A THESIS

FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

Repellency and insecticidal activity of *Zanthoxylum* oil constituents against *Stomoxys calcitrans* L. (Diptera: Muscidae)

산초나무속(*Zanthoxylum*) 식물 성분들의 침파리(*Stomoxys calcitrans* L.)에 대한 기피력 및 살충활성

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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 stable fly, *Stomoxys calcitrans* L., is one of the important insect pests as a blood-sucking ectoparasite that mainly bites cattle, horses, pigs, and dogs, but also bites humans. In this study, an assessment is made of the repellency of 22 plant essential oils (EOs), *Zanthoxylum* oil constituents, and the mixtures with tamanu, *Calophyllum inophyllum*, nut oil (CI-NO) and the olfactory responses of the fly antennae to *Zanthoxylum*-derived constituents. The fumigant toxicity and possible insecticide mode of action of the constituents also were evaluated.

In a repellency test using the EOs at a dose of 0.5 mg/cm^2 with six human male volunteers based on protection time (PT) to first bite, patchouli, clove bud, lovage root, and clove leaf EOs were the most effective (PT, 3.20–3.62 h) but were less active than *N*,*N*-diethyl-*m*-toluamide (DEET) (PT, 4.35 h). Strong repellency was also produced by thyme white EO (PT, 2.07 h), while moderate repellency was obtained from *Z. piperitum* pericarp steam distillate (ZP-SD), geranium, thyme red, and oregano EOs (PT, 1.01–1.12

h). *Z. armatum* seed oil (ZA-SO) and bergamot EO exhibited weak repellency (PT, 0.60 and 0.57 h). At 0.25 mg/cm^2, protection time of patchouli, savory, thyme white and ZP-SD (PT, ≈0.6 h) and clove bud, lovage root and clove leaf EOs (PT, ≈1.0 h) were also shorter than that of DEET (PT, ≈2.0 h). Binary mixtures of the active EOs and CI-NO produced a significant increase in the protection time, although the repellency of each EO was weaker than that of DEET. PT of binary mixtures (PT, 2.17–2.64 h) of five EOs (lovage root, patchouli, clove bud, thyme white, and clove leaf) and CI-NO (0.25 + 2.0 mg/cm^2) was similar to that of DEET + CI-NO mixture (PT, 2.69 h) at the same doses and showed longer protection time than that of either DEET alone (PT, 2.03 h), the constituted EOs (PT, 0.54–1.07 h), or CI-NO alone (PT, 0.54 h). With the exception of savory, the other EOs, CI-NO, and binary mixtures did not induce any adverse effects on the human volunteers at 0.5 mg/cm^2. ZP-SD and ZA-SO volatile constituents, cuminaldehyde, cuminyl alcohol, geraniol, limonene, linalool, methyl cinnamate, neral, peperitone, and α-phelandrene alone gave short protection time (PT, ≈0.5 h) and were significantly less effective than DEET alone (PT, ≈2.0 h) at 0.25 mg/cm^2. However, the binary mixtures of the four compounds (limonene, α-phellandrene, cuminyl alcohol, and cuminaldehyde) and CI-NO (0.25 + 1.0 mg/cm^2) provided strong protection (PT, 2.36 – 2.57 h), similar to that of the DEET + CI-NO mixture (PT, 2.52 h), and gave significantly stronger effects than that of DEET alone. Two major fatty acids of CI-NO such as oleic acid and linoleic acid, and the fatty acid methyl esters such as methyl oleate and methyl linoleate were found to provide longer protection than CI-NO. In a repellency test using eight aerosol formulations containing ZP-SD or ZA-SO and CI-NO as active ingredients,

the repellency of the formulations against *S. calcitrans* females was comparable with that of DEET + CI-NO mixtures and DEET alone.

The behavioral and olfactory response patterns to 1,8-cineole, citronellal, cuminaldehyde, linalool oxide, linalool, neral, piperitone, and terpinen-4-ol from *Zanthoxylum* plants in combination with the attractants such as 1-octen-3-ol and butyric acid were investigated on the fly antenna. The patterns of behavioral and olfactory responses were significantly altered depending on the ratios of 1-octen-3-ol or butyric acid to the EOs or compounds in the air mixtures. The present study demonstrated that *Zanthoxylum* EOs lowered the levels of response of flight behaviors of the stable fly toward the test attractants. The results indicate that an olfactory receptor on the fly antenna detects directly the repellent chemicals.

In the vapor-phase mortality assay with ZP-SD and ZA-SO, their 28 constituents, and eight structurally related compounds against female stable fly, cuminaldehyde, thymol, (1*S*)-(–)-verbenone, (–)-myrtenal, carvacrol, (*S*)-(*Z*)-verbenol, ZP-SD, cuminyl alcohol, ZA-SO, piperitone, (–)-(*Z*)-myrtanol, and citronellal (LC_{50}, 0.075–0.456 μg/cm^3) showed strong activity, although their magnitude was less toxic than either chlorpyrifos or dichlorvos. Structure–activity relationship investigation indicated that structural characteristics such as carbon skeleton, degrees of saturation and types of functional groups, and vapor pressure parameter, appear to play a role in determining toxicities of the test compounds. In the acetylcholinesterase (AChE) inhibition assay, citronellyl acetate, α-pinene, thymol, carvacrol, and α-terpineol (IC_{50}, 1.20–2.73 mM) inhibited the fly head AChE but their activities were weaker than that of chlorpyrifos and dichlorvos. However, octopamine and dopamine remarkably stimulate adenylate cyclase in brain of

stable flies. At 100 nmol/ml, octopamine increased cAMP level 2.7-fold compared with control. The octopamine-stimulated cAMP level significantly reduced 13.7-fold and 3.9-fold by citronellal and thymol at 100 nmol/ml, respectively, and 2.0–2.9-fold by cuminaldehyde, cuminyl alcohol, (–)-myrtenal, and (1*S*)-(–)-verbenone. These results indicate that the possible target site for neurotoxicity of EO fumigants is the octopamine-sensitive adenylate cyclase in the nervous system.

In conclusion, global efforts to reduce the level of highly toxic synthetic repellents or insecticides in the agricultural environment justify further study on plant EOs and *Zanthoxylum*-derived constituents described as potential biocides for the control of stable fly populations as repellents or insecticides with fumigant action.

Key words: *Stomoxys calcitrans*, plant essential oil, *Zanthoxylum piperitum*, natural repellent, natural fumigant, acetylcholinesterase inhibition, cAMP immunoassay, olfactory response

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List of abbreviations

AChE	acetylcholinesterase
ATChI	acetylthiocholine iodide
ATP	adenosine 5′-triphosphate
BA	butyric acid
cAMP	cyclic adenosine 3′,5′-monophosphate
CI-NO	tamanu (*Calophyllum inophyllum*) nut oil
Conc.	concentration
DEET	*N*,*N*-diethyl-*m*-toluamide or *N*,*N*-diethyl-3-methylbenzamide

EAG	electroantennography
ELISA	enzyme-linked immunosorbent assay
EO	essential oil
GC	gas chromatography
GC-MS	gas chromatography-mass spectrometry
LA	linoleic acid
ML	methyl linoleate
MO	methyl oleate
MT	monoterpenoid
OA	oleic acid
Oct	1-octen-3-ol
OD	optical density
OP	organophosphate
PT	protection time
RH	relative humidity
SEM	scanning electron microscopy
TChI	thiocholine iodide
ZA-SO	*Zanthoxylum armatum* seed oil
ZP-SD	*Zanthoxylum piperitum* pericarp steam distillate
ZS-SD	*Zanthoxylum schinifolium* fruit steam distillate
USEPA	United State Environmental Protection Agency
USDA	United States Department of Agriculture
VP	vapor pressure

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INTRODUCTION

The stable fly (*Stomoxys calcitrans* Linnaeus, 1758) is one of the most serious haematophagous insect pests of confined livestock, pastured cattle, wild animals, and human throughout the world (Newson, 1977; Campbell, 1993; Miller, 1995). The painful bites of stable flies cause stress and discomfort to both large domestic and wild mammals. In extreme cases, stable flies have caused death in large domestic mammals in Africa (Zumpt, 1973; Kunz and Monty, 1976; Elkan *et al*., 2009). In addition, heavy infestation causes annoyance and reduced milk production and weight gain, as well as occasional secondary infection (Newson, 1977; Campbell, 1993; Miller, 1995). They are also known to transmit various livestock diseases (Newson, 1977; Campbell, 1993; Miller, 1995). Annual economic impact caused increasingly by the stable flies on cattle industries in the United States (US) was estimated to be more than $US 2.2 billion (Taylor *et al*., 2012).

Increasing public concern for the environmental effects of pesticides, human health effects, and undesirable effects on nontarget organisms intensifies when repeated applications of conventional pesticides become necessary. In addition, the number of approved insecticides or repellents will probably be reduced in the near future in the US by the US Environmental Protection Agency (EPA) as registration occurs under the 1996 Food Quality and Protection Act (Anonymous, 2011). Registration requirement is also a concern in other regions including in the European Union, where it is under the control of the Commission Regulation (EC) No 1048/2005 (Anonymous, 2005), and in Republic of Korea (ROK) where it falls under the purview of the Agrochemicals Control Act (Anonymous, 2006). The removal of conventional insecticide or repellent products from markets due to the

increase in insecticide resistance or welfare concerns will have a serious impact on the proliferation of the flies. There is, therefore, a critical need for the development of selective control alternatives with novel target sites in order to establish an efficient management strategy and tactics for stable flies, particularly those with fumigant action, which allow effective application in indoor environments that reach deep harborages missed by many commercially-available insecticides, or repellents.

Essential oils (EOs) mostly extracted by steam distillation or hydrodistillation from plants have been suggested as alternative sources for arthropod control products, largely because they constitute a potential source of bioactive chemicals that pose fewer risks to the environment, with minimal impacts to animal and human health (Peterson and Coats, 2001; Ahn *et al*., 2006; Isman, 2006; Ditzen *et al*., 2008). They often act at multiple and novel target sites (Kostyukovsky *et al*., 2002; Priestley *et al*., 2003), thereby reducing the potential for resistance. They are widely available, with some being relatively inexpensive compared with plant extracts (Isman, 2006). Many are commonly used as fragrances and flavoring agents for foods, beverages, and cosmetics (Bruneton, 1995; Lawless, 2002) and are generally regarded as safe to human and environment. No information, however, is available concerning the potential use of EOs for the control of stable flies, despite their excellent pharmacological or pesticidal actions (Lawless, 2002).

In this study, an assessment is made of the repellency of 22 EOs from nine different plant families and 29 volatile constituents from *Zanthoxylum piperitum* (Rutaceae) pericarp steam distillate (ZP-SD) and *Zanthoxylum armatum* seed oil (ZA-SO) as well as binary mixtures of the most effective EOs or potential constituents and *Calophyllum inophyllum* nut oil to female stable flies. The repellencies of the test compounds were

compared with those of a currently available repellent *N*,*N*-diethyl-*m*-toluamide (DEET) (Fradin, 1998) to assess their use as future commercial fly repellents. The stable fly's perception of attractive cues in the presence of the potential repellent constituents from ZP-SD and ZA-SO was investigated using an electrophysiological recording technique and compared with that of two major attractant volatiles 1-octen-3-ol and butyric acid. In addition, the fumigant and topical toxicities of ZP-SD and ZA-SO, their 29 constituents, and seven structurally related compounds to the female stable flies were examined and compared with those of two organophosphorus insecticides (OPs) chlorpyrifos and dichlorvos. Possible insecticide mode of action and quantitative structure–activity relationship (QSAR) of the test compounds are also discussed.

LITERATURE REVIEW

1. Stable fly

The stable fly is a member of the subfamily Stomoxynae (Zumpt, 1973). This subfamily is a part of the large family Muscidae comprising about 4,500 described species and is classified in 180 genera (De Carvalho *et al*., 2005). The genus *Stomoxys* Geoffroy (1762) consists of at least 18 different species of parasitic flies (Table 1) that are of medical and economic importance (Zumpt, 1973).

Among these species, 17 species have a tropical distribution and only the stable fly (*S. calcitrans*) is the most important and cosmopolitan species native to Africa, Europe, and Asia (Zumpt, 1973; Moon, 2002). It is well-known as a haematophagous and pestiferous fly that becomes a major pest of humans and livestock in many part of the world (Campbell *et al*., 1987; Hogsette and Farkas, 2000). At least three other common names are used regionally for the stable fly, known as the beach fly because of its outbreaks on recreational beaches, the dog fly because it pesters dogs, and the lawn-mower fly because the larvae have been found in damp, matted grass on the undersides of lawn mowers (Moon, 2002). The stable fly is also misleadingly called the biting housefly because it is similar with the housefly (*Musca domestica* L.) in size and color (Moon, 2002; Koehler and Kaufman, 2006).

Table 1. Distributions of 18 *Stomoxys* species

Species	Distribution
S. bengalensis Picard, 1908	India to Java
S. boueti Roubaud, 1911	Benin (ex-Dahomey), Congo
S. calcitrans Linnaeus, 1758	Cosmopolitan
S. indicus Picard, 1908	Oriental region and neighbouring Paleartic territories
S. inornatus Grünberg, 1906	Tropical Africa
S. luteolus Villeneuve, 1934	Central and East Africa
S. niger Macquart, 1851	
S. niger niger	Ethiopian and Madagascan regions
S. niger bilineatus	Ethiopian and Madagascan regions
S. ochrosoma Speiser, 1910	Central and East Africa
S. omega Newstead, Dutton & Todd, 1907	Ethiopian Region
S. pallidus Roubaud, 1911	Tropical Africa
S. pullus Austen, 1909	India
S. sitiens Rondani, 1873	Ethiopian and Oriental regions, in Egypt it reaches the Palearctic region
S. stigma Van Emden, 1939	Uganda–Congo
S. taeniatus Bigot, 1888	Ethiopian region
S. transvittatus Villeneuve, 1916	Southern and Central Africa
S. uruma Shinonaga & Kano, 1966	Oriental region
S. varipes Bezzi, 1907	East and Central Africa southward to Rhodesia
S. xanthomelas Roubaud, 1937	Congo–Tanzania–Ugand

2. Biology, habits, and life cycle

The adult stable fly can be easily distinguished from the housefly by its piercing mouthpart for sucking blood (Moon, 2002). In addition, it has four longitudinal dark stripes on its grey thorax and seven circular black sports on top of its gray abdomens (Masmeatathip *et al*., 2006). Both sexes of the flies are obligate blood feeders (Anderson, 1978). Stable flies inflict extremely painful bites to both livestock and wildlife while the animals are feeding or resting (Schofield and Torr, 2002). They prefer to bite mainly on the lower legs of livestock (Moon, 2002; Koehler and Kaufman, 2006). However, when large populations occur during summer, stable flies may also feed on the sides (belly) or the backs of their hosts (Hogsette and Farkas, 2000). The flies typically feed once per day and remain on their host for 2–5 min to obtain a full blood meal (Moon, 2002). According to Hoffman (1968), stable flies have a bimodal pattern of feeding at 10 AM and 4 PM.

