *Ae*. *aegypti* and -resistant *An*. *sinensis* larvae, suggesting that these constituents and the pyrethroid and OP 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 constituents, although the octopaminergic and γ-aminobutyric acid receptors have been suggested as novel target sites for some monoterpenoid essential oil constituents by Kostyukovsky *et al*. and Priestley *et al*. respectively (Kostyukovsky *et al*., 2002; Priestley *et al*., 2003). This original finding indicates that materials derived from *M. denudata* seed may hold promise for the development of novel and effective mosquito larvicides towards mosquito field populations.

Our current findings clearly indicate that binary mixtures of honokiol with linoleic acid (1:3, 2:1, 1:2, 1:1, and 3:1 ratios) exhibited synergistic action against both insecticide-susceptible and -resistant *Ae. albopictus* and *An. sinensis* larvae, based on the SF values. Particularly, 2:1 mixture was significantly more effective than either honokiol or linoleic acid alone. This original finding indicates that these mixtures may hold promise for the development of novel and effective mosquito larvicides even toward currently insecticide-resistant mosquito populations. The estimated Co-toxicity coefficient (CC) values were used to designate the following responses: (a) CC <80 was considered as antagonistic; (b) CC between 80 and 120 as additive; and (c) CC> 120 as synergistic. The co-toxicity

coefficient (CC) values of the HK+LA mixtures were estimated to reveal that the mixtures had pronounced synergistic effects against both *Ae. albopictus* and *An*. *sinensis* under study. The co-toxicity coefficient (CC) values of the honokiol and linoleic acid mixtures against the larvae of *Ae. albopictus* (Table 4) revealed that apart from a HK: LA ratio of 1:1, 2:1, 3:1, 1:2, 1:3 ratios produced synergistic effects, having the CC values of 229, 306, 290, 274 and 189, respectively. The same were true for honokiol and linoleic acid mixtures, having the CC values of 245, 338, 300, 256 and 189, respectively at 1:1, 2:1, 3:1, 1:2, 1:3 ratios against the larvae of *An*. *sinensis*. 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* Linnaeus (Annonaceae) and *Pongamia glabra* Vent. (Fabaceae) toward *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 toward *Spodoptera litura* (Fabricius, 1775) larvae (Hummelbrunner and Isman, 2001). Investigations on the joint toxic action mechanisms of binary mixtures and 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, 1776) 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 P_{450} 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, 1828. They suggested that synergism might be due to plant extract inhibiting some factors, such as detoxifying enzymes in mosquito larvae. In the current study, linoleic acid has acted as a powerful synergist, enhancing the effectiveness of both linoleic acid and honokiol toward both insecticide-susceptible and -resistant *Ae. albopictus* and *An. sinensis* larvae.

In conclusion, the *M. denudata* seed-derived products containing 2,4-di-*tert*-butylphenol, honokiol, linoleic acid and palmitic acid, as well as the binary mixtures of honokiol and linoleic acid could be useful as larvicides in the control of mosquito populations, particularly in the light of their activity towards insecticide-resistant mosquito larvae. This plant is a fast-growing deciduous tree with the potential for high seed production in East

and Southeast Asia (Xu *et al*., 2008). There is a potential source of seed hydrodistillation available as an eco-product. For practical use of these products as novel mosquito larvicides to proceed, further research is needed to establish their safety to humans, although *M. denudata* seeds are carminative and diaphoretic (Duke *et al*., 1985). In addition, their effects on non-target aquatic organisms including larvivorous fishes and the aquatic environment need to be established. Lastly, detailed tests are needed to understand how to improve larvicidal potency and stability for eventual commercial development.

CHAPTER Ⅲ

Acetylcholinesterase Inhibition and Histopathological Effects of Phyotochemicals on the Midgut Epithelium of Mosquito Larvae

INTRODUCTION

Studies on the mode of action of insecticides 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. Lastly, 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, 1987).

All 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 acetylcholinesterase (organophosphates and carbamates) (Constant *et al*., 2000). Another possible target for phytochemicals might be the octopaminergic system of insects. Octopamine [2-amino-1-(4-hydroxyphenyl) ethanol] plays a key role in regulation of many physiological and behavioral processes (Kostyukovsky et al., 2002; Rattan, 2010). In addition, histopathological study in insect response to a variety of toxic substances demonstrates that the midgut are the main target organs for many xenobiotics, which include dietary substances from plants (Berenbaum, 1988). Rey *et al*. (1999) observed that tannic acid posttreatment in third instar larvae of *Cx. p. pipiens* clearly caused 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.

The aim of the study is to assess the biochemical properties acetylcholinesterase (AChE) inhibitory and octopamine sensitive adenylate cyclase activities as well as the histopathological alterations in midgut epithelial cells in the third instar larvae of *Ae. aegypti* following exposure to osthole, imperatorin, and honokiol using fluorescent microscopy, confocal laser scanning microscopy, and transmission electron microscopy (TEM).

MATERIALS AND METHODS

Chemicals and reagents

Osthole, imperatorin and honokiol were obtained from the fruit of *C. monnieri* and the seed of *M. denudata*, as stated previously. Acetylthiocholine iodide (ATChI), 5,5′-dithio-bis(2-nitrobenzoate) (DTNB), eserine salicylate, tetrabutylammonium phosphate, and octopamine were purchased from Sigma-Aldrich (St.Louis, MO, USA). Glutaraldehyde, osmium tetroxide, sodium cacodylate buffer, propylene oxide, epon or elastic material, ortho periodic acid methylene blue, and uranyl acetate were supplied by Eletron Microscopy Science (Hatfield, PA, USA). Bovine serum albumin (BSA) and cyclic AMP (cAMP) Biotrak Enzymeimmunoassay system were purchased from Sigma-Aldrich and GE Healthcare (Little Chalfont, Buckinghamshire, UK), respectively. Mouse monoclonal antibody and horseradish peroxidase-labeled cAMP were purchased from R&D Systems

(Minneapolis, MN, USA). All of the other chemicals and reagents used in this study were of analytical grade quality and available commercially.

Acetylcholinesterase inhibition assay

Microplate AChE assay was carried out following the method of Hemingway *et al*. adapted from Ellman *et al*. (1961). Third instar larvae of *Ae. aegypti* kept –20°C were homogenized in ice-cold 0.1 M phosphate buffer (pH 8.0). After filtering through cheese cloth, the homogenate was centrifuged at 1000 × *g* for 5 min. The supernatant fraction was used directly as the enzyme source for AChE. Protein concentrations were determined using the Bradford dye method (1976) (Fig. 37). The reaction mixture consisted of 50 µL of the crude enzyme preparation (3.7–3.8 µg protein equivalents), 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 at 30°C, the reaction was stopped by adding 20 µL of 5 mM eserine salicylate. The absorbance was recorded at 412 nm using VersaMax microplate reader (Molecular Devices, Sunnyvale, CA, USA). Results were expressed as mean ± SE of triplicate samples of three independent experiments (Fig. 38).