The life cycle of stable fly from egg to adult requires about 3–4 weeks during warm weather and up to 9 weeks when temperatures are cooler (Foil and Hogsette, 1994; Masmeatathip *et al*., 2006). The female flies oviposit in all kinds of decomposing organic matter like silage, rotting hay, garden compost, and even in sea grass. Animal feces mixed with fermenting vegetal matter are also common site for oviposition (Jeanbourquin and Guerin, 2007a). Each female fly lives about 20–35 days and may lay 500–600 eggs (1 mm long) in 4 separate batches in fermenting organic matter. Eggs hatch in 2–5 days into small, first instar larvae. The larvae develop within 14–26 days (Moon, 2002). The pupae develop approximately 7–14 days and then the adult flies emerge. After emerging, adult male and female flies start seeking host for blood meal to successfully mate after 3–5 days old. The females require several blood meals to complete their reproductive

function and start to lay eggs after 5–8 days old (Jones *et al*., 1992; Moon, 2002). Nevertheless, the males require at least one blood meal to produce seminal fluid and to stimulate sexual drive (Klowden, 1996). The fly's larvae may slowly develop in breeding areas below the frost line during winter (Campbell *et al*., 1987). Stable flies overwinter as larvae or pupae in piles of larval breeding materials (Powell and Barringer, 1995).

Stable flies are also a significant nuisance and readily attack humans on beaches and in residential areas near agricultural areas (Foil and Hogsette, 1994; Hogsette and Farkas, 2000). Adult stable flies are strong fliers. They can fly over 1–5 km in search of blood meals that seems to be a normal movement characteristic of the fly (Hogsette *et al*., 1987). However, adult stable flies can fly up to 112 km and more than 200 km over several days (Hogsette and Ruff, 1985; Koehler and Kaufman, 2006). Wind-assisted active migration or passive wind-borne dispersion was reported to play a role in the movement patterns of the stable fly (Hogsette and Ruff, 1985). The stable flies could not survive for a few days without a blood meal (Foil and Hogsette, 1994). They are found to feed on nectar from flowers (Jones *et al*., 1992) as an energy source for long distance flight and thus increase their chances of finding blood meals and ultimately reproducing (Hogsette and Ruff, 1985).

3. Medical and economic impact

The greatest economic impact appears to be the result of annoyance, fatigue, and stress on animals caused by painful feeding by the flies. The behavioral responses of animals to dislodge the flies such as bunching of the herd to protect their front legs, foot stomping, tail twitching, and head throwing lead to reduced weight gains, feed efficiency,

milk production, and injured calves (Dougherty *et al*., 1993; Campbell *et al*., 2001). The suggested economic threshold is 5 stable flies per cow per leg (Mc Neal and Campbell, 1981). Heavy infestations of more than 50 flies per animal can reduce weight gain by 25% and milk production by 40–60% (Powell and Barringer, 1995). Annual economic impact caused by the stable flies on cattle industries in the US was estimated to be approximately 608 million USD (Byford *et al*., 1992), while total losses to the Canadian cattle industry in 1994 from stables flies were estimated to be about 27 million USD (Gibson, 1998). In north America alone, total annual expenses for stable flies control in the cattle industry have been estimated to cost more than $US 100 million (Campbell, 1993) and around $1 billion dollars in the damage to the cattle industry (Taylor and Berkebile, 2006). Total impact to the cattle industry in the United State was estimated to be approximately US $2 billion per year (USDA-NAS, 2008) and more than $2.2 billion in 2009 (Taylor *et al*., 2012). This makes stable flies the most damaging insect pest of cattle in the United States.

Stable flies act as an intermediate host of spiruid nematodes such as *Habronema microstoma*, *H. muscae* and *Draschia megastoma*, which cause gastric and cutaneous habronemiasis in horse (Moon, 2002). The flies can harbor bovine leukemia virus (BLV) (Buxton *et al*., 1985; Weber *et al*., 1988) and lumpy skin disease virus (LSDV) (Chihota *et al*., 2003) but the ability of stable flies to transmit BLV and LSDV was not found. Stable flies can also harbor and are capable of mechanically transmitting several other viruses such as African swine fever (Mellor *et al*., 1987); bovine virus diarrhea (Tarry *et al*., 1991); vesicular stomatitis virus (Ferris *et al*., 1955); and Rift Valley fever (Turell *et al*., 2010). Johnson *et al*. (2010) and Doyle *et al*. (2011) have reported that stable flies may be capable of mechanically transmitting West Nile virus (WNV) but no evidence of

the viral replication in the stable flies was observed. Stable flies are also mechanical vectors for *Bacillus anthracis* (Turell and Knudson, 1987).

4. Integrated pest management (IPM) for stable flies

Proper sanitation and manure management procedures are the most important steps for reducing stable fly population in confined livestock, cattle feedlots, and dairies (Foil and Hogsette, 1994). The elimination of stable fly's larval breeding habitats such as garden compost, moist grasses and rotting hay or decaying feed mixed with old manure can help to prevent the larval development. However, these controls alone often fail to keep adult stable fly population below the economic injury threshold (Powell and Barringer, 1995; Gerry *et al*., 2007).

The IPM is commonly combined with the biological control agents and the utilization of insect traps with safe and judicious use of pesticides as well (Powell and Barringer, 1995). Using fly traps could be a useful and effective way to reduce stable fly populations in isolated sites (Taylor and Berkebile, 2006). Williams (1973) and Broce (1988) have developed alsynite fiberglass panel trap covered with sticky substance for monitoring stable fly number in various feedlot habitats. The mechanism of attraction for alsynite traps is demonstrated that the flies are attracted to the electromagnetic energy reflected by the panels (Zacks and Loew, 1989). Mihok *et al*. (2006) has developed triangular blue/black cloth trap and evaluated the performance of the Nzi traps made from appropriate fabrics in different blue dyes for monitoring population of biting flies such as stable flies, tsetse flies (*Glossina* spp.), horse flies (Tabanidae), and *Aedes* mosquitoes (Diptera: Culicidae). According to Beresford and Sutcliffe (2006), coroplast sticky traps

were more efficiency in catching stable flies than alsynite traps. The proper use of modified traps with either treated targets or solar-powered electrocution grids can also be effective (Foil and Hogsette, 1994).

Along with sanitation and trapping programs, biological control agents are also used. Several parasitic wasps such as *Muscidafurax raptor*, *M. raptorellus*, and *Spalangia cameroni*, solitary primary ectoparasitoids of various Diptera pupae have been studied for biological control of house flies and stable flies (Foil and Hogsette, 1994).

5. Impact of current-use pesticides on stable fly control

Commercial insecticides applied to agricultural protection were often used for controlling biting flies. Pyrethroid and organophosphate insecticides are commonly used for fly control programs, and other insecticide classes such as organochlorines, carbamates, and cyclodienes may sometimes be used (Elliott *et al*., 1978). These insecticide mode of actions rely primarily on three target sites in insects (Ware, 1991), (*i*) the gated sodium channel (pyrethroids and DDT), (*ii*) inhibition of γ-aminobutyric acid (GABA) receptors (cyclodienes), and (*iii*) the inhibition of AChE (organophosphates and carbamates).

For the fly control methods, wet sprays on the legs, dust bags, insecticide impregnated ear tags, and oral larvicides are typically used. However, they are not really effective in controlling stable fly populations below economic levels, because stable flies almost spend a short time (2–5 min) for blood feedings on their hosts. Therefore they may feed on their hosts before being killed by the insecticides sprayed or soared on the animal's feet, legs and belly. It is also difficult to administer insecticides with enough

residual activity to kill the fly (Dadour, 2006; Gerry *et al*., 2007). Moreover, resistance to the current-use insecticides has resulted in a resurgence of insect pests, and raising human and environmental concerns about insecticide toxicity (Pang *et al*., 2012). In addition, livestock behavior of walking through water and surfaces covered with wet vegetation and wet manure on feedlot or dairy lot quickly washes pesticides out of their bodies (Campbell, 2006).

DDT was used to control stable flies in Nordic countries from 1950 to 1965, but resistant strains to DDT and related insecticides such as DDD and methoxychlor were found soon after that (Stenersen, 2004). Pyrethroid insecticides are commonly used for the control of live-stock pests, including biting flies. However, there was still lack of information on stable fly resistance to permethrin (Cilek and Greene, 1994) and organophosphates such as diazinon or diazinon plus chlorpyrifos-ethyl (Guglielmone *et al*., 2004). The common uses of the insecticides have resulted in the development of varying levels of pyrethroid resistance of many muscoid pests in field populations including the housefly, *M. domestica* (Soderlund and Knipple, 2003) and the horn fly, *Haematobia irritans* (Guglielmone *et al*., 2001). A laboratory-selected stable fly strain was recently reported to exhibit a 15-fold increase in permethrin resistance (Pitzer *et al*., 2010). Thus, Olafson *et al*. (2011) has also established a rapid bioassay to identify a mutation associated with permethrin resistance in the para-type sodium channel of the stable fly. The uses of organophosphate and carbamate insecticides for several consecutive years have also resulted in several resistant insect species whose altered AChEs are less sensitive to these insecticides (Cilek and Greene, 1994). In most of insects, AChE insensitive to organophosphate and carbamate insecticides has been

identified as a major resistance mechanism and various resistances to these compounds result from point mutations in the encoding gene (Vontas *et al*., 2002). Therefore, rapid identification of mutations that specifically increase the organophosphate hydrolase activity are essential efforts in pesticide resistance management to prevent fixation of the mutations in pest populations (Temeyer and Chen, 2012).

6. Medicinal and aromatic plants as natural insecticidal and repellent resources

Medicinal and aromatic plants from many different families contain different volatile compounds that give them their own characteristic flavor and odor. Their extracts have mainly become the natural sources for medicines, seasonings, colorings, and preservatives in both traditional and modern purposes throughout the world. Essential oils (EOs) from aromatic plants are the most frequently used in food and medicinal industries and fragrances in perfume industry (Lawless, 2002). Plant EOs also hold great promise for pest control because of their effective and safe use. Many EO volatiles mainly comprised of terpenes and their derivatives affect biting behavior of arthropods in many ways such as kairomones, feeding deterrents, feeding stimulants, and repellent (Isman, 2000; Ahn *et al*., 2006; Nerio *et al*., 2010). For example, EOs obtained from many plant species of Asteraceae, Rutaceae, Poaceae, Myrtaceae, Apiaceae, Geraniaceae, Lamiaceae, and Santalaceae were known to possess repellent and/or insecticidal activities to several insect and arthropod pests such as human head louse (Yang *et al*., 2004a), house dust mites (Ahn *et al*., 2006), mosquitoes (Kim *et al*., 2004a; Trongtokit *et al*., 2005), cockroaches (Jang *et al*., 2005), stable flies (Hieu *et al*. 2010a; Zhu *et al*., 2010) and stored-product insect pests (Kim and Ahn, 2001; Kim *et al*., 2003). The EO compounds

such as cuminaldehyde, cuminyl alcohol, geraniol, limonene, linalool, methyl cinnamate, and α-phellandrene were reported to show potent repellent against mosquitoes (Kwon *et al*., 2011), cockroaches (Jung *et al*., 2007; Phillips *et al*., 2010), stable flies (Hieu *et al*., 2010b), and house dust mites (Watanabe *et al.*, 1989).

6.1. Medicinal plants from the genus of Zanthoxylum

The genus *Zanthoxylum* (Rutaceae) commonly called prickly ash or Sichuan pepper is an economically important genus found in the Himalaya region, Central, South, South East and East Asia with over 200 species ranging from small shrubs to large trees (Austin and Felger, 2008). Many medicinal species have ethnobotanical properties such as relief of dental problems, treatment of malaria, gastrointestinal disorders, gonorrhea and lung diseases, emmenagogue action, effective for rheumatism, anthelmintic use in animals and humans, aphrodisiac, analgesic, action against various skin diseases, febrifuge, antihemorrhagic, effective for genitourinary diseases, anticancer, anti-convulsive, and tonic (Patino *et al*., 2012). Several species such as *Z. piperitum*, *Z. bungeaum*, *Z. schinifolium*, and *Z. nitidum* are used as a spice in Korea, Japan and China (Tshin, 2011). EOs from leaves, fruits and inflorescences of *Zanthoxylum* plants are also used in perfumery and food industry (Patino *et al*., 2012). EOs are most abundant in pericarps and the dried pericarps are often used for flavouring stews, soups and meats (Tshin, 2011; Verma and Khosa, 2012). They have been also used as insecticides, insect repellents, and feeding deterrents (Bowers *et al*., 1993; Tiwary *et al*., 2007; Hieu *et al*., 2012).

Z. piperitum is widely distributed in Korea, Japan, and China (Austin and Felger, 2008; GRIN, 2012), while *Z. armatum* was found in the hot valleys of the Himalayas

from Jammu to Bhutan and in India's Eastern coast (Verma and Khosa, 2012). Besides their use as a spice, *Z. piperitum* and *Z. armatum* are also traditionally applied for treatment of tuberculosis, stomachic, dyspepsia, diarrhea, and anthelmintic (Verma and Khosa, 2012). Extracts obtained from fruits and leaves of the plants possess antioxidative activity (Verma and Khosa, 2012). *Z. piperitum* and *Z. armatum* fruit EOs have repellent properties against several mosquito genera of *Aedes*, *Anopheles*, *Armigeres*, *Culex* and *Mansonia* (Kamsuk *et al*., 2007) and also showed insecticidal and repellent properties to female stable fly (Hieu *et al*., 2010b).

6.2. Medicinal plants of the genus Calophyllum

The genus *Calophyllum* (Clusiaceae) is a large group of tropical trees consisting of approximately 180–200 different species distributed in the tropical areas (Filho *et al*., 2009) and most of them native to the Indo-Pacific (Stevens, 1980). Several species of the genus are known to be valuable sources of bioactive coumarins and xanthones with relevant biological activities such as inhibitory activity against the HIV-1 and its virally-encoded reverse transcriptase (Kashman *et al*., 1992; Spino *et al*., 1998; Dharmaratne *et al*., 2001), anti-leishmanial activity (Brenzan *et al*., 2007), antimicrobial activity (Pretto *et al*., 2004), and antifungus (Morel *et al*., 2002).