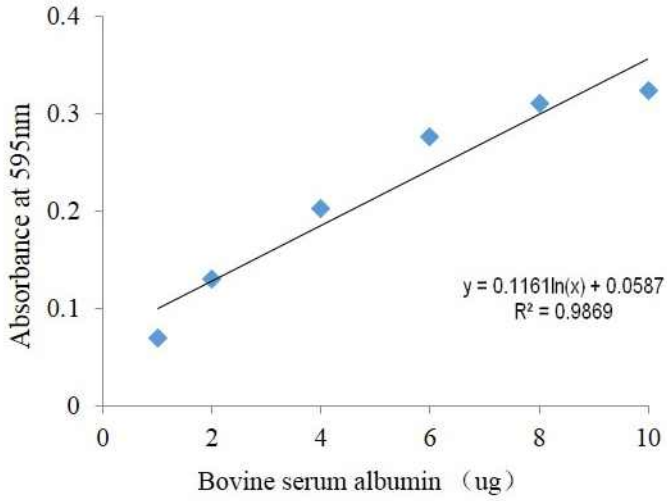


Fig. 37. Protein standard curve.

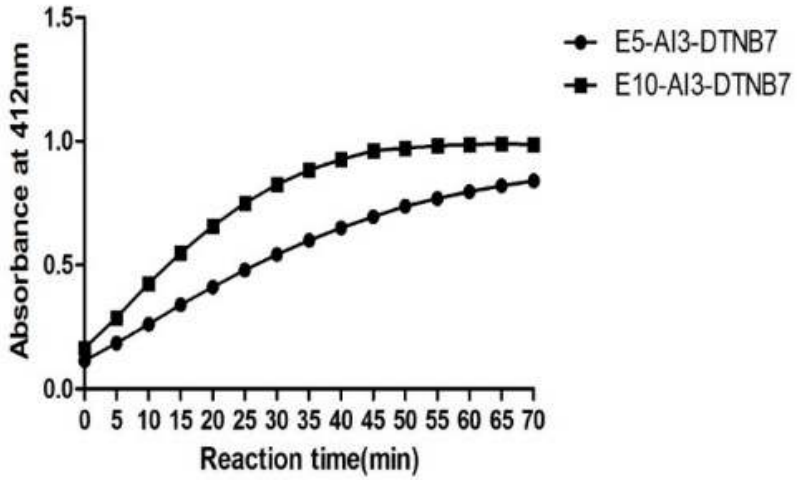


Fig. 38. Reaction intensity of various concentrations of AChE enzyme concentration standard curve.

Light microscopic analysis

The test compound-treated and -untreated (control) third instar *Cx. p. pipiens* larvae were

put on microscope slides at room temperature for light microscopy. Morphological observations were made with a Leica EZ4 HD equipped with an Integrated 3.0 Mega-Pixel CMOS camera (Heerbrugg, Switzerland).

Histological analysis by Cason's trichome staining

The treated and control third instar *Cx. p. pipiens* larvae were immediately fixed in Bouin's fluid (Hong *et al*., 2011) at 4°C for 24 hours. Larvae were then dehydrated in an ethanol-tetrahydrofuran-xylene series and embedded in Paraplast X-tra (Sigma-Aldrich). Embedded preparations of the larvae were sectioned at 5 μm thickness by using a Thermo Scientific Microm HM 340E rotary microcotome (Walldorf, Germany). Sections were dried at 40°C overnight and subsequently deparaffinized with Fisher Scientific CitriSolv (Fair Lawn, NJ, USA) and rehydrated with a series of ethanol to phosphate-buffered saline (PBS) solution as described previously (Kwon *et al*., 2006). Samples were stained in Weigert's iron hematoxylin for 30 s, followed by Cason's trichrome staining procedures (Kiernan, 1990). This staining protocol stains the columnar and goblet cells of the midgut blue and red, respectively (Lacham-Kaplan and Trounson, 1995). Sections were dehydrated, cleared in xylene, and mounted in EMS permount (Hatfield, PA, USA). Images were observed and captured using a Nikon compound microscope (Tokyo, Japan) equipped with Nomarski Optics (Nomarski, Wetzlar, Germany).

Transmission electron microscopic analysis

The midgut of the test compound-treated and control third instar *Cx. p. pipiens* larvae was primarily fixed in Karnovsky's fixative (2 % glutaraldehyde and 2% paraformaldehyde in 0.05 M sodium cacodylate buffer pH 7.2) at 4°C in darkness for 2–4 hours, and were washed with the same buffer three times (Kim and Park, 2007). The specimens were postfixed with 1% (w/v) osmium tetroxide in the same buffer at 4°C for 2 hours, and washed with distilled water three times. The postfixed specimens were then dehydrated through a graded series of ethanol increasing concentrations up to 100% for 15 min as a transitional fluid, and embedded in Spurr's resin (Wallis and Griffin, 1973). Finally, they were dried using hexamethyldisilazane. Ultrathin sections (approximately 50 nm thickness) were cut with a Leica UC6 ultra-microtome (Leica, Wetzlar, Germany), stained in 2% aqueous uranyl acetate for 7 min at room temperature, and with Reynolds lead citrate (Reynolds, 1963) for 7 min. The sections were mounted on copper grids, and the micrographs were obtained from a Philips CM120 transmission electron microscope (Philips Electronics, Amsterdam, the Netherlands) at 80 kV. Images were captured using an Olympus-SIS MegaView III digital camera (Olympus-SIS, Lakewood, CO, USA). Observations were taken of five larvae under the transmission electron microscope.

Determination of cyclic AMP level

The *in vitro* octopamine sensitive adenylate cyclase activity was investigated according to the modified method of Pratt and Pryor (Pratt and Pryor, 1986). The whole bodies of 75 third instar *Ae. aegypti* larvae (106.2 mg) were homogenized in 500 µL of 2 mM Tris-

maleate buffer (pH 7.4) containing 0.8 mM ethylene glycol tetraacetic acid (EGTA). Adenylate cyclase activity was measured using a cAMP Biotrak Enzymeimmunoassay system according to the manufacturer's instructions. The assay was conducted in a total volume of 100 μL containing 80 mM Tris-maleate buffer, 5 mM theophylline (to inhibit phosphodiesterase activity), 2 mM $MgSO_4$, 0.5 mM adenosine triphosphate (ATP), 0.2 mM EGTA, 50 μL of whole body homogenate (equivalent to 4.22 μg protein), and 1 μL of the test compounds in Tris-maleate buffer containing 0.2% ethanol. After incubation for 5 min at 20°C, the reaction was initiated by the addition of ATP. Incubation was carried out at 30°C for 3 min in a shaking water bath. The reaction was terminated by boiling for 2 min, and then the assay tube was cooled and centrifuged for 10 min at 8000 × *g*. The 50 μL aliquots of the supernatant were assayed for cAMP level.