C. inophyllum L. so called Alexandrian laurel is commonly used in folk medicine (Friday and Okano, 2006). The large tropical tree is indigenous to Southeast Asia found in Thailand, Vietnam, Myanmar, Malaysia, South India, Sri Lanka, and throughout the numerous islands of Melanesia and Polynesia (Kilham, 2004), on the east coast of Africa, in Mandagasca, and the Hawaiian island (Friday and Okano, 2006). Tamanu (*C.*

inophyllum) nut oil has been traditionally used to cure aching joints, rheumatism, gonorrhea, itching, skin diseases, and insect bites (Halpern and Weverka, 2003). The oil is a significant skin healing agent with anti-inflammatory, antimicrobial, antioxidant properties (Halpern and Weverka, 2003; Kilham, 2004). Especially, it is recommended for treatment of burns from sunburn to chemical burns and first aid treatment in case of serious burns (Kilham, 2004). Tamanu oil was known to be a rich source of costatolide and inophyllum P acting as potent HIV reverse transcriptase inhibitors (Spino *et al*., 1998) and anti-tumor-promoting agents (Itoigawa *et al*., 2001), and also a source of xanthones with both antimicrobial and antitumoral cytotoxic agents (Yimdjo *et al*., 2004). The nut oil contains high essential fatty acids such as oleic acid and linoleic acid, which exhibit as sun protection factors without cytotoxic on the human conjunctival epithelial cells (Crane *et al*., 2005; Said *et al*., 2007). Seed and leaf extracts of *C. inophyllum* also possess larvicidal activities on the juveniles of *Culex quinquefasciatus*, *Anopheles stephensi*, and *Aedes aegypti* (Pushpalatha and Muthukrishnan, 1999).

7. Potency and mode of action of plant EO constituents

Many volatile components from botanical EOs have been screened for toxic contact and/or fumigant as well as attractant, repellent, feeding deterrent and ovipositional stimulant activities against various insect species and other arthropods. Although incomplete understanding the mode of action of the EOs in insects, many EOs or their constituents that cause insecticidal symptoms were confirmed as a neurotoxic mode of action (Grundy and Still, 1985; Coats *et al*., 1991; Enan, 2001; Kostyukovsky *et al*., 2002; Picollo *et al*., 2008).

Many monoterpenoids possess inhibitory activities to AChE preparations from several insect species such as housefly and Madagascar roach (Grundy and Still, 1985), head louse (Picollo *et al*., 2008), rice weevil (Lee *et al*., 2001; Abdelgaleil *et al*., 2009), rust red flour beetle (Abdelgaleil *et al*., 2009), German cockroach (Yeom *et al*., 2012), American cockroach (Anderson and Coats, 2012), and stable fly (Hieu *et al*., 2012). Several studies reported that there is no direct correlation between insect toxicity and the *in vitro* AChE inhibition by terpenoids (Grundy and Still, 1985; Lee *et al.*, 2001; Picollo *et al*., 2008; Abdelgaleil *et al*., 2009). However, Ryan and Byrne (1988) reported a relationship between insecticidal toxicity and electric eel AChE inhibitory activities of terpenoids. In the stable flies, some of terpenoids such as carvacrol, cuminaldehyde, α-terpineol, thymol, and (1*S*)-(–)-verbenone were reported to possess both potent AChE inhibiton and insecticidal activity, whereas citronellyl acetate, α-copaene, and α-pinene were potent inhibitors of the enzyme but exhibited low to no toxicity (Hieu *et al*., 2012). In turn, (–)-myrtenal, citronellal, (*S*)-(Z)-verbenol, (–)-(*Z*)-myrtanol, cuminyl alcohol, and piperitone were toxic to stable fly but exhibited low to no AChE inhibitory activity, indicating that AChE was not the major site of action for these compounds (Hieu *et al*., 2012). A direct correlation were also found between insect toxicity and AChE inhibition by carvacrol and dihydrocarvone to German cockroach, while α-pinene was pronounced to be strong AChE inhibitor, but weak fumigant or contact toxicity to the roach (Yeom *et al*., 2012). Similar results were also reported on rice weevil with the direct correlation by some monoterpenes such as cuminaldehyde, 1,8-cineole, (–)-limonene and (L)-fenchone (Abdelgaleil *et al*., 2009). However, Lee *et al*. (2001) did not find the direct correlation by menthone or β-pinene on the product-stored insect. Thus, there are differences of

insecticidal activities of terpenoids between different insect species and of correlations between insecticidal toxicity and *in vitro* AChE inhibition. These differences could be due to the penetration rate, detoxifying enzyme activity, and the relative sensitivity to the toxicants at the target site (Terriere, 1984; Graham-Bryce, 1987). Of several terpenoids that showed potential toxicity, but no AChE inhibitory activity, toxic action of the compounds could be mediated through other pathways such as GABA receptors (Priestley *et al*., 2003; Tong and Coats, 2012), octopaminergic receptors (Kostyukovsky *et al*., 2002; Enan, 2001 and 2005), and tyramine receptor (Lei *et al*., 2010). There is another suggestion that some monoterpenes may inhibit cytochrome P450-dependent monooxygenases (De-Oliveira *et al*., 1997).

Further detailed investigations of the *in vivo* toxicity of various biologically active EOs are necessary to improve our knowledge of the target specificity of EO volatiles, which could represent a new alternative in the control of insect pests. Notably, several studies characterizing additional potential modes of action of terpenes in insects, including octopamine receptors, sodium channels, nicotinic acetylcholine receptors and GABA receptors are currently underway, in an effort to better understand the primary sites of action of the compounds.

8. Olfactory perception of volatile chemicals by the stable fly

8.1. Stable fly antennae

The odors emitting from the body surface and the breath of warm-blooded animals play an important role in the behavior of blood-sucking insects. Stable flies are extremely sensitive to cow rumen odor (Jeanbourquin and Guerin, 2007a) and active with human breath (Warnes and Finlayson, 1986). They are also attracted by skin odor (Gatehouse and Lewis, 1973). Several host-associated odorants such as indole, dimethyl trisulphide, phenol, *p*-cresol, 2-heptanone, acetic acid, butyric acid, isovaleric acid, and hexanoic acid associate with cattle manure and urine (Birkett *et al*., 2004; Jeanbourquin and Guerin, 2007b).

The stable fly's perception of these odors is accomplished by receptors mainly located on the third segments (funicle) of its antennae (Lewis, 1971; Tangtrakulwanich *et al*., 2011). Each antenna consists three segments: a short basal segment (scape) with no sensilla; the second segment (pedicel) bears stout articulated bristles with a probable tactile function and the Johnston's organ serving as a flight speed indicator; the third segment (funiculus) is relatively large covered with quite a number of olfactory sensilla belonging to four major types: trichoid (over 16,000 sensilla); basiconic (about 1,200 sensilla); clavate (approximately 200 sensilla); and coeloconic (more than 50 sensilla). An arista arises from outer face of the third segment and bears no sense organs. Three types of sensilla (basiconic, clavate and coeloconic) could have potential olfactory chemoreceptor function while trichoid sensilla are likely to be involved in mechano-reception only due to no spore structure on any of this type. (Tangtrakulwanich *et al*., 2011)

8.2. Insect olfactory system

In most insect species, olfactory system remarkably sensitive and capable to detect a vast array of odors is decisive for the control of several behavioral patterns such as orientation and avoidance or movement toward target sites such as food, habitat, oviposition sites, and species-specific recognition (Hinldebrand, 1995; Rützler and Zwiebel, 2005; Lu *et al*., 2007). The olfactory signals (smells) are received by olfactory receptor neurons (ORNs) and transmitted into the central nervous system (CNS) where they were then deciphered to lead to behavioral responses (Hinldebrand 1995; Clyne *et al.*, 1999). The insect olfactory sensilla are comprised of cuticular and cellular components (Chapman, 1998). The cuticular part is characterized by the presence of numerous pores (Fig. 1) that allow odors to enter the interior of the sensillum and reach the sensory cell membrane (Rützler and Zwiebel, 2005; Sachse and Krieger, 2011).

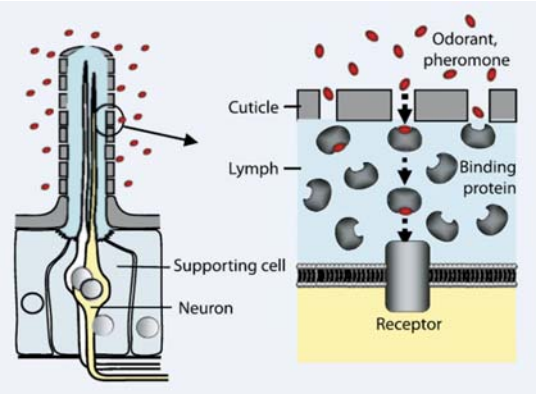

Fig. 1. Structure of a sensilla hair. Olfactory sensory neurons (OSNs) are surrounded by supporting cells. The OSN dendrites project into the aqueous sensillum lymph. Airborne odorant molecules (odorant, pheromone) are dissolved by binding proteins in the lymph and transported to the dendritic membrane (Sachse and Krieger, 2011).

There are various olfactory receptor types in insects. Olfactory sensory neurons (OSNs) of basiconic and trichoid sensilla were found to respond to general odorants. Each of the OSNs expresses one distinct odorant receptor (OR) and one general olfactory co-receptor (Orco). Especially, pheromone-sensitive OSNs have been found only in trichoid sensilla. These OSNs possess a specific pheromone receptor type and also express the Orco. The OSNs of coeloconic sensilla express two types of ionotropic olfactory receptors and they were suggested to respond to amines, alcohols, acids, ammonia and also water vapor. The odorant receptor (OR) and the Orco are supposed to form heteromeric complexes, whereby the OR subunit binds a specific ligand while Orco functions as an ion channel. (Yao *et al*., 2005; Sachse and Krieger, 2011)

8.3. Electrophysiological methods

Electroantennogram (EAG) technique is used to measure the electrical potential of the antenna to brain for a given odor. The technique was invented by Schneider (1957) and used to demonstrate that olfactory receptors were present on insect antennae (Chapman, 1998). EAG is widely used in screening of insect pheromones by examining the responses to fractions of a compound mixture separated using gas chromatography (Tangtrakulwanich *et al*., 2011). Several odor compounds such as indole, dimethyl trisulphide, 1-octenol-3-ol, phenol, *p*-cresol, 2-heptanone, acetic acid, butyric acid, isovaleric acid, and hexanoic acid elicited EAG responses from both male and female stable flies. Among these attractants, 1-octen-3-ol was reported to induce strongest EAG responses, followed by phenol, *p*-cresol, dimethyl trisulphide, and indole (Tangtrakulwanich *et al*., 2011). 1-octen-3-ol has proven to be an olfactory attractant for

various biting insects (Gibson and Torr, 1999) and evoked significantly upwind movement of the stable flies (Tangtrakulwanich *et al*., 2011).

9. Future perspectives

The discovery and utilization of chemicals for pest control in agriculture have contributed in part to the tremendous increase and stabilization in crop and livestock yields. However, the negative impacts of synthetic pesticides such as residues, resistance, and environmental pollution have raised the public concerns over human health leading to the discovery and development of plant-based pesticides (Dayan *et al*., 2009). Especially, many essential oils (EOs) mainly from different parts of plants emit a mixture of volatile components such as low-molecular-weight terpenes and phenolics. These compounds provide repellent and/or growth inhibitory properties against insect at different life stages. They also inhibit the enzymes involved in key processes of insect behavior and its life (Nerio *et al*., 2010; Regnault-Roger *et al*., 2012). The use of EOs and their volatile constituents have become one of the most recent trends in insect pest management and crop protection, because of their broad spectrum of activity against insect and mite pests (Isman, 2000; Ahn *et al*., 2006), plant pathogenic fungi (Chang *et al*., 2008; Bajpai and Kang, 2012), and nematodes (Oka *et al*., 2000; Kong *et al*., 2007). However, most effective repellents are high vapor pressure EOs and compounds and therefore they provide a short protection time (Ahn *et al*., 2006). Traditionally, many EOs are often used to blend with carrier oils (or vegetable oils) for natural skin care and plant-based repellent (Maia and Moore, 2011). Most vegetable oils are edible and have low evaporation rate. Some possess insect repellent or toxic properties such as cotton seed oil (Butler *et al*.,

1991), soybean oil (Frandin and Day, 2002), coconut oil *Cocos nucifera* (Amstrong, 2005), and tamanu oil *C. inophyllum* (Friday and Okano, 2006). Therefore, several mixtures of EOs and vegetable oils have enhanced repellency against biting insects in compared with that of DEET. For example, *Lantana camara* flower extract in coconut oil provides good protection against *Aedes* moquitoes (Dua *et al*., 1996). Several EOs and compounds in binary mixtures with tamanu oil are described as potential repellents against stable flies (Hieu *et al*., 2010a and 2010b).

In this respect, phytochemicals that possess repellents and insecticidal properties act at multiple and novel target sites (Kostyukovsky *et al*., 2002; Isman, 2006), thereby reducing the potential for resistance. Furthermore, Enan (2001) and Kostyukovsky *et al*. (2002) reported that many EO constituents, especially monoterpenoids possess toxic effect on several insect species by blocking octopamine receptors. Interestingly, the lack of the corresponding receptors in vertebrates showed that the compounds are active in invertebrates but almost inactive in vertebrates (Isman, 2001; Roeder, 2005). Therefore, EOs and their constituents may be developed and used for protection of humans and animals from biting flies.

Chapter 1

Repellency of plant essential oils, *Zanthoxylum* oil constituents, and *Calophyllum inophyllum* nut oil against *Stomoxys calcitrans*

Introduction

Repellents are one of the most effective tools for protecting humans and domestic animals from bites by nuisance arthropods (Curtis *et al*., 1990; Rozendaal, 1997; Barnard, 2000; Peterson and Coats, 2001; Isman, 2006), as continued or repeated use of conventional insecticides has disrupted natural biological control systems and led to resurgences in insect populations, has often resulted in the development of resistance (Cilek and Greene, 1994; Kunz and Kemp, 1994; Rozendaal, 1997), and raises serious human health and environmental concerns (Hayes and Laws, 1991; Kunz and Kemp, 1994). The most widely used repellent products are based on DEET (Fradin, 1998), which continues to be effective. However, this compound has many problems such as unpleasant odor, damage to certain plastics, synthetic rubber and painted surfaces, and medical issues that include central nervous system depression, urticaria, contact dermatitis, and potential encephalopathic toxicity which, rarely, results in death (Brown and Hebert, 1997; Knowles, 1991; Katz *et al*., 2008). There is, therefore, the need for the development of new improved repellents and strategies for protection from the stable fly attacks.