The polystyrene microplates (1 strip of 8 wells) coated with a goat anti-mouse polyclonal antibody were used. Fifty microliters of primary antibody (mouse monoclonal antibody) solution was added to each well except the blank wells (or the nonspecific binding (NSB) wells). The wells covered with the adhesive strip were incubated for 1 h at 25°C in a shaking incubator (480 rpm), followed by four times washing steps, each with 400 μL of wash buffer. The 50 μL of the test samples for cAMP determination and cAMP standard were added to wells. Control, blank (NSB), and zero standard wells were added with 50 μL of the diluent RD5-55 buffer. The 50 μL of cAMP conjugate (horseradish peroxidase-labeled cAMP) was then added to wells. The plate covered with a new adhesive strip was incubated for 2 h at 25°C on the shaker, followed by the four times washing steps. Then, 200 μL of substrate solution (equal volume of stabilized hydrogen peroxide and stabilized chromogen) was added to each well, and the test plate was incubated for 30 min at 25°C

on the benchtop in darkness. Finally, the reaction was stopped by adding 100 μL of stop solution (2 N sulfuric acid) to each well. Optical densities at 450 and 540 nm were measured using the VersaMax microplate reader. The readings at 540 nm were subtracted from the readings at 450 nm. The cAMP concentrations were expressed as nM μg protein^{-1}. Results were expressed as mean ± SE of duplicate samples of three independent experiments.

RESULTS

Acetylcholinesterase inhibitory activity

AChE inhibition by the test coumarins was assayed against third instar larvae of *Ae. aegypti* (Table 30). There was no significant inhibition by the coumarins (IC_{50}, $>1 \times 10^{-3}$ M).

Table 30. Acetylcholinesterase inhibitory activity of test coumarins against third instar larvae of *Ae. aegypti*

Coumarin	IC_{50} (M)
Osthole	$>1 \times 10^{-3}$
Imperatorin	$>1 \times 10^{-3}$
IsoImperatorin	$>1 \times 10^{-3}$
7-Ethoxycoumarin	$>1 \times 10^{-3}$
Angelicin	$>1 \times 10^{-3}$
Osthole	$>1 \times 10^{-3}$

AChE inhibition by honokiol and two fatty acids was assayed against third instar larvae of *Ae. aegypti* (Table 31). Honokiol and linoleic acid exhibited potent AChE inhibitory activity.

Table 31. Acetylcholinesterase inhibitory activity of the isolated principles from the seed of *Magnolia denudata* against third instar larvae of *Ae. aegypti*

Compound	IC_{50} (mM)
Honokiol	0.92
Linenic acid	0.80
Palmitic acid	$>1000 \times 10^{-3}$

Light microscopic analysis

A microscopic picture shows that the whole body of the larvae changed into white after the treatment of osthole. Fat tissue layers cannot be shown clearly. Clear alimentary canal region is shown in untreated larvae (Fig. 39). However, complete damage was occurred after the treatment of isolated compounds imperatorin. These figures indicate that damage was occurred by the treatment of isolated compounds. The results of isolates compounds after 24 hours of treatment, which drove into next level of experiment, show clear observation of mode of delivery of compounds.

Fig. 39. Light microscopy picture of the untreated (A), osthole treated (B), imperatorin treated(C) larvae.

Histopathological effect on anterior and posterior midgut

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* were shown clear cells and undamaged (Fig. 40A, 41A, 42A). The most characteristic effects were disruption of peritrophic membrane, striated border, secretory cells, regenerative cells, and longitudinal muscles. Nuclei of the epithelial cells disintegrated as compared to the control. The columnar cells were moderately disturbed. Overall tissue destruction leaving gaps and lacunae at various layers was observed. Apical parts of adjacent untreated dark cells are showing long microvilli, dense cytoplasm with mitochondria and polysomes, and normal junctional complex.

The treated larvae were undergone histopathological sections under transmission electron microscope. Midgut-region of compounds treated cells were shown in Fig. 43. Midgut tissues showed cytopathic effects on treated mosquitoes. The compound was destroyed all clear cells and degeneration of dark cells, particularly cells were surrounded in the nucleus and cytoplasmic cells which were destroyed completely (Fig. 43, 44) .

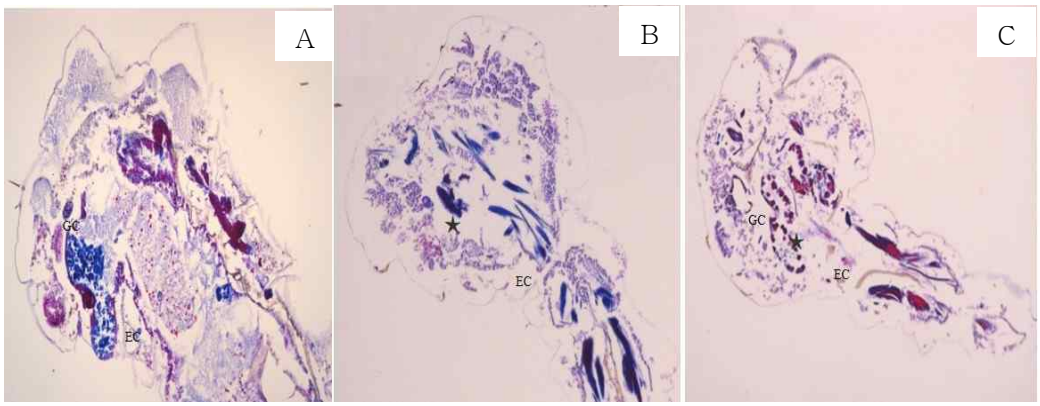


Fig. 40. Thorax and anterior mid-gut region of the untreated (A), osthole (B), imperatorin treated (C) larvae after 24 h.

It 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. 40A, 41A, 42A). Posterior midgut-region of compounds treated were shown in Fig. 40B. The compound was destroyed all clear cells and also degeneration of polysomes cells, particularly cells basal lamina and lumen regions were completely destroyed. After 24 h of treatment, the posterior midgut was almost entirely damaged, bearing only residues inside the intestinal lumen cells (Fig. 43C).

Following treatment of osthole and imperatorin, we observed an acceleration in the blebbing of the midgut epithelium into the gut lumen and eventual lysis of the epithelium. The circular muscles and longitudinal muscles were apparently disrupted. (Fig. 43B, 44B)

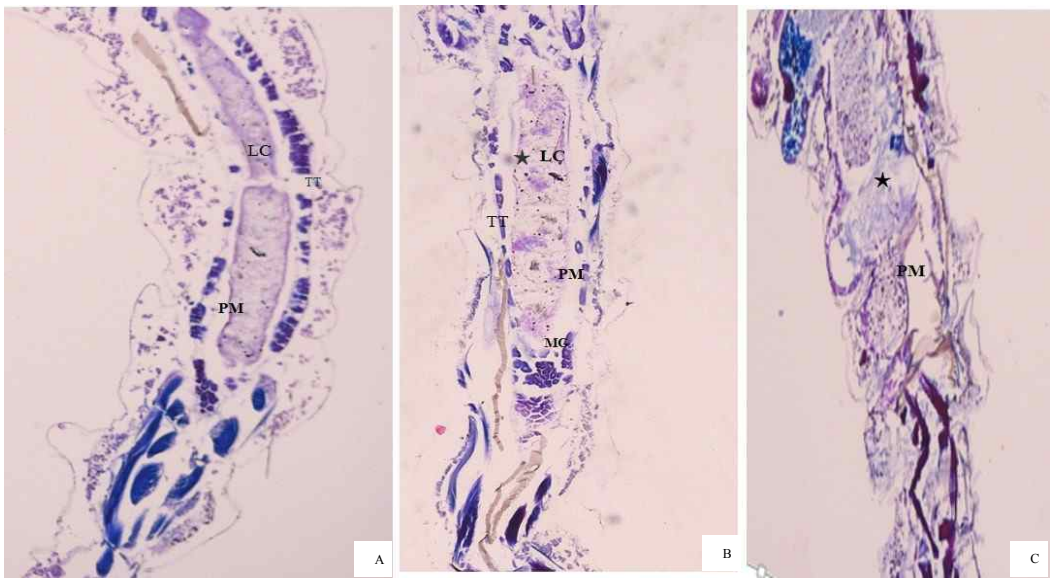


Fig. 41. Light micrographs of posterior mid-gut region of third instar *Ae. aegypti* larvae without (A) and with treatment with LC_{50} of natural imperatorin treated after 12 h (B) and 24 h (C). ×35 magnification. Abbreviations: LC- lumen contents, PM- Peritrophic m embrane, TT- Tracheal tube.

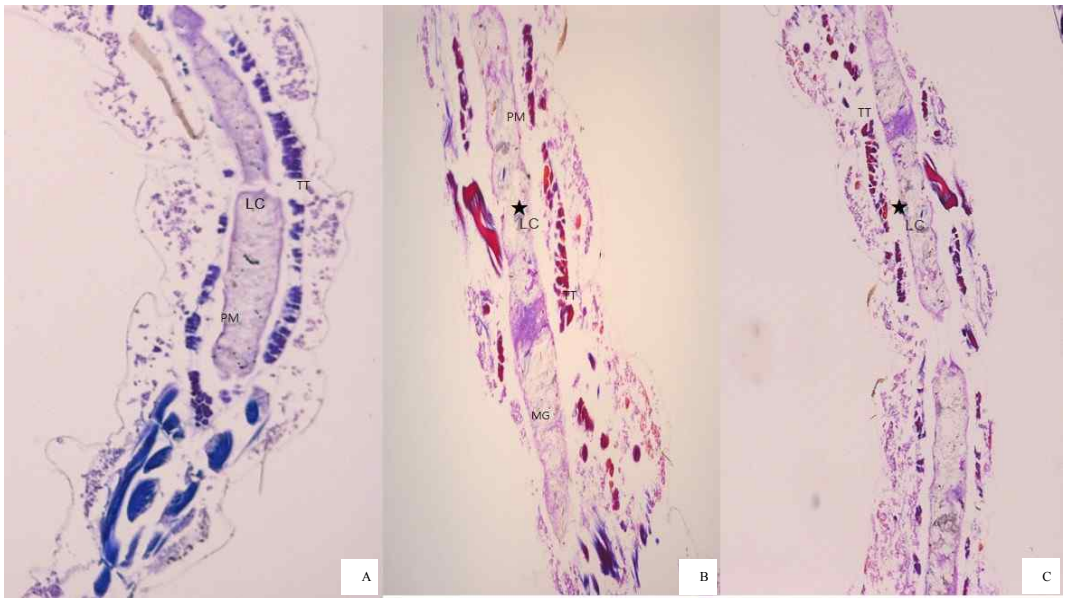


Fig. 42. Light micrographs of posterior mid-gut region of third instar *Ae. aegypti* larvae without (A) and with treatment with LC_{50} of natural osthole treated after 12 h (B) and 24 h (C). ×35 magnification. Abbreviations: LC- lumen contents, PM- Peritrophic membra ne, TT- Tracheal tube.

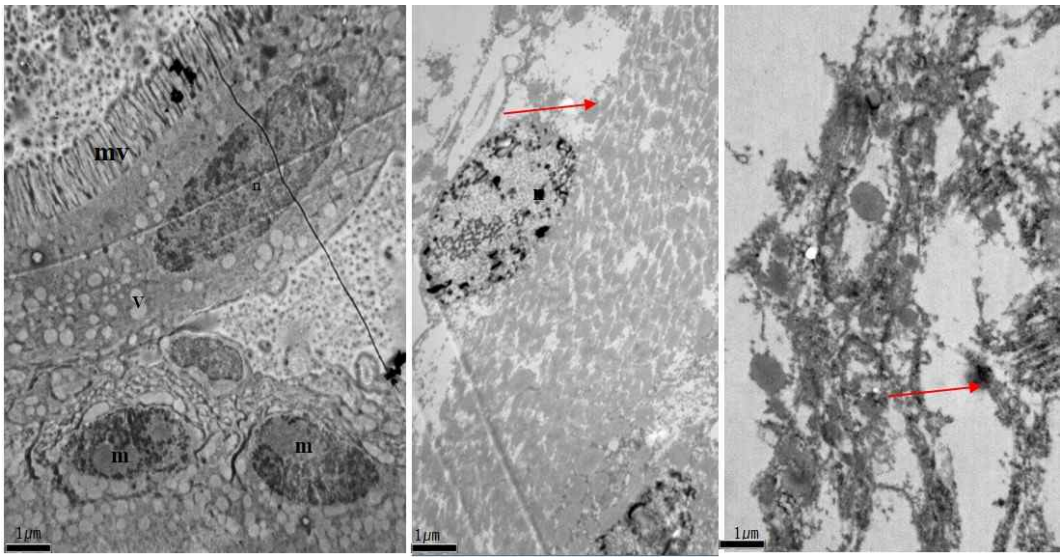


Fig. 43. TEM pictures of midgut regions of third instar *Ae. aegypti* larvae without (A) and with treatment with LC_{50} of natural imperatorin after12 hs (B) and 24 hs (C). m, mitochondria; mv, microvilli; n, nucleus; v, vacuole.