This chapter was aimed at assessing the potential of EOs and volatile constituents for use as future commercial repellents against stable fly. The potential of ZP-SD and its 19 constituents and ZA-SO and another 10 previously known constituents (Tiwary *et al*., 2007) was assessed. The repellencies of 12 selected EOs, including ZP-SD and ZA-SO, and their bioactive constituents alone or in combination with tamanu nut oil (CI-NO) were compared with those of DEET or binary mixture of DEET and CI-NO. The repellency of twelve aerosol formulations containing ZP-SD (5 and 10%), ZA-SO (5 and 10%), and

binary mixtures of ZP-SD or ZA-SO (2.5 and 5%) and CI-NO (2.5 and 5%) was also compared with that of aerosols containing 5 and 10% DEET alone or in combination with CI-NO.

Materials and methods

1.1. Plant samples and steam distillation

The fresh fruits of *Z. schinifolium* and *Z. piperitum* were collected in mid-August 2007 and in early September 2008 from the South Forest Research Center, Korea Forest Research Institute (Jinju, Gyeongnam Province, ROK), respectively. Voucher specimens (ZS-01) and (ZP–02) were deposited in the Research Institute for Agriculture and Life Sciences, College of Agriculture and Life Sciences, Seoul National University (SNU) (Seoul, ROK). Fresh fruits (130 g) of *Z. schinifolium* were finely ground and air-dried pericarp (342.3 g) of *Z. piperitum* was pulverized using a blender. They were subjected to steam distillation at 100°C for 2 h by using a Clevenger-type apparatus. Each volatile oil was dried over anhydrous sodium sulfate and stored in a sealed vial at 4°C until use. The yields of *Z. schinifolium* steam distillate (ZS-SD) and *Z. piperitum* steam distillate (ZP-SD) were 0.46 and 6.38% based on dried weight of the fruit and the pericarp, respectively.

1.2. Essential oils and chemicals

Twenty commercial essential oils and two steam distillates (ZP-SD and ZS-SD) used for skin repellency tests are listed in Table 2. ZA-SO was provided by Seema International (Delhi, India). The other 19 EOs were purchased from Berje´ (Bloomfield, NJ).

Table 2. List of twenty commercial plant essential oils and two steam distillates of *Zanthoxylum* plants tested for repellency

Family	Common name	Plant species
Apiaceae	Coriander	*Coriandrum sativum* L.
	Lovage root	*Levisticum officinale* L. Koch
Asteraceae	Armoise	*Artemesia vulgaris* L.
Geraniaceae	Geranium	*Pelargonium graveolens* L'Héritier de Brutelle
Lamiaceae	Lavender	*Lavandula officinalis* Chaix
	Marjoram	*Origanum majorana* L.
	Oregano	*Origanum vulgare* L.
	Patchouli	*Pogostemon cablin* (Blanco) Bentham
	Rosemary	*Rosmarinus officinalis* L.
	Sage, Clary	*Salvia sclerea* L.
	Savory	*Satureja monata* L.
	Thyme red	*Thymus vulgaris* L.
	Thyme white	*Thymus vulgaris* L.
Myrtaceae	Clove bud	*Eugenia caryophyllata* Thunberg
	Clove leaf	*Eugenia caryophyllata* Thunberg
	Eucalyptus	*Eucalyptus globules* Labillardiére
Poaceae	Citronella	*Cymbopogon nardus* (L.) Rendle
Rutaceae	Bergamot	*Citrus bergamia* (Risso) Wright and Walder-Arnott
	ZA-SO	*Zanthoxylum armatum* de Candolle
	ZP-SD	*Zanthoxylum piperitum* de Candolle
	ZS-SD	*Zanthoxylum schinifolium* Siebold and Zuccarini
Santalaceae	Sandalwood	*Santalum album* L.

Nineteen compounds identified in the ZP-SD and another 10 previously known ZA-SO constituents (Tiwary *et al*., 2007) used in this study are listed in Table 3, along with their purities and sources.

Table 3. Compounds used for repellency tests

Compound	CAS	Purity (%)	Source
Carveol	99-48-9	97	S-A[a]
β-Caryophyllene	87-44-5	80	S-A
β-Caryophyllene oxide	1139-30-6	99	S-A
1,8-Cineole	470-82-6	99	S-A
Citronellal	106-23-0	98	FA[b]
Citronellol	106-22-9	95	FA
Citronellyl acetate	150-84-5	95	TCI[c]
α-Copaene	3856-25-5	90	S-A
Cuminaldehyde	122-03-2	98	S-A
Cuminyl alcohol	536-60-7	98	TCI
p-Cymene	99-87-6	99	S-A
Geraniol	106-24-1	98	S-A
Geranyl acetate	105-87-3	98	S-A
α-Humulene	6753-98-6	96	S-A
Limonene	5989-27-5	97	S-A

Linalool	78-70-6	97	S-A
Linalool oxide	60047-17-8	97	TCI
Methyl cinnamate	103-26-4	99	S-A
β-Myrcene	123-35-3	90	S-A
Neral[d]	-	75	KI[e]
Nerol	106-25-2	98	TCI
α-Phellandrene	4221-98-1	65	TCI
α-Pinene	7785-70-8	99	S-A
β-Pinene	18172-67-3	99	S-A
Piperitone	89-81-6	94	TCI
Terpinen-4-ol	2438-10-0	98	FA
α-Terpinene	99-86-5	95	S-A
γ-Terpinene	99-85-4	97	S-A
α-Terpineol	10482-56-1	96	S-A

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

[b] Purchased from Fluka (Buchs, Switzerland).

[c] Purchased from Tokyo Chemical Industry (Tokyo, Japan).

[d] A mixture of neral (75.4%) and geranial (21.8%) is described in the previous study of Lee *et al*. (2008).

[e] Obtained from the Korea Forest Research Institute (Seoul, ROK).

Diazomethane (CH_2N_2) in diethyl ether is a methylating agent prepared by NICEM (National Instrumentation Center for Environmental Management, SNU). Oleic, linoleic, linolenic, palmitic and stearic acids (99%), methyl and ethyl oleates, and linoleates (98~99%), and DEET (97% purity) were purchased from Sigma-Aldrich. All of the other chemicals were of reagent-grade quality and available commercially. Tamanu (*C. inophyllum*) nut oil (CI-NO) was purchased from Binh Minh (Hochiminh, Vietnam).

1.3. Stable flies

A colony of stable flies was collected at the animal farm of SNU (Suwon, Gyeonggi Province, ROK) in August 2007 and early October 2008. Adult flies were maintained on a 8% sucrose solution in cotton mesh cages (40 × 40 × 40 cm) and fed twice daily on bovine blood (Hyupsin Food Company, Anyang, Gyeonggi Province) soaked in cotton pad place on a plastic dish (7 × 7 × 1 cm). Larvae were reared in glass beakers (2 L) containing 200 g of cow diet (DaeHan Livestock & Feed, Inchon, ROK). The feed consisted of crude protein (> 16.0%), crude lipid (> 2.5%), crude fiber (< 15.0%), crude ash (< 10.0%), Ca (> 0.80%), P (< 0.60%), and total digestible nutrition (70.0%). Amount of 50% Lignocel® hygienic animal bedding (JRS - J. Rettenmaier & Söhne, Roenberg, Germany) was then added to the feed for air ventilation. They were reared at 27 ± 1°C and 60–70% relative humidity (RH) under a 16:8 h light:dark cycle. Under these conditions, longevity of eggs, larvae, pupae, and adults was ca 2.3, 6.3, 6.5, and 19.5 days, respectively. The newly hatched adults were used for repellency tests (Fig. 2).

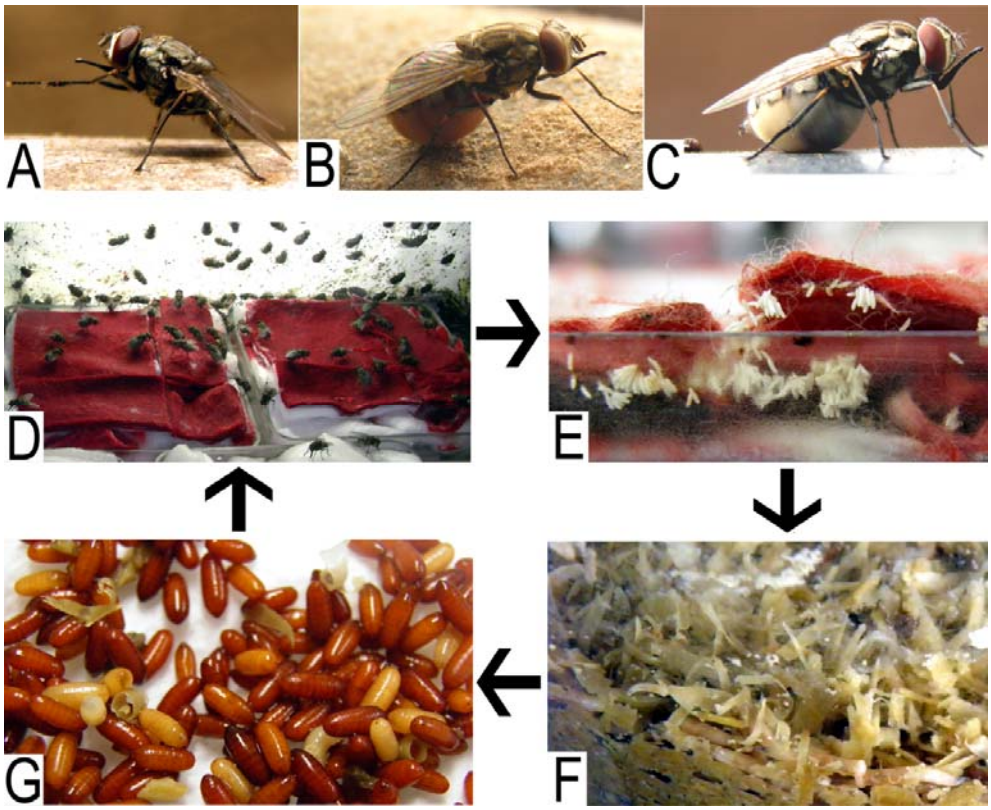

Fig. 2. Laboratory rearing of the stable fly. A, a hungry female fly; B & C, two full with cow blood; D, 5–7 days old adults fed with cow blood in a cotton cage; E, egg batches deposited on cotton pads soaked with cow blood and hatched after 1–2 days; F, larvae reared in a beaker and developed within 6–7 days and started to pupa; G, 1–2 days old pupae developed in 6–7 days and hatched into adult flies (D).

1.4. Experimental repellent formulations

The 12 aerosols containing ZP-SD, ZA-SO, and DEET alone or in combination with CI-NO in aluminum cans are listed in Table 4. These aerosols are not yet products.

Table 4. Twelve aerosol formulations containing *Z. piperitum* pericarp steam distillate (ZP-SD), *Z. armatum* seed oil (ZA-SO), and DEET alone or in combination with tamanu nut oil (CI-NO)

Aerosol formulation	Content (%)			
	EO[a]	CI-NO	Ethanol	LPG[b]
ZP-SD-5%	5		75	20
ZA-SO-5%	5		75	20
DEET-5%	5		75	20
ZP-SD-10%	10		70	20
ZA-SO-10%	10		70	20
DEET-10%	10		70	20
ZP-SD-2.5% + CI-NO-2.5%	2.5	2.5	75	20
ZA-SO-2.5% + CI-NO-2.5%	2.5	2.5	75	20
DEET-2.5% + CI-NO-2.5%	2.5	2.5	75	20
ZP-SD-5% + CI-NO-5%	5	5	70	20
ZA-SO-5% + CI-NO-5%	5	5	70	20
DEET-5% + CI-NO-5%	5	5	70	20

[a] Essential oil: ZP-SD or ZA-SO.

[b] Liquefied petroleum gas.

1.5. Gas chromatography analysis

An Agilent 6890N gas chromatograph (Santa Clara, CA, USA) equipped with a split injector and a flame ionization detection (FID) system was used to separate and detect the constituents of ZP-SD. Analytes were separated with a 30 m × 0.25 mm ID (*d*f = 0.25 μm) DB-1 capillary column (J&W Scientific, Folsom, CA). The oven temperature was programmed from 40°C (1 min isothermal) to 250°C at 6°C/min (held for 4 min at final temperature). The linear velocity of the helium carrier gas was 34 cm/s at a split ratio of 1:10. Oil constituents were identified by coelution of authenticated samples following coinjection.

1.6. Gas chromatography-mass spectrometry analysis

1.6.1. Z. piperitum pericarp steam distillate

Gas chromatography-mass spectrometry (GC-MS) analysis of ZP-SD was performed using an Agilent 6890N gas chromatograph-Agilent 5973N MSD mass spectrometer. The capillary column and temperature conditions for the GC-MS analysis were the same as described in Section 1.5 for GC analysis. Helium carrier gas was used at a column head pressure of 15.7 psi (108.4 kPa). The ion source temperature was 230°C. The interface was kept at 280°C, and mass spectra were obtained at 70 eV. The sector mass analyser was set to scan from 25 to 800 amu every 0.35 s. Chemical constituents were identified by comparison of mass spectra of each peak with those of authentic samples in a mass spectrum library (The Wiley registry of mass spectral data, 2000).

1.6.2. Tamanu nut oil

Tamanu nut oil (CI-NO) (1 μg) was methylated overnight at 25°C with diazomethane (CH_2N_2) in 200 μl of diethyl ether. Fatty acid methyl esters (FAMEs) were prepared by acid-catalyzed transmethylation of the lipids (Meltcalfe and Schmitz, 1961). The FAMEs were analyzed on a Shimadzu GC 9A chromatograph (Columbia, MD, USA) equipped with a FID system, stainless steel column (152.4 cm × 3.17 mm) packed with 20% diethyleneglycol succinate on 80–100 mesh Chromosorb W support, at a column temperature of 180°C, the injection port and FID at 210°C under a nitrogen flow rate of 40 ml/min. The peak area and relative percentage of FAMEs were obtained with a Shimadzu integrator. The component of each peak was identified on the basis of a calibration curve or retention times versus equivalent chain length, and by comparison with those of authentic methyl ester standards.

These FAMEs were analyzed using a HP 6890 gas chromatograph-JMS-600W mass spectrometer, a Finnigan Trace 2000 gas chromatograph-Finnigan TSQ mass spectrometer, and an Agilent 6890N gas chromatograph-Agilent 5973N MSD mass spectrometer. The capillary column and temperature conditions for the GC-MS analysis were the same as described above for GC analysis. Helium carrier gas was used at a column head pressure of 15.7 psi (39.2 kPa). The ion source temperature was 230°C, and mass spectra were obtained at 70 eV. The oil constituents were identified by comparison of mass spectra of each peak with those of authentic samples in a mass spectra library (The Wiley registry of mass spectra data, 2000).

1.7. Exposed human hand bioassay

An exposed human hand bioassay with six human volunteers was used to evaluate the repellency of EOs, test compounds, and binary mixtures of EOs or compounds with CI-NO to 24 h-blood-starved female stable flies (3–6 days old). Every bioassay was conducted within the time zone of 10:00–17:00 h because both male and female stable flies mostly feed on the legs of domestic animals and on humans in the daytime (Newson, 1977; Rozendaal, 1997). Groups of 50 female stable flies were transferred into each cotton cage (40 × 40 × 40 cm) because the biting density plays an important role (Schreck, 1995; Rozendaal, 1997).

The test EOs and compounds (0.5 and 0.25 mg/cm^2), each in 100 µl of ethanol, were directly applied to the exposed skin of the back of the left hand through a 5 × 5 cm hole made on the back part of a nylon glove. Controls received 100 µl of ethanol. After drying in the air for 1 min, the treated hands of each volunteer were exposed to the group of 50 female flies in the cotton cage (Fig. 3) for 5 min, 10 min after test material application, and then every 20 min until the test volunteer received a stable fly bite at the same conditions used for colony maintenance. DEET served as a positive control for comparison in repellency tests. Each assay was replicated three times and conducted at 25 ± 2°C and 60–70% RH. If a test material caused > 0.50 h of protection time (PT) at 0.5 mg/cm^2, further bioassays were done at 0.25 mg/cm^2.

Fig. 3. An exposed (5 × 5 cm) human hand skin bioassay used for evaluating the repellency of test materials to 50 female stable flies in a cotton cage.

In binary mixture experiments with six volunteers, the repellency of selected EOs, compounds, and DEET (0.25 mg/cm^2) alone or in combination with CI-NO (2.0, 1.0 or 0.5 mg/cm^2) were examined as stated above. The binary mixtures were then directly applied to each human skin. CI-NO only was used as control. CI-NO was selected for synergy tests because mature fruit of tamanu is burned for mosquito repellency (Friday and Okano, 2006).

The repellencies of 12 aerosol formulations were similarly examined using four volunteers. Each test was conducted as stated above. Aerosols were applied in sufficient amounts evenly to cover the skin from wrist to elbow. After air drying for 1 min, the treated hand of each volunteer was exposed to stable flies in the cotton cages (40 × 40 × 40 cm) containing 100 blood-starved females for 5 min. Control volunteers received the liquefied petroleum gas and ethanol. The numbers of biting females were recorded and each assay was replicated three times using 100 female stable flies per replicate. All volunteers wore shirts and cotton gloves to protect against excessive attack by stable flies.

1.8. Dermatological test

An EPA toxicity classification (USEPA, 2010) was used to determine whether treatment with the test EOs, CI-NO, test compounds, and binary mixtures induce dermal irritation. The test materials were applied evenly to the skin below the elbow and the knees of six human male volunteers (22–40 years old).

1.9. Data analysis

Protection time (PT) was recorded according to the method of Gillij *et al*. (2008). PT was the time elapsed between the test material application and the observation period immediately preceding that in which a confirmed bite was obtained. The Bonferroni multiple-comparison method was used to test for significant differences among the treatments (SAS OnlineDoc®, version 8.01, Cary, NC, 2004). Means ± standard error (SE) of untransformed data are reported. The repellent efficiency was classified as follows:

- PT (> 3.0 h): very strong repellency
- PT (> 2.0–3.0 h): strong repellency
- PT (> 1.0–2.0 h): moderate repellency
- PT (> 0.5–1.0 h): weak repellency
- PT (≤ 0.5 h): very weak repellency.

Results

1.1. Chemical composition of *Z. piperitum* pericarp steam distillate

ZP-SD was comprised of 5 major and 19 minor constituents by comparison of mass spectral data and coelution of authenticated samples following coinjection (Fig. 4). The 5 major constituents were limonene, cryptone, 1,8-cineole, citronellal, and geranyl acetate, and comprised 26.0, 16.8, 11.3, 7.1, and 6.6% of the steam distillate, respectively (Table 5). Together they constituted ca 68% of total constituents of the ZP-SD.

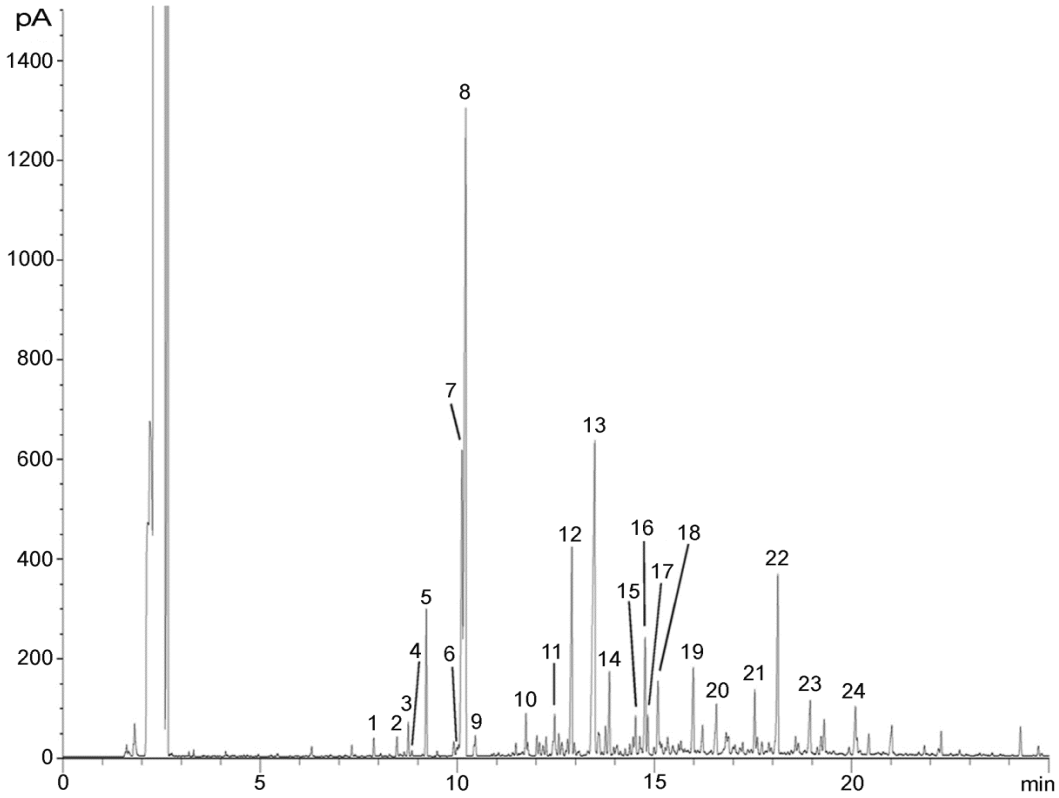

Fig. 4. Gas chromatograph of *Z. piperitum* pericarp steam distillate

Table 5. Boiling points (BP) and chemical constituents of *Z. piperitum* pericarp steam distillate (ZP-SD) identified by gas chromatography and gas chromatography-mass spectrometry and another ten previously known *Z. armatum* seed oil (ZA-SO) constituents

Peak no.	Compound	BP[d] (°C/760 mmHg)	RT[h] (min)	% Area
1	α-Pinene[a]	154–156	7.88	0.42
2	1-Penten-3-one[b]	68–70[e]	8.47	0.54
3	Sabinene[b]	163–164	8.76	0.82
4	β-Pinene[a]	164–165	8.85	0.14
5	β-Myrcene[a]	166–167	9.21	3.69
6	*p*-Cymene[a]	176–178	10.03	0.31
7	1,8-Cineole[a]	176–177	10.12	11.25
8	Limonene[a]	175–177	10.21	26.01
9	2,6-Dimethylhepten-5-en-1-al[b]	116–124[f]	10.45	0.79
10	Linalool[a]	198–200	11.74	1.15
11	Linalool oxide[a]	188	12.47	1.65
12	Citronellal[a]	206–207	12.90	7.10
13	Cryptone[b]	198	13.48	16.78
14	α-Terpineol[a]	214–224	13.86	2.51
15	Carveol[a]	231–232	14.52	1.22
16	Cuminaldehyde[a]	235–236	14.76	3.93
17	Citronellol[a]	225	14.83	1.15
18	Piperitone[a]	233–235	15.09	2.64
19	Cuminyl alcohol[a]	246–248	15.98	3.18
20	Unknown		16.52	1.84

21	Citronellyl acetate[a]	229	17.54	1.89
22	Geranyl acetate[a]	240–245	18.13	6.62
23	α-Copaene[a]	246–251	18.95	2.48
24	β-Caryophyllene oxide[a]	–[g]	20.09	1.89
	β-Caryophyllene[c]	256–259		
	Geraniol[c]	229–230		
	α-Humulene[c]	166–168		
	Methyl cinnamate[c]	260–262		
	Neral[c]	229–230		
	Nerol[c]	225–227		
	α-Phellandrene[c]	175–176		
	Terpinene-4-ol[c]	212		
	α-Terpinene[c]	173–175		
	γ-Terpinene[c]	181–183		

[a] Constituents identified co-elution of authenticated samples following co-injection.

[b] Constituents identified by GC-MS without authentic sample injection.

[c] Constituents of *Z. armatum* seed oil (ZA-SO) reported by Tiwary *et al*. (2007).

[d] Boiling points obtained from http://www.thegoodscentscompany.com

[e] 200 mmHg.

[f] 100 mmHg.

[g] No data available.

[h] Retention time.

Peak numbers (1–24) of ZP-SD compounds were showed in Fig. 4.

1.2. Free fatty acid constituents of tamanu nut oil

Methylation of fatty acids in CI-NO showed that the nut oil consists of 4 major fatty acids (> 8%) by comparison of mass spectral data and coelution of standard samples following coinjection (Fig. 5). The 4 major constituents were 9-octadecenoic acid (or oleic acid), 9,12-octadecadienoic acid (or linoleic acid), hexadecanoic acid (or palmitic acid), and octadecanoic acid (or stearic acid), and comprised 28.3, 11.0, 9.7, and 8.4% of the nut oil, respectively (Table 6). Together they constituted 57.4% of free fatty acids of the CI-NO.

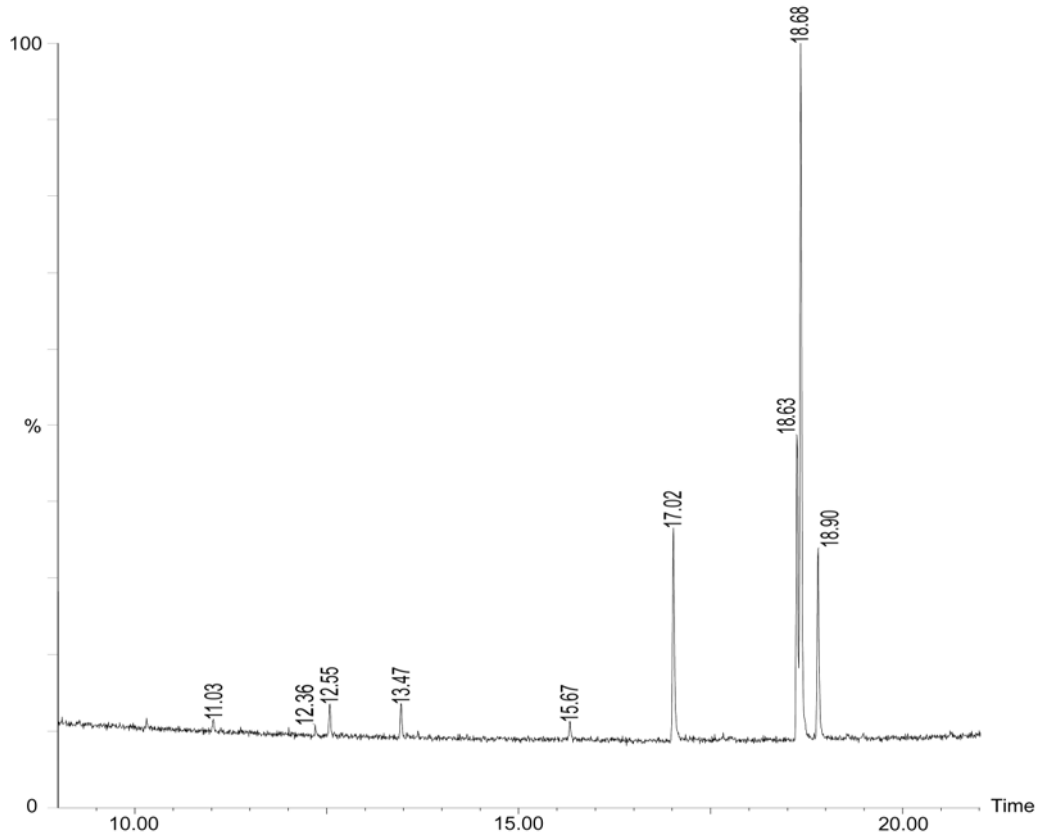

Fig. 5. Gas chromatography of tamanu nut oil containing fatty acids

Table 6. Fatty acid composition of tamanu nut oil (CI-NO) identified by gas chromatography and gas chromatography-mass spectrometry

Peak no.	Compound	MF[b]	RT[c] (min)	% Area
1	1-Hexanol	$C_6H_{14}O$	11.03	0.7
2	Acetamide	C_2H_5NO	12.36	0.8
3	Pentanoic acid	$C_5H_{10}O_2$	12.55	1.5
4	3-Undecene	$C_{11}H_{22}$	13.47	1.5
5	Oxalic acid	$C_2H_2O_4$	15.67	1.0
6	Hexadecanoic acid[a]	$C_{16}H_{32}O_2$	17.02	9.7
7	9,12-Octadecadienoic acid[a]	$C_{18}H_{32}O_2$	18.63	11.0
8	9-Octadecenoic acid[a]	$C_{18}H_{34}O_2$	18.68	28.3
9	Octadecanoic acid[a]	$C_{18}H_{36}O_2$	18.90	8.4

[a] Constituents identified coelution of authenticated samples following coinjection.