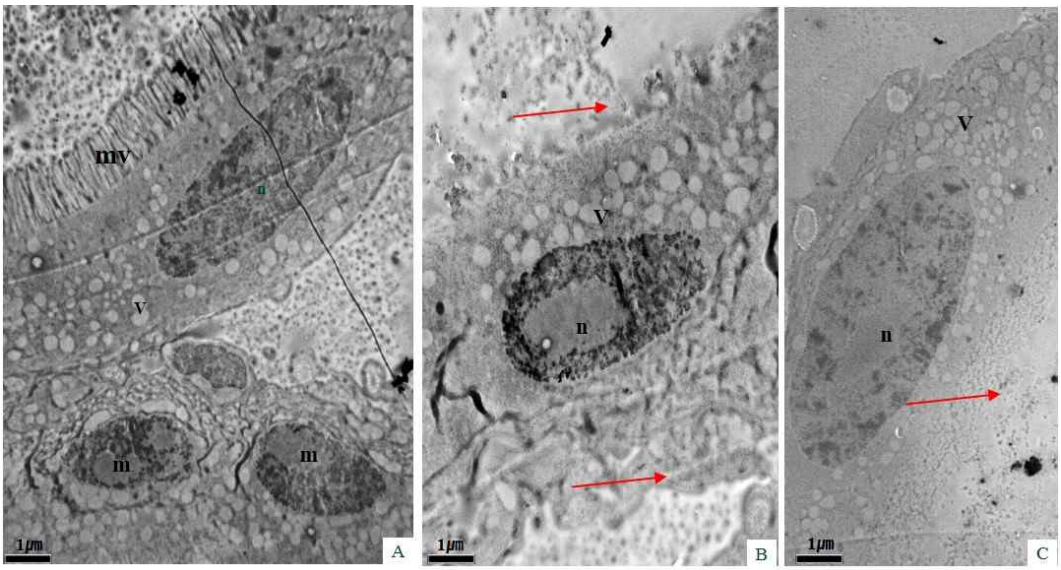


Fig. 44. TEM pictures of midgut regions of third instar *Ae. aegypti* larvae without (A) and with treatment with LC_{50} of natural osthole (B) after12 hs (B) and 24 hs (C). m, mitochondria; mv, microvilli; n, nucleus; v, vacuole.

Effect of coumarins on cAMP production

Based on a competitive ELISA in the immunoassay system, there is an inverse correlation between cAMP concentration in the sample and the assay signal intensity. Low levels of cAMP result in a high OD intensity, while a high concentration of cAMP results in a low signal (Fig. 45).

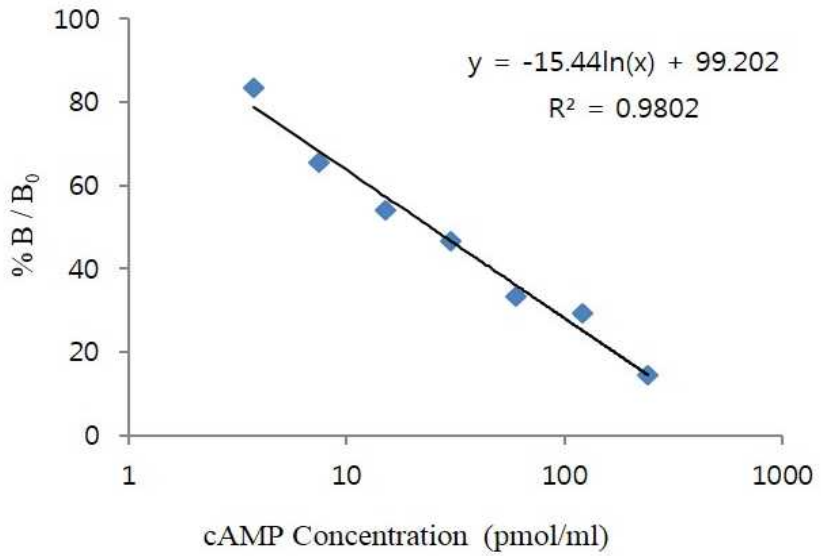


Fig. 45. cAMP standard curve. Assays were performed with the cAMP standard.

The effects of octopamine on the cAMP production in the preparation were investigated (Fig. 46). Dose dependences in cAMP levels were observed as octopamine concentrations increased. cAMP levels slightly increased in the presences of octopamine at 5–10 nM mL^{-1}, respectively. However, significant increases in cAMP levels were found in the presences of octopamine at 10–1000 nM mL^{-1}, respectively. The drug concentration showed remarkably decrease in OD values during the period of 10–100 nM mL^{-1} octopamine, respectively. Therefore, the octopamine preparation (100 nM mL^{-1}), which

gave OD value 0.22 was used for the adenylate cyclase activity.

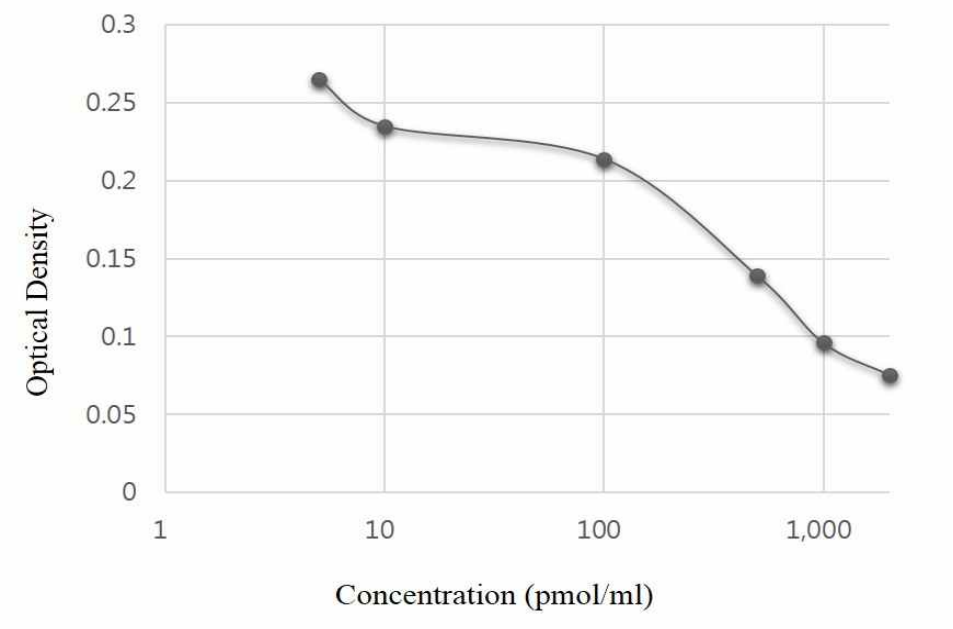


Fig. 46. Effects of octopamine at 5, 10, 100, 500, and 1000 nM mL^{-1} on cAMP levels in the nervous system of the *Ae. aegypti* larvae.

The effects of two linear furanocoumarins, two simple coumarins, one Furanocoumarins on cAMP levels of whole body homogenates from third instar *Ae. aegypti* larvae were examined and compared with those induced by octopamine alone (Fig. 47). There were significant differences ($F = 2107$; $df = 6, 14$; $p < 0.0001$) in the cAMP levels by the test compounds. At a concentration of 100 μM, the cAMP levels induced by osthole, 7-Ethoxycoumarin, imperatorin, isoimperatorin, and angelicin were significantly lower than that induced by octopamine alone. This finding indicates that both test coumarins might act on octopaminergic receptor. Detailed test are needed to fully understand the exact mode of action.

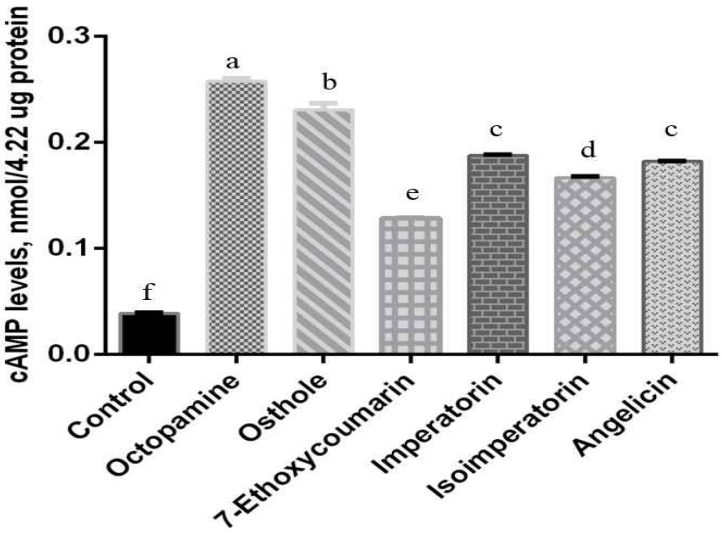


Fig. 47. Effect on cyclic AMP levels. A whole body homogenate from third instar *Aedes aegypti* larvae was assayed for adenylate cyclase activity, in the presence of 100 μM of two linear furanocoumarins (osthole, 7-ethoxycoumarin), two simple coumarins (imperatorin, isoimperatorin), one angular furanocoumarins (angelicin). The effects of the test compounds on cAMP levels of the homogenate were compared with those induced by octopamine (100 μM) alone. Data were expressed as nM 4.22 μg protein^{-1}. Each bar represents the mean ± standard error of duplicate samples of three independent experiments. (* p = 0.05, according to Bonferroni multiple-comparison method)

DISCUSSION

Investigations on the modes of action of naturally occurring compounds 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). The modes of insecticidal action of naturally occurring compounds are mainly due to AChE inhibition and interference with the octopaminergic system (Isman, 2006). Certain terpenoids such as pulegone-1, 2-epoxide and 1, 8-cineole inhibit AChE from *A. aegypti* larvae (IC_{50}, 1.45–74.33 mM) (Lee and Ahn, 2013), housefly and Madagascar roach (Grundy and Still, 1985), head louse (IC_{50}, 77 mM) (Picollo *et al*., 2008), and three stored-product insect pests (López and Pascual-Villalobos, 2010). 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 and Still, 1985; Picollo *et al*., 2008). The octopaminergic and gamma aminobutyric acid receptors have also been suggested as novel target sites for some monoterpenoid essential oil constituents in *Helicoverpa armigera* (Hübner, 1809) (Kostyukovsky *et al*., 2002) and *Drosophila melanogaster* Meigen, 1830 (Priestley *et al*., 2003), respectively.

Many of the physiological functions of octopamine appear to be mediated by multiple pharmacologically distinct subclasses of octopamine receptor, which are coupled to different second messenger systems (Evans, 1981; Howell *et al*., 1998). The receptor subtypes differ in their tissue and cellular localization, their affinity for octopamine, and in the conducted secondly messenger pathways (Nathanson, 1985). It was proposed to

designate the octopamine receptors responsible for the slowing of the myogenic rhythm in this preparation the octopamin-1 subtype receptors in locust (Evans, 1981). As well as the octopamine-2A and octopamine -2B receptors, which mediate their effects via the activation of adenylate cyclase activity (Evans *et al*., 2005). More recently, octopamine receptors that mediate changes in cyclic AMP levels in the locust central nervous system have been distinguished pharmacologically as OAR subtype (OAR3) (Roeder *et al*., 1993). These receptors were subsequently shown to be likely to mediate their effects via a mechanism that elevated intracellular calcium levels (Howell *et al*., 1998). A number of essential oil compounds have been demonstrated to act on octopaminergic system of insects. In the current study, the cAMP levels induced by the phytochemicals were lower than that induced by octopamine alone. The unsaturated two linear furanocoumarins (osthole, 7-ethoxycoumarin), two simple coumarins (imperatorin, isoimperatorin), one angular furanocoumarins (angelicin) were ineffective at inhibiting AChE but caused decrease in cAMP levels, indicating that the mechanism of insecticidal action of these coumarins might be due to interference with the octopaminergic system. Linoleic acid and honokiol moderately inhibited AChE. This finding indicates that Linoleic acid and honokiol might act on AChE suggested that they might act as a natural cholinesterase inhibitor. Detailed tests are needed to fully understand the exact modes of action of these phytochemicals.

The current microscopic analysis clearly indicates that osthole and imperatorin caused histopathological alterations in thorax, midgut regions of third instar larvae of *Ae. aegypti*. Investigations on the modes of action and the resistance mechanisms of plant-based biocides are of practical importance because they may provide useful information on the most appropriate formulations to be adapted for their future commercialization and for