[b] Molecular formula.

[c] Retention time.

1.3. Repellency of test EOs and binary mixtures

The repellency of 22 EOs and DEET against female stable flies was evaluated by the exposed human hand bioassay (Table 7). Based on the protection time (PT), clove leaf, lovage root, clove bud, and patchouli EOs were the most effective EOs (PT, 3.20–3.62 h). Thyme white exhibited strong repellency (PT, 2.07 h). Moderate repellency was produced by oregano, thyme red, geranium, and ZP-SD EOs (PT, 1.01–1.12 h). ZA-SO and bergamot exhibited weak repellency (PT, 0.60 and 0.57 h). Very weak repellency was observed with the other 10 EOs (PT, 0.44–0.11 h). Overall, these EOs were less effective than DEET (PT, 4.35 h). Stable fly bites occurred within the 3 min in the ethanol treated controls (average biting pressure, 91 ± 3.2% females per person per 5 min).

Based on their potent repellency above, 12 selected EOs at 0.25 mg/cm^2 were likewise compared (Table 7). Effective PT of clove bud, lovage root, and clove leaf EOs was 1.12, 1.08, and 1.05 h, respectively. Weak PT of patchouli, savory, thyme white and ZP-SD were 0.61, 0.60, 0.58, and 0.56 h, respectively, whereas that of DEET was 2.06 h. The other 5 EOs exhibited very weak repellency (PT, < 0.5 h).

Table 7. Repellency of twenty commercial essential oils, two *Zanthoxylum* steam distillates, and DEET against female stable flies using the exposed human hand bioassay

Essential oil	Protection time[a] (mean ± SE, h)[b] at conc. (mg/cm^2)	
	0.5	0.25
Patchouli	3.62 ± 0.169 b	0.61 ± 0.031 e
Clove bud	3.48 ± 0.184 b	1.12 ± 0.082 d
Lovage root	3.31 ± 0.098 b	1.08 ± 0.092 d
Clove leaf	3.20 ± 0.084 b	1.05 ± 0.086 d
Savory	–[c]	0.60 ± 0.044 e
Thyme white	2.07 ± 0.035 c	0.58 ± 0.055 e
ZP-SD	1.12 ± 0.044 d	0.56 ± 0.020 e
Geranium	1.08 ± 0.102 d	0.41 ± 0.043 ef
Thyme red	1.05 ± 0.060 d	0.38 ± 0.043 ef
Oregano	1.01 ± 0.067 d	0.37 ± 0.024 ef
ZA-SO	0.60 ± 0.058 e	0.26 ± 0.043 fgh
Bergamot	0.57 ± 0.024 e	0.24 ± 0.036 fgh
Sage, Clary	0.44 ± 0.059 ef	
Lavender	0.42 ± 0.040 ef	
ZS-SD	0.35 ± 0.063 efg	
Armois	0.28 ± 0.035 fgh	

Citronella	0.25 ± 0.048 fgh	
Sandal wood	0.24 ± 0.039 fgh	
Rosemary	0.15 ± 0.019 gh	
Coriander	0.15 ± 0.025 gh	
Marjoram	0.12 ± 0.026 h	
Eucalyptus	0.11 ± 0.020 h	
DEET	4.35 ± 0.135 a	2.06 ± 0.043 c

[a] Time (hour) to first bite of the stable fly

[b] Means followed by the same letter are not significantly different (P = 0.05, Bonferroni method).

[c] Data were not available because of skin irritation of the EO at 0.5 mg/cm^2.

The repellency to blood-starved female stable fly of binary mixtures of the 12 selective EOs (0.25 mg/cm^2) and CI-NO (2 mg/cm^2) was likewise compared with that of DEET (0.25 mg/cm^2) alone and DEET (0.25 mg/cm^2) mixed with CI-NO (2 mg/cm^2) (Table 8). The exposed human hand bioassays revealed that CI-NO synergized the repellency of each EO tested. For example, the binary mixtures of lovage root EO with CI-NO produced strong repellency (PT, 2.64 h), similar to that of DEET and CI-NO mixture (PT, 2.69 h), and significantly greater repellency than either lovage root EO (PT, 1.07 h), CI-NO (PT, 0.54 h), or DEET (PT, 2.03 h) alone. The repellency of binary mixtures of each EO (ZP-SD, savory, clove leaf, thyme white, clove bud, and patchouli) with CI-NO (PT, 2.02–2.23 h) was almost identical to that of DEET alone (PT, 2.03 h). Effective PT of binary mixtures of bergamot, oregano, thyme red, ZA-SO, and geranium EOs with CI-NO was 1.48–1.60 h.

Table 8. Repellency of 12 selected essential oils alone or in combination with tamanu nut oil (CI-NO) and DEET against female stable flies using the exposed human hand bioassay

Treatment	Protection time[a] (mean ± SE h)[b] at conc. (mg/cm^2)	
	0.25	0.25 + 2.0 CI-NO
Lovage root	1.07 ± 0.051 e	2.64 ± 0.100 a
Patchouli	0.59 ± 0.049 fg	2.23 ± 0.068 ab
Clove bud	1.05 ± 0.060 e	2.21 ± 0.072 ab
Thyme white	0.54 ± 0.049 fgh	2.19 ± 0.073 ab
Clove leaf	1.06 ± 0.040 e	2.17 ± 0.053 ab
Savory	0.71 ± 0.062 f	2.04 ± 0.106 bc
ZP-SD[c]	0.61 ± 0.055 fg	2.02 ± 0.096 bc
Geranium	0.40 ± 0.039 ghi	1.60 ± 0.098 cd
ZA-SO[d]	0.30 ± 0.035 i	1.59 ± 0.072 cd
Thyme red	0.39 ± 0.040 ghi	1.51 ± 0.053 d
Oregano	0.35 ± 0.054 hi	1.50 ± 0.069 d
Bergamot	0.27 ± 0.034 i	1.48 ± 0.082 d
CI-NO	0.10 ± 0.017 j	0.54 ± 0.024 fgh
DEET	2.03 ± 0.081 bc	2.69 ± 0.073 a

[a] Time (hour) to first bite of the stable fly.

[b] Means followed by the same letter are not significantly different (P = 0.05, Bonferroni method).

[c] *Z. piperitum* pericarp steam distillate. [d] *Z. armatum* seed oil.

1.4. Repellency of two *Zanthoxylum* oil constituents and binary mixtures

The repellency of 29 individual constituents identified in either ZP-SD or ZA-SO and DEET to blood-starved female stable flies was likewise examined (Table 9). At 0.25 mg/cm^2, methyl cinnamate, cuminaldehyde, limonene, and cuminyl alcohol alone gave weak PT (0.51–0.55 h); their repellency quickly and significantly decreased over time. Very weak PT (≤ 0.5 h) was produced by the other 20 compounds, while α-humulene, β-caryophyllene, β-caryophyllene oxide, α-copaene, and β-myrcene gave no PT at the test concentration. The boiling point parameter of all these volatiles (Table 5) appears not to be greatly associated with repellency to stable flies and all these test compounds were less effective than DEET.

However, CI-NO was also found to synergize the repellency of volatile compounds in that the binary mixtures of each volatile compound with CI-NO resulted in significantly greater repellency than either compounds, CI-NO, or DEET alone. The effectiveness and duration of repellency of the binary mixtures of the test compounds and CI-NO were comparable with that of the DEET and CI-NO mixture. The repellency of nine selected active compounds (each 0.25 mg/cm^2) combined with CI-NO (1.0 mg/cm^2) was similarly examined (Table 9). The binary mixture of compounds (cuminaldehyde, cuminyl alcohol, α-phellandrene, and limonene) and CI-NO provided strong protection (PT, 2.36–2.57 h), similar to that of DEET and CI-NO mixture (PT, 2.52 h), and significantly stronger than that of DEET alone (PT, 1.95 h). The other 5 compounds (linalool, geraniol, methyl cinnamate, peperitone, and neral) in binary mixtures with CI-NO produced moderate repellency (PT, 1.15–1.96 h), similar to or slightly weaker than that of DEET alone.

Table 9. Repellency of 29 test compounds and DEET alone or in combination with tamanu nut oil (CI-NO) to female stable flies using the exposed human hand bioassay

Treatment	Protection time[a] (mean ± SE, h)[b] at conc. (mg/cm^2)	
	0.25	0.25 + 1.0 CI-NO
Cuminaldehyde	0.52 ± 0.053 efg	2.57 ± 0.176 a
Cuminyl alcohol	0.55 ± 0.039 ef	2.45 ± 0.178 ab
α-Phellandrene	0.43 ± 0.028 fg	2.40 ± 0.160 ab
Limonene	0.54 ± 0.055 ef	2.36 ± 0.100 ab
Linalool	0.42 ± 0.049 fgh	1.96 ± 0.088 bc
Geraniol	0.41 ± 0.040 fgh	1.75 ± 0.068 c
Methyl cinnamate	0.51 ± 0.040 efg	1.52 ± 0.072 cd
Piperitone	0.41 ± 0.049 fgh	1.18 ± 0.092 d
Neral	0.40 ± 0.064 fgh	1.15 ± 0.101 d
α-Terpineol	0.35 ± 0.035 ghi	
Nerol	0.34 ± 0.019 ghi	
α-Pinene	0.32 ± 0.040 ghi	
Terpinen-4-ol	0.32 ± 0.053 ghi	
Carveol	0.31 ± 0.034 hi	
1,8-Cineole	0.30 ± 0.048 hi	
Citronellal	0.25 ± 0.054 ij	

Citronellol	0.25 ± 0.044 ij	
Citronellyl acetate	0.24 ± 0.040 ij	
p-Cymene	0.23 ± 0.031 ij	
β-Pinene	0.21 ± 0.024 ij	
γ-Terpinene	0.20 ± 0.025 ij	
α-Terpinene	0.20 ± 0.035 ij	
Geranyl acetate	0.12 ± 0.020 j	
Linalool oxide	0.12 ± 0.024 j	
α-Humulene	0 k	
β-Caryophyllene	0 k	
β-Caryophyllene oxide	0 k	
α-Copaene	0 k	
β-Myrcene	0 k	
CI-NO	0.12 ± 0.015 j	0.38 ± 0.053 hi
DEET	1.95 ± 0.073 bc	2.52 ± 0.173 a

[a] Time (hour) to first bite of the stable fly.

[b] Means followed by the same letter are not significantly different ($P = 0.05$, Bonferroni method).

1.5. Repellency of fatty acids identified in tamanu nut oil and related compounds

The binary mixtures of CI-NO and EOs or volatile compounds increase significantly repellency against the stable flies, whereas this did not appear in any formulae of EOs or compounds alone which tend to turn into vapor quickly and lost repellency rapidly compared to the volatility of DEET. The binary mixture formulae raised the question to the efficiency of CI-NO in the insect repellents, even though the nut oil itself produced low repellency to the stable flies. Repellencies of 4 major fatty acids, oleic, linoleic, stearic, and palmitic acids identified in CI-NO, one related fatty acid (linolenic acid) and four fatty acid esters (methyl oleate, methyl linoleate, ethyl oleate, and ethyl linoleate) against female stable fly bites were evaluated and compared with that of CI-NO (Table 10). At 1 mg/cm^2, repellency of oleic acid, linoleic acid, methyl oleate, and methyl linoleate was stronger (PT, 0.88–0.94 h) than that of CI-NO (PT, 0.33 h). Ethyl oleate and ethyl linoleate displayed significant lower repellency (PT, 0.55 and 0.52 h, respectively) than the corresponding methyl esters. Repellency of oleic acid and linoleic acid was meaningfully stronger than that of linolenic acid (PT, 0.40 h), whereas palmitic and stearic acid showed no repellency to the flies at the exposed concentration. Palmitic (C16:0) and stearic (C18:0) acids were found to easily turn into a waxy layer on the treated skin at room temperature due to the characteristics of saturated fatty acids. However, unsaturated fats such as OA (C18:1n9), LA (C18:2n6) and linolenic acid (C18:3n3), and the ethylated and methylated derivatives basically remain as a liquid film layer on the treated skin.

Table 10. Repellency to female stable flies of the fatty acids identified in tamanu nut oil (CI-NO) and related compounds using the exposed human hand bioassay at the concentration of 1 mg/cm^2

Treatments	Formula	BP (°C/760 mmHg)	Protection time[a] (mean ± SE, h)[b]
Oleic acid	C18:1	360	0.94 ± 0.064 a
Linoleic acid	C18:2	365–366	0.90 ± 0.058 a
Linolenic acid	C18:3	230–232[c]	0.40 ± 0.044 b
Palmitic acid	C16:0	204–220	0 c
Stearic acid	C18:0	196–211	0 c
Methyl oleate		351–353	0.92 ± 0.083 a
Methyl linoleate		373–374	0.88 ± 0.062 a
Ethyl oletate		205–208	0.55 ± 0.073 b
Ethyl linoleate		388–389	0.52 ± 0.082 b
CI-NO			0.33 ± 0.043 b

[a] Time (hour) to first bite of the stable fly.

[b] Means followed by the same letter are not significantly different (P = 0.05, Bonferroni method).

[c] At 1 mmHg.

Due to the repellency of the fatty acids and their methyl esters, the repellencies of binary mixtures of fatty acids or methyl esters (0.5 mg/cm^2) and three selected monoterpenoids (MTs) or DEET (0.25 mg) were evaluated and compared with those of the fatty acids, methyl esters, three MTs, and DEET alone (Table 11). The binary mixtures of MTs and fatty acids or methyl esters produced repellencies (PT, 1.70–2.05 h) similar to those binary mixture of DEET and fatty acids or methyl esters (PT, 1.87–2.10 h) and significantly stronger than those of DEET alone (PT, 1.50 h), the fatty acids and the methyl esters alone (PT, 0.45–0.55 h) and 3 MTs alone (PT, 0.5–0.7 h), except for those binary mixtures of α-phellandrene and methyl esters gave similar repellency (PT, 1.55 and 1.56 h) to that of DEET alone. In general, the binary mixtures containing fatty acids were slightly stronger repellent than those of harboring methyl esters.