future resistance management. In addition, they may contribute to the development of selective mosquito control alternatives with novel target sites and low toxicity (Ahn *et al*., 2006. Isman, 2006). It has been reported that osthole and imperatorin is effective against *Cules pipiens pallens* larvae with high levels of resistance to AChE inhibitors such as chlorpyrifos, fenitrothion, and fenthion as well as axonic nerve poisons such as α-cypermethrin and deltamethrin (Wang *et al*., 2012). These results suggest that the coumarins osthole and imperatorin and OP insecticides do not share a common mode of action. In addition, histopathological investigations indicate that the midgut epithelium is the site of action of plant preparations and PSMs in *Papilio polyxenes* and *Papilio glaucus* (Steinly and Berenbaum, 1985), some aquatic dipteran larvae (Rey *et al*, 1999; Al-Mehmadi, 2001), and *Schistocerca gregaria* and *Locusta migratoria* (Reynolds, 1963), *Rhodnius prolixus* (Park *et al*., 2012), and several species of Acridoidea (Clements, 1992). Midgut is known to have functions such as ionic and osmotic regulation (Kiernan, 1990.), lipid and carbohydrate storage (Clements, 1992.) control of the midgut lumen pH, and secretion of digestive enzymes and absorption of nutrients (Gonzalez and Garcia, 1992). The histopathological effects differ qualitatively according to the localization of organs along the midgut and quantitatively according to the concentration of test material examined, the duration of the treatment, and the taxon (Reynolds, 1963). The mosquito midgut epithelium is one of the first physical barriers encountered by ingested pathogens; it is composed of a single layer of polarized epithelial cells supported by an underlying basal lamina (Hecker *et al*., 1977, Reinhardt, 1973). In mosquito-arboviral systems, midgut infection and escape barriers that have been described include inhospitable chemical environment of midgut lumen that destroys incoming virions (e.g., proteolytic enzymes, pH), lack of epithelial

receptors for viral attachment and/or entry, dose dependence of epithelial cell infection, and relative abundance of organelles necessary for virions assembly (Higgs, 2004).

Nasiruddin and Mordue reported that azadirachtin caused some of the initial effects on necrosis, particularly associated with the swelling of the cell and organelles, vesiculation of membranes, and dilation of rough endoplasmic reticulum in locusts. It has been also reported that tannic acid caused dramatic degenerative response of the midgut through a sequential epithelial disorganization in *Cx. pipiens* larvae (Rey, 1999). Our current study revealed that osthole and imperatorin caused dramatic degenerative responses in thorax and anterior and posterior midgut regions of *Ae. aegypti* larvae by targeting ion transporting cells in gastric caeca of thorax region and epithelial cells of anterior and posterior midgut region where osmoregulation-related machineries such as, H+V-ATPase are highly expressed in the anterior midgut of *Ae. aegypti* larvae (Nishiura, 2007).

In conclusion, osthole and imperatorin caused degenerative responses in the cell organelles of thorax, midgut regions, possibly by targeting with osmoregulation system. The alkaloid acts as a potential mosquito larvicide with a specific target site for the control of mosquito populations.

CONCLUSION

Mosquitoes are prevalent worldwide and common and serious disease vectoring insect pest. It is most important single group of insects well known for their public health importance, as they act as vector for many tropical and subtropical diseases such as dengue fever, yellow fever, malaria, filariasis and encephalitis of different types including Japanese encephalitis. Mosquito larval abatement has been achieved principally by the use of organophosphorus (OP), permethirn, and other types of insecticides, insect growth regulators, and bacterial larvicides, which continue to be effective larvicides. In this study, we have done PCR identification of *Anopheles* species (*An. sinensis*), and insecticide resistance monitoring was assessed of identified *Anophiline* mosquito strain. The toxicity evaluation of mosquito larvicidal insecticides against filed collected *An. sinensis* and *Culex pipiens pallens* from different parts of Korea. The two coumarins toxicity derived from *Cnidium monnieri* fruit , three *Magnolia denudata* derived constituents and *Magnolia denudata* steam distillate were assayed against larvae of four mosquito species. The possible mode of action and delivery of isolated active principles are also examined.

The *C. monnieri* fruit-derived preparations containing imperatorin and osthole may hold promise for the development of novel and effective mosquito larvicides even against currently insecticide-resistant mosquito populations, although imperatorin (LC_{50} = 3.14 mg L^{-1} for KS-CP *C. p. pallens* and 2.88 mg L^{-1} for *A. aegypti*) and osthole (13.11 and 13.14 mg L^{-1}) fail to meet the stage 3 criteria (LC_{50} < 1 mg L^{-1}). For the mode of action study, AChE inhibition assay was performed, results clearly shown, the isolated active constituents are not strong AChE inhibitor against mosquito larvae. But caused decrease in cAMP levels, indicating that the

mechanism of insecticidal action of these coumarins might be due to interference with the octopaminergic system. Further study requires finding out exact mode action of all identified compounds. In histopathological effect on midgut epithilium observations at osthole and imperatorin posttreatment intervals 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 this preliminary study indicated that osthole and imperatorin compound showed very strong active and dramatic degenerative response in the cell organelles, which indicates osthole and imperatorin acts as potential larvicides which helps to improve further study about specific target site and mode of delivery to target insects.

In conclusion, further research is needed to establish their safety issues in relation to human health, non-target aquatic organisms, and the aquatic environment. Additionally, their larvicide modes of action need to be established and formulations for improving larvicidal potency and stability, thereby reducing costs, need to be developed.

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국문 초록

황열 모기, 이집트 숲모기; 아시아 타이거 모기, 흰줄숲모기; 말라리아 모기, 중국얼룩날개모기, 그리고 노던 집모기, 빨간집모기은 활열, 말라리아, 웨스트 나일 바이러스와 같은 다양한 질병을 매개하는 곤충이다. 현재 100 여개 국가에서 뎅기열 감염 가능성이 높은 지역에 거주하는 인구만 25 억 명에 달하는 것으로 알려져 있으며, 매해 5 천만-1 억 명이 감염된다고 추산한다. 그의 의한 사망자는 약 2 만명 정도 이며, 대부분은 아동이다. 최근에는 128 개 국가의 약 40 억명이 노출됐다고 보고되고 있다. 미국에서 1999 년부터 2010 까지, 웨스트나일 바이러스 감염자 37088 건 가운데 16196 명은 심각한 신경계 질환으로 번져 1549 명이 숨졌다. 지구온난화 등 환경의 변화와 더불어 국제간의 교역 및 여행 증가 등으로 한동안 줄어들던 각종 모기 매개 질병 발생율이 점차 높아지고 있습니다. 해충종합관리의 하나의 수단으로서 살충제 저항성발달 등이 문제가 되고 있다. 현재 말라리아나 뎅기열에 특별한 백신은 없기 때문에 새로운 작용대상에 작용하며, 모기 종합 관리 전략 마련이 시급합니다.

벌사상자 열매에서 분리 동정한 쿠마린 화합물 그리고 이들 화합물과 구조적으로 유사한 11 종 화학물, 백목련 종자 정유의 17 동 구성성분, 백목련 종자 추출물에서 honokiol, linoleic acid 및 palmitic acid 대하여 살충제 감수성 빨간집모기, 감수성 계통 빨간집모기(KS-CP)와 살충제에 저항성을 가지는 야외 계통 빨간집모기, 집트숲모기, 흰줄숲모기 그리고 중국얼룩날개모기 3 령 유충을 대상으로 독성을 직접-접촉 생물검정법을 이용하여 살충활성을 조사하였다. 에집트숲모기 및 빨간집모기 3 령유충에 대한 살충활성을 조사하여 정량적 구조-활성관계를 분석하였다. 아울러 이들 화합물의 살충메카니즘을 조사한 내용이다. 벌사상자 열매에서 분리 동정한 imperatorin, osthole 및 기타 11 종 유사 coumarin 계열 화합물질에 대하여 에집트숲모기,

감수성 계통 빨간집모기(KS-CP)와 살충제에 저항성을 가지는 야외 계통 빨간집모기 3 령 유충을 대상으로 독성을 직접-접촉 생물검정법을 이용하여 살충활성을 조사하였다. 결과는 temephos 와 비교하였다. 본 연구에서는 imperatorin 과 osthole 유사체를 대상으로 QSAR (정량 적구조활성상관관계)를 이용하여 활성치를 예측하고자 하였다. coumarin 계열의 살유충활성 물질 Imperatorin 및 Osthole 을 질량분석 (MS)과 핵자기공명 (NMR) 과 같은 분광분석을 통하여 구조를 동정하였다. 화합물 처리 24 시간 후, 반수치사농도값(LC_{50})을 기준으로 imperatorin (2.88−3.14 mg L^{-1})이 가장 높은 독성 화합물로 나타났으며, osthole (13.11−13.14)에서 모기에 대한 살충활성이 확인되었다. 탄소 원자(8 위치)로 methox 기(OCH3)의 치환, 탄소 원자(8 위치)에 alkoxyl 사슬의 길이, 탄소 원자(7 위치)에 methoxy 의 치환 등의 상관 성질이 모기 2 종에 대한 살충 활성을 결정하는 역할을 한다. 분자량, 소수성 파라미터, log P, 분자 굴절 등 물리적 특정은 살유충활성에 큰 영향이 없었다.

백목련 종자 정유 화합물의 17 종 의 빨간집모기, 에집트숲모기, 흰줄숲모기, 중국얼룩날개모기 3 령 유충 대한 독성을 직접-접촉 생물검정법을 이용하여 살충활성을 조사하였다. 결과는 Fenitrothion, fenthion, temepho, deltamethrin로 비교하였다. 백목련 종자 정유는 반수치사농도(LC_{50}) 기준으로 ($LC_{50,}$ 19.30-23.30 ppm)의 좋은 활성을 나타내었다. 액상 제형에 대한 실험은 화합물 종자 정유를 함유한 약제를 10, 20, 30 및 50 mg L^{-1} 농도 4 가지를 제조하여 시중에 판매되는 temephos 200 g L^{-1} 유제화 상품과 함께 살충효력을 평가하였다. 높은 독성 화합물은 2,4-Di-*tert*-butylphenol ($LC_{50,}$ 1.98-3.90 ppm)이고 그 다음으로 nerolidol, (±)-limonene, α-terpinene and ɣ-terpinene (LC_{50}, 9.84-36.42 mg L^{-1}) 이었으며, 13 개 2,4-Di-*tert*-butylphenol 계열 화합물의 정량적 분자구조와 저감활성 관계분석(QSAR) 도 같이 분석 하였다.

백목련 종자 메탄올 추출물에서 살충성분은 리그닌 화합물인 honokiol 그리고 지방산 화합물인 linoleic acid 및 palmitic acid 으로 확인 하였고. 살충제-감수성 빨간집모기, 에집트숲모기, 야외에서 채집한 흰줄숲모기 그리고 다양한 살충제에 저항성을 가지는 중국얼룩날개모기의 3 령기 유충을 이용하여 실시하였으며 honokiol (LC_{50}, 6.32, 6.51, 6.13, 7.37 ppm)은 linoleic acid 그리고 palmitic acid 보다 각각 1-1.2 배, 5.1-14.8 배 높은 독성을 보였다. Honokiol, linoleic acid 및 palmitic acid 의 독성평가를 Honokiol 을 독립적으로 사용하였을 때와 honokiol 을 linoleic acid, palmitic acid 와 1:1, 1:2, 1:3, 2:1, 그리고 3:1 비율로 혼합한 이진 화합물로 처리하였을 때, 야외에서 채집한 흰줄숲모기, 살충제-저항성 중국얼룩날개모기 각각의 3 령기 유충에 대한 독성평가를 직접-접촉 생물검정을 통해 실시하였다. Honokiol 과 linoleic acid (2:1 비율) 이진 혼합물은 흰줄숲모기 유충과 중국얼룩날개모기 유충에서 각각 2.11ppm, 2.19ppm 으로 상당히 높은 독성을 보였으며, honokiol [흰줄숲모기(6.13ppm), 중국얼룩날개모기(7.37ppm)]과 linoleic acid [흰줄숲모기(7.28ppm), 중국얼룩날개모(7.50ppm)] 을 단독적으로 사용하였을 때보다 독성이 높음을 확인하였다.

선발된 화합물에 대한 살충메커니즘을 밝히기 위해 아세틸콜린에스터라제 (AChE)의 저해성를 조사하고, 조직병리학적으로 화합물 처리시 모기유충 위장의 변화를 관찰하였다. 쿠마린 계열 화합물 osthole, 7-ethoxycoumarin, imperatorin, isoimperatorin, angelicin 는 AChe 저해성를 확인할 수 없지만 모기 세포 내 정보전달 물질인 cyclic AMP 에서는 변화를 일으켰다. cAMP 의 변화는 이들 화합물에 의한 옥토파민 수용체의 활성에 경쟁 가능성을 가르킨다. honokiol 과 linoleic acid 은 AChe 저해성를 확인 할 수 있었으나, 조직병리학적인 연구에서는 imperatorin 과 osthole 을 처리하였을 때 유충 내의 세포소기관이 관통, 치환 그리고 파괴되는 것을 투과전자현미경(TEM) 사진을 통해 확인할 수 있었다.

이상의 결과를 바탕으로 본 논문의 연구는 벌사상자 및 백목련에 함유된 활성본체들을 분리 동정 하였고, 활성 물질의 작용기구를 연구했는데 그 의의가 있고, 구성 물질에 대한 생물검정을 통해 향후 모기 방제에 있어 살유충, 특히 살충제 저항성 모기에 대한 그들의 활성 관점에서 유용할 수 있다.

주요어: 살충제, 모기, 빨간집모기, 중국얼룩날개모기, 에집트숲모기, 흰줄숲모기, 식물유래살충제, 정유, 이원혼합물, 상승효과, 백목련 종자, 벌사상자 열매, 아세틸콜린에스터라제 저해, adenlylate cyclase, 옥토파민, cyclic AMP immunoassay, 조직병리학

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