Table 11. Repellency of CI-NO components mixed with selected monoterpenoids against the bite of female stable flies using the exposed human hand bioassay

Treatment	Rate, mg/cm^2	Protection time[a] (mean ± SE, h)[b]
OA[c] alone	0.5	0.55 ± 0.063 d
LA[d] alone	0.5	0.52 ± 0.049 d
MO[e] alone	0.5	0.47 ± 0.038 d
ML[f] alone	0.5	0.45 ± 0.073 d
Cuminyl alcohol alone	0.25	0.70 ± 0.042 d
Cuminaldehyde alone	0.25	0.65 ± 0.044 d
α-Phellandrene alone	0.25	0.50 ± 0.054 d
Cuminyl alcohol + OA	0.25+ 0.5	2.05 ± 0.104 ab
+ LA	0.25+ 0.5	2.02 ± 0.112 ab
+ MO	0.25+ 0.5	1.76 ± 0.068 abc
+ ML	0.25+ 0.5	1.73 ± 0.055 abc
Cuminaldehyde + OA	0.25+ 0.5	2.04 ± 0.125 ab
+ LA	0.25+ 0.5	2.00 ± 0.048 ab
+ MO	0.25+ 0.5	1.72 ± 0.100 abc
+ ML	0.25+ 0.5	1.70 ± 0.025 abc

α-Phellandrene + OA	0.25+ 0.5	1.90 ± 0.058 abc
+ LA	0.25+ 0.5	1.88 ± 0.082 abc
+ MO	0.25+ 0.5	1.56 ± 0.095 bc
+ ML	0.25+ 0.5	1.55 ± 0.073 bc
DEET + OA	0.25+ 0.5	2.10 ± 0.098 a
+ LA	0.25+ 0.5	2.07 ± 0.082 a
+ MO	0.25+ 0.5	1.91 ± 0.101 abc
+ ML	0.25+ 0.5	1.87 ± 0.078 abc
DEET alone	0.25	1.50 ± 0.092 c

[a] Time (hour) to first bite of the stable fly.

[b] Means followed by the same letter are not significantly different (P = 0.05, Bonferroni method).

[c] Oleic acid.

[d] Linoleic acid.

[e] Methyl oleate.

[f] Methyl linoleate.

1.6. Repellency of aerosol formulations

The repellency of 12 different experimental aerosols against blood-starved female stable flies in the exposed human hand bioassay was compared with those of DEET-5% and DEET-10% aerosols (Table 12). In a laboratory test with 6 human volunteers, effectiveness and duration of repellency of the ZP-SD-2.5% + CI-NO-2.5% and ZA-SO-2.5% + CI-NO-2.5% aerosols were stronger than those of ZP-SD-5% and ZA-SO-5% aerosols and comparable to those of ZP-SD-10%, ZA-SO-10%, and DEET-5% aerosols, but slightly lower than that of DEET-2.5% + CI-NO-2.5% aerosol. However, the ZP-SD-5% + CI-NO-5% and ZA-SO-5% + CI-NO-5% aerosols had similar repellent effect to that of DEET-10% aerosol, but they were slightly less effect than DEET-5% + CI-NO-5% aerosol.

1.7. Allergic reaction

The test EOs, CI-NO, and their binary mixtures did not induce any allergic reactions at 0.5 mg/cm^2 with the exception of savory EO. The EO induced slightly dermal irritation (EPA toxicity category IV) to one of six volunteers at 0.5 mg/cm^2 but no dermal irritation was observed at 0.25 mg/cm^2.

Table 12. Repellency to female stable flies of 12 different experimental aerosol formulations of *Z. piperitum* pericarp steam distillate (ZP-SD), *Z. armatum* seed oil (ZA-SO), and DEET alone or in combination with tamanu nut oil (CI-NO) using the exposed human hand bioassay

Aerosol formulation	Protection time[a] (mean ± SE, h)[b]
ZA-SO-5%	0.68 ± 0.039 e
ZP-SD-5%	0.80 ± 0.063 e
ZA-SO-10%	1.14 ± 0.055 d
ZP-SD-10%	1.31 ± 0.045 d
DEET-5%	1.21 ± 0.072 d
ZA-SO-2.5% + CI-NO-2.5%	1.30 ± 0.025 d
ZP-SD-2.5% + CI-NO-2.5%	1.34 ± 0.040 d
DEET-2.5% + CI-NO-2.5%	1.48 ± 0.078 cd
DEET-10%	1.90 ± 0.051 abc
ZA-SO-5% + CI-NO-5%	1.92 ± 0.130 abc
ZP-SD-5% + CI-NO-5%	1.95 ± 0.144 ab
DEET-5% + CI-NO-5%	2.34 ± 0.099 a

[a] Time (hour) to first bite of the stable fly.

[b] Means followed by the same letter are not significantly different ($P = 0.05$, Bonferroni method).

Discussion

Essential oils consist of highly complex mixtures of the hydrocarbons such as terpenes (monoterpenes, sesquiterpenes, and diterpenes), and the oxygenated compounds such as esters, aldehydes, ketones, alcohols, phenols, and oxides (Sellar, 2001; Lawless, 2002). They jointly or independently contribute to behavioral efficacy such as repellency and feeding deterrence, and physiological efficacy such as acute toxicity and developmental disruption against various arthropod species (Isman, 2006). Many plant extracts and EOs manifest repellency against various arthropod species (Curtis *et al*., 1990; Rozendaal, 1997; Yang *et al*., 2004b; Ahn *et al*., 2006; Isman, 2006; Hieu *et al*., 2010a). Mehlhorn *et al*. (2005) reported that seed extract from *Vitex agnus castus* L. (Verbenaceae) was effective as a repellent against eight blood-sucking arthropods including stable fly. These potential new arthropod repellents can be applied to human and animal skin, clothing, and livestock barns and stables in the same manner as the repellents currently used. Little work has been done to consider the potential of EOs to manage stable fly, although their repellency to mosquitoes has been well noted (Curtis *et al*., 1990; Quarles, 1996; Das *et al*., 2003; Yang *et al*., 2004b). In the current study with female stable fly, repellency varied according to EO and exposure dose tested. As judged by the PT, potent activity was observed with clove bud, clove leaf, geranium, lovage root, oregano, patchouli, thyme red, thyme white, and ZP-SD EOs at 0.5 mg/cm^2 without adverse effects on six human volunteers. This original finding indicates that the EOs described may hold promise as novel and effective repellent products against stable fly.

Investigations on physical and physiological characteristics of naturally occurring

repellents are of practical importance for stable fly control because they may give useful information on the most appropriate formulations and delivery means to be adopted for future commercialization. Several products based on EOs such as citronella, fennel, geranium, lavender, and rosemary, have been commercialized (Curtis *et al*., 1990; Brown and Hebert, 1997). The effectiveness and duration of inherent repellency of EOs or chemicals depend on the type of active ingredients, the frequency formulation of application, test conditions such as tested arthropod species and involved volunteers, loss due to removal by perspiration and abrasion, and the numerical density of arthropods (Schreck, 1995; Rozendaal, 1997). Because EOs and their constituents are high volatility, many commercial products or formulations based on EOs are usually effective against arthropods only for a relatively short period, typically less than 1 h under both laboratory and field conditions (Rozendaal, 1997; Barnard, 2000; Fradin and Day, 2002; Kim *et al*., 2004b; Isman, 2006). As little as 3.3 µl of *Z. piperitum* EO gave a median PT of 0.5–1 h against female *Aedes aegypti* L. (Choochote *et al*., 2007) and 10% *Z. limonella* (Dennst.) Alston seed and fruit oils gave complete protection for 0.5 h (Trongtokit *et al*., 2005). MossZero aerosol containing 5% fennel oil, MossZero cream containing 8% fennel oil and MeiMei cream containing citronella and geranium oils produced 84, 70, and 57% repellency, respectively, at 1.5 h after exposure, whereas Repellan S aerosol containing 19% DEET gave 89% repellency at 3.5 h (Kim *et al*., 2004a). Although available information on stable fly repellency is limited, Mehlhorn *et al*. (2005) reported that spray application of a carbon dioxide extract from the seeds of monk's pepper, *Vitex agnus castus* L., provided PT of 3 h against stable fly. In the current study with six human volunteers, clove bud, clove leaf, lovage root, and patchouli EOs gave protection from

female stable fly bites for 3–4 h at 0.5 mg/cm^2, although the PT of the EOs was slightly shorter than that of DEET. ZP-SD, geranium, oregano, thyme red, and thyme white EOs provided protection from the fly bites for ≈1 h, while ZA-SO and bergamot EO gave PT within 0.5–1 h at the same dose. This different PT may be attributed to the difference in the quantitative loss due to the volatility of chemical constituents of the EOs tested.

Z. piperitum and *Z. armatum*, and several other species such as *Z. bugeanum*, *Z. limonella*, *Z. alatum*, and *Z. schinifolium* have been used as insecticides, insect repellents, and feeding deterrents (Dube *et al*., 1990; Bowers *et al*., 1993; Tiwary *et al*., 2007; Kwon *et al*., 2011, Hieu *et al*., 2012). These natural insect repellent *Zanthoxylum* species have been particularly used for centuries as spices in Asian cuisine and traditional Asian medicine (Perry, 1980; Cho *et al*., 2003). This indicates that the plant species would possess several insect repellent constituents, which would be safe to humans and environment. In the current study, the repellent constituents of ZP-SD were determined to be the monoterpenoids cuminaldehyde, cuminyl alcohol, and limonene. This is the first report of the repellency of *Z. piperitum* and *Z. armatum* constituents to stable fly. Of the 29 individual compounds examined, cuminaldehyde, cuminyl alcohol, limonene, and methyl cinnamate were the most active. The bioactive constituents were effective against stable fly with complete protection of ca ≈0.5 h at 0.25 mg/cm^2. At the concentration, the effective protection of the individual active compounds was similar to that of ZP-SD and more pronounced than ZA-SO. In our previous report, cuminyl alcohol, limonene, cuminaldehyde, and methyl cinnamate at 0.2 mg/cm^2, gave 100% protection at 10 min post-treatment but respectively 82, 74, 74 and 64% repellency following a 30 min posttreatment interval; their repellency significantly decreased over time (Hieu *et al*.,

2010b). The differences in repellency noted may be attributable to the differences in the quantitative losses due to the differential volatility of the ZP-SD and ZA-SO repellent constituents. The ability of a chemical vapor to repel is related to its boiling point, with boiling points between 230 and 260°C at atmospheric pressure being the most desirable range for an effective repellent (Brown and Hebert, 1997). EO constituents are somewhat volatile (Lawless, 2002; Isman, 2006), whereas DEET is almost nonvolatile (111^{o}C/1 mmHg) (Knowles, 1991). For example, DEET volatilization for 13 days at 24°C is less than 1% (Inchem, 1990). Of the 12 constituents with low boiling points (≤ 200°C), limonene was the most active. Of the 16 compounds with high boiling points (> 200°C), cuminaldehyde, cuminyl alcohol, and methyl cinnamate were all significantly more effective than the other 13 constituents. The present findings indicate that other physicochemical parameters also appear to be associated with the repellency of these compounds against stable fly, although the boiling points of cuminaldehyde, cuminyl alcohol, and methyl cinnamate coincide with the optimal range and were significantly more effective than the 12 compounds with lower boiling points.

Various controlled-release formulations have been developed to increase repellency effectiveness and duration (Khan *et al*., 1975; Gupta and Rutledge, 1989; Sharma and Ansari, 1994; Dua *et al*., 1996). The flower extract of *Lantana camara* L. added in coconut oil provides 94.5% protection from *Aedes albopictus* (Skuse) and *Aedes aegypti* (L.) without adverse effects on the human volunteers for a 3-month period after the application (Dua *et al*., 1996). Sharma and Ansari (1994) reported that a 1% neem oil and kerosene mixture may provide economical personal protection from mosquito bites. A 50%

liquid formulation containing natural *p*-menthane-3,8-diol as an active ingredient was reported to provide a high level of protection against stable fly for at least 5 h (Trigg and Hill, 1996). Khan *et al*. (1975) reported that the mixtures of DEET and vanillin (1:1, 1:2, and 1:3) against mosquitoes increased from 5 to 12–14 h compared with DEET application alone in the protective lasting time. It has been reported that a mosquito repellent of 10% citronella oil lotion containing emulwax and 5% vanillin provided PT of 4.8 h (Songkro *et al*., 2012). In the current study, the increase in the PT was produced by binary mixtures of 12 EOs and CI-NO (1:8 by weight) against female stable fly compared with the constituted oil alone and DEET alone. Also, the effectiveness of complete PT was significantly promoted by the binary mixtures of nine selected active compounds and CI-NO (1:4 by weight) against female stable flies compared with the individual compounds alone and DEET alone. The PT of the EOs or compounds and CI-NO binary mixtures were similar to or slightly lower than that of the DEET and CI-NO mixture depending on EO or compound used. The improved effectiveness of repellency might be attributed to the lower evaporation rate and better skin persistence of these EOs and volatile compounds in the combined presence of CI-NO.

Although CI-NO gave a short PT, the unsaturated fatty acid constituents produced potential repellency against stable fly. Oleic acid, linoleic acid, methyl oleate, and methyl linoleate were the strongest compounds, while linolenic acid, ethyl oleate, and ethyl linoleate showed weak repellency. The saturated fatty acids, stearic and palmitic acid, was found to have no repellency to the fly. Vegetable oils are readily available worldwide and may provide a simple, inexpensive, and effective alternative. Oleic acid and linoleic acid are unsaturated fatty acids, generally found to be abundant in many vegetable oils

such as grape seed, sunflower, soybean, olive and corn oils, which may provide the most potential benefit to the skin barrier, and also used in pharmacy as an emulsifier (Darmstadt *et al*., 2002; Young, 2002). Vegetable oils may enhance epidermal barrier function in neonates by forming a physical barrier and providing γ-linolenic acid, which decreases cutaneous inflammation (Darmstadt *et al*., 2002). Oleic acid and linoleic acid have become increasingly popular in the beauty products industry because of its beneficial properties on the skin. Several studies clearly pointed to anti-inflammatory, acne reductive, and moisture retentive properties of LA when applied topically on the skin (Letawe *et al*., 1998; Darmstadt *et al*., 2002). The mammalian stratum corneum lipids in mammalian skin are known to be important regulators of skin permeability (Lampe *et al*., 1983; Hsu *et al*., 2004). In the present study, oleic acid, linoleic acid, and their methylated derivatives in CI-NO may interact with the human stratum corneum lipids (Hsu *et al*., 2004) and therefore provide anti-inflammation and moisture retention properties used as a vehicle to carry and maintain repellent EOs and volatile compounds in our test formulations of binary mixtures to protect human skin again the stable fly.

In addition, the repellencies of ZP-SD-2.5% + CI-NO-2.5% and ZA-SO-2.5% + CI-NO-2.5% aerosol products were comparable with that of DEET-5%. In turn, ZP-SD-5% + CI-NO-5% and ZA-SO-5% + CI-NO-5% aerosols gave similar repellency with that of DEET-10%. However, the repellency of EO + CI-NO aerosol was slightly lower than that of DEET + CI-NO at the same concentrations. This original finding indicates that binary mixtures of ZP-SD, ZA-SO or their bioactive constituents and CI-NO may hold promise for the development of novel and effective stable fly repellent products.

Chapter 2

Olfactory response of *Stomoxys calcitrans* to *Zanthoxylum* oil constituents and their mixtures with attractants

Introduction

Insect olfactory and gustatory systems are well-capable to efficiently perceive meaningful signals of its host from a complex odor background. In haematophagous insects, a large and divergent population of olfactory receptor neurons (ORNs) located on the antennae and palps respond to chemical cues such as CO_2, 1-octen-3-ol, ammonia, lactic acid, and carboxylic fatty acids in host seeking and orientation behaviors (Hallem *et al*., 2006; Lu *et al*., 2007; Syed and Leal, 2009). Recent studies on determining the chemical specificities and functional properties of olfactory receptors have provided insight into the mechanisms underlying odor coding in insects (Hallem *et al*., 2006; Kwon *et al*., 2006; Kent *et al*., 2009). Research with *Drosophila melanogaster* L. has provided tremendous insight into the molecular basis of insect chemoreception (Hallem *et al*., 2006; Ditzen *et al*., 2008; Gardiner *et al*., 2009). However, there are significant gaps in the understanding of the neural and molecular mechanisms underlying olfactory-driven behaviors in medical insects, although recent characterization of the mosquito genomes have boosted several lines of olfactory-related research in vector insect species (Hallem *et al*., 2004; Zwiebel and Takken, 2004; Kwon *et al*., 2006; Bohbot *et al*., 2007). Due to the incomplete understanding of olfactory function in medical insects, stable fly was used as a model insect to determine olfactory responses and behaviors in the presence of repellent air flow mixed with that of attractant. The stable fly is obligate blood feeder in both sexes known as significant blood-sucking pest of human and livestock in many parts of the world (Bruce and Decker, 1958; Anderson, 1978; Campbell *et al*., 1987), which extremely sensitive to cow rumen odor (Jeanbourquin and Guerin, 2007a) and human

breath, rumen volatiles (Warnes and Finlayson, 1986; Alzogaray and Carlson, 2000; Jeanbourquin and Guerin, 2007b), and human skin odors (Gatehouse and Lewis, 1973).

Plant essential oils (EOs) have been extensively tested to assess their repellent properties as a valuable natural resource against many insects (Isman, 2000; Ahn *et al*., 2006; Nerio *et al*., 2010). In most cases, many EOs and their constituents manifest repellency against mosquitoes (Trongtokit *et al*., 2005; Choochote *et al*., 2007; Gillij *et al*., 2008). Several EOs alone or in combination with tamanu nut oil were found to protect human skin from the bite of stable fly (Hieu *et al*., 2010a). In particular, ZP-SD, ZA-SO, and their constituents such as cuminaldehyde, cuminyl alcohol, limonene, and methyl cinnamate possess repellency against female stable flies (Hieu *et al*., 2010b).

In the present study, behavioral experiments and electrophysiological recording techniques were conducted to examine how the EOs and volatiles constituents affect the perception of the stable flies to two major attractant volatiles, 1-octen-3-ol (Oct) and butyric acid (BA), general attractants of blood-sucking insects (Gibson and Torr, 1999; Smallegange *et al*., 2009). The study mainly focused on the question how behavioral and antennal olfactory responses of adult female flies are modulated by various air mixtures of different concentrations of EOs and attractant chemicals. This may lead to increase the understanding of the perception of attractive cues in the presence of repellent odorants, which would provide better strategies to control this pest insect.

Materials and methods

2.1. Materials

ZP-SD, ZA-SO, 29 volatile constituents, and DEET used in this study were obtained as stated in Chapter I. Butyric acid (99% purity) and 1-octen-3-ol (98%) were purchased from Sigma-Aldrich. All of the other chemical compounds used in this study were analytical grade and commercially available.

2.2. Stable flies

A colony of stable flies was maintained in the laboratory as stated in Chapter I. Female flies (3–5 days old) reared with 8% sugar solution in cotton mesh cages without exposed to cow bovine were used for behavioral and electrophysiological experiments.

2.3. Scanning electron microscopy

The fly heads were dehydrated overnight at 40°C. They were primarily fixed in Karnovsky's fixative (2% glutaraldehyde (v/v) and 2% paraformaldehyde (v/v) in 0.05 M sodium cacodylate buffer pH 7.2) (Kim and Park, 2007). The samples were incubated at 4°C in darkness for 2–4 h. They were then washed three times with the same buffer. Second fixing was performed with 1% OsO_4 (w/v) in the same buffer at 4°C for 2 h. Fixed samples were washed twice with the same buffer and distilled water. The samples were then dehydrated in a graded series of ethanol increasing concentrations up to 100% for 10 min. Finally, samples were substituted to hexamethyldisilazane and dried in a Bio-Rad E3000 critical point drying machine (Cambridge, MA). Specimen was photographed using a Jeol JSM 5410LV scanning electron microscope (Tokyo, Japan) at 3.00 kV.

2.4. Behavioral bioassay

Behavioral experiments were conducted using a release-in-cage (Fig. 6) described previously (Kim *et al*., 2004a). Fifty female flies (3–5 days old) were first released in the fly release cage (R). Two amounts of the materials (5 and 10 mg per cage) were tested based on the previous report of toxic effects at higher concentrations (Hieu *et al.*, 2012). All test materials in 150 µl ethanol were applied to 55 mm diameter Whatman no. 2 filter papers (Maidstone, UK). For behavioral tests with attractants, BA and Oct were applied at concentrations of 0.2 and 2 mg. Control filter papers received 150 µl of ethanol only. After air-drying for 1 min, the treated filter paper was attached on central wall of the treated cage (T), while a control filter paper was attached on central wall of the control cage (C). After 1 min of treatment, the two doors of the R cage were gently lifted up in order to expose the test flies to both of the T and C cages. In the experiments conducted with mixtures of repellent and attractant, two filter papers (one treated with repellent and the other with attractant) were attached on central wall of the T cage. The numbers of flies flying into each cage were counted at 5 and 15 min time points. All the bioassays were carried out in triplicate and conducted under the same conditions used for colony maintenance. The behavioral index (BI) was calculated according to the following formula (Schreck, 1995): BI = $[(C - T)/(C + T)]$ x 100, where C is the number of stable flies in the control cage and T is the number of stable flies in the treated cage. Based on the index, average ± SE of repellent or attractant percentage was recorded.

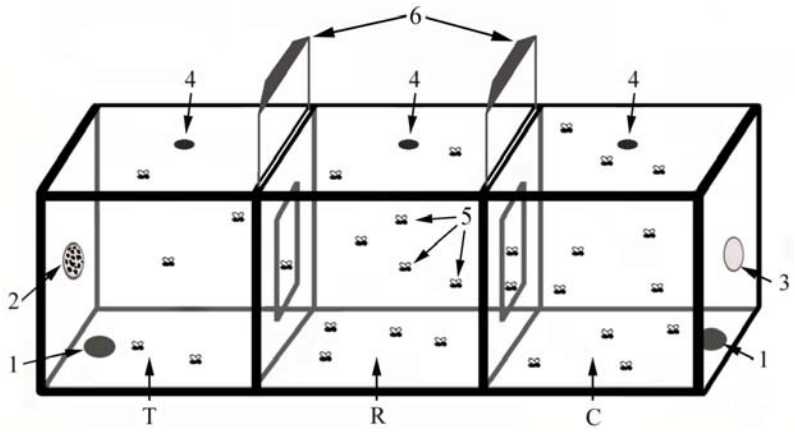

Fig. 6. A release-in-cage for behavioral test of stable flies with three connected cages, each 30 x 30 x 30 cm. The treated cage (T), the fly release cage (R), and control cage (C) were separated by 2 rectangular doors (15 x 15 cm). Two cotton dishes (1) containing 8% sugar solution were placed in the T and C cage. A treated filter paper (2) was attached on the wall of the T, and a control filter paper (3) on the wall of the C. On the top of each cage has a ventilation hole (4), each 2 cm in diameter sealed with gauze no. 3. The test flies (5) were released in the R cage and the two doors (6) were closed. The doors (6) were lifted up to expose the flies to test materials when the test behavior started.

2.5. Electroantennography analysis

ZP-SD, ZA-SO, 29 major volatile constituents, DEET, Oct, and BA were diluted in mineral oil (v/v), except for *β*-caryophyllene oxide and methyl cinnamate (w/v) from 10^0 to 10^6 times. Amount of 25 μl each test dilution was transferred to an Advantec paper disk (0.8 cm diameter × 0.1 cm thickness) (Toyo Roshi, Japan), after which the paper disk was placed into a 15 cm Pasteur pipette (Fig. 7A and B). The preparations of air mixtures of repellent and attractant are shown in Fig. 7C. Control stimulation was conducted by 25 μl mineral oil only. A humidified continuous air stream (100 ml/min) was delivered to a fly through a plastic pipette using a Syntech CS-55 stimulus controller (Hilversum, The Netherlands). Each diluted odorant was delivered in a 1 second air pulse to continuous air stream through Pasteur pipette to fly antennae. The procedure of fly preparation for EAG recording was modified from the method of Ayer and Carlson (1991). In brief, a female adult fly was allowed to climb into the pipette tip. The end of the pipette tip was trimmed and only the fly's head was exposed for EAG recording (Fig. 7D). Glass electrodes were prepared by using a Sutter horizontal electrode puller P-97 (Novato, CA, USA) filled with 0.1 M KCl. A WPI 1.5/0.84 mm OD/ID recording borosilicate glass electrode (Sarasota, FL, USA) was placed in contact with the epithelium of the antenna (Fig. 7E and F). The close-up position on stable fly antennae for EAG recording is shown in Fig. 7G. A reference glass electrode was similarly prepared and placed into the fly compound eye. The procedure of EAG recordings was modified from EAG procedures described previously (Ayer and Carlson, 1992). Recorded EAG data were conveyed to a 4-channel IDAC-UAB and were analyzed with Syntech EAG2000 software (Hilversum).

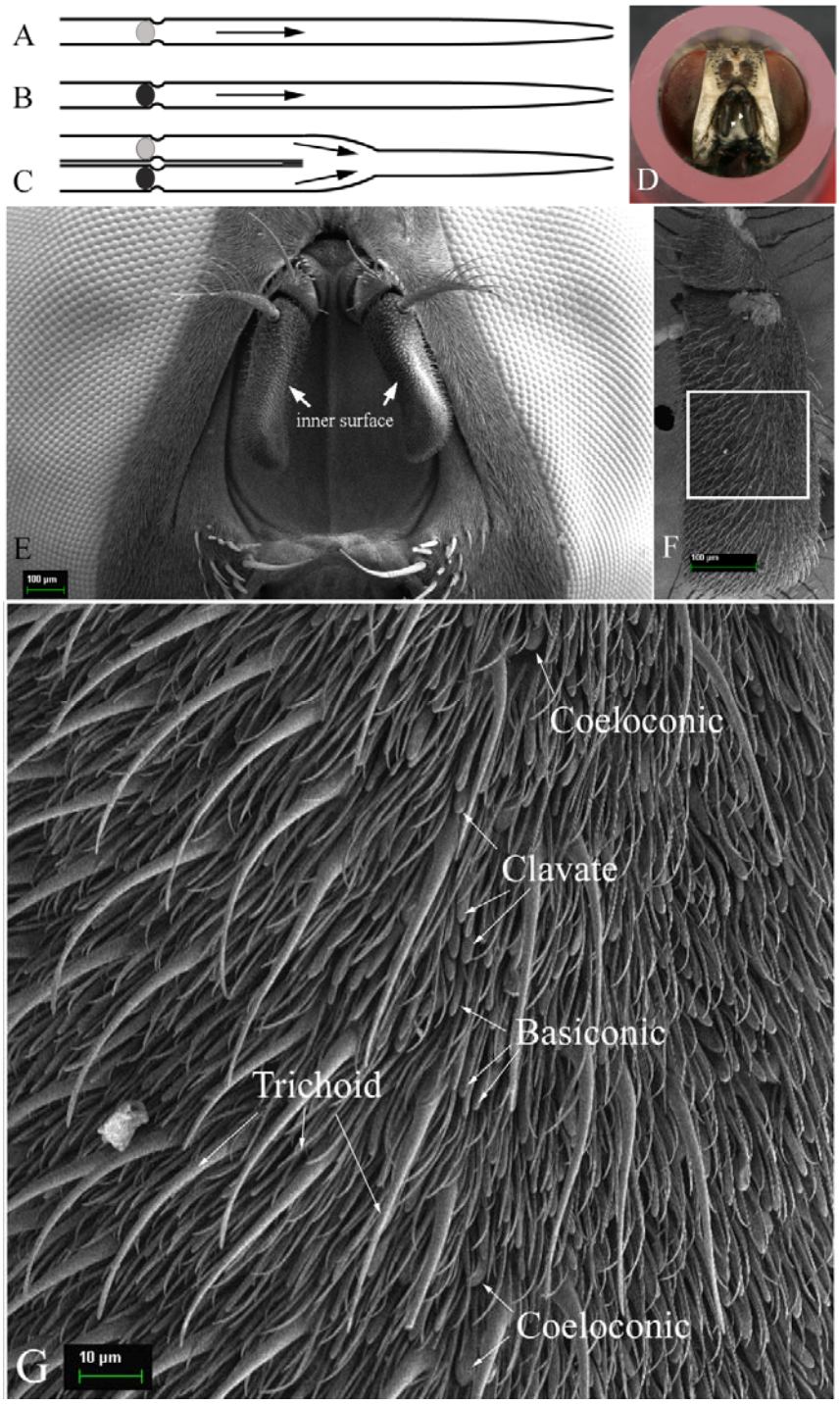
A
B
C
D
inner surface
E
100 µm
F
100 µm
Coeloconic
Clavate
Basiconic
Trichoid
Coeloconic
G
10 µm

Fig. 7. Preparations for electroantennography (EAG) recordings. Stimulus Pasteur pipettes containing filter treated with (A) repellent, (B) attractant, and (C) preparation of the air mixtures of repellent and attractant. The arrows show the air flows and the air mixture. (D) EAG preparation of stable fly head in the pipette tip; (E) scanning electron micrograph of the stable fly head shows inner surface of the antennae. (F and G) scanning electron micrography image of a left antenna showing the position of the recording electrode. (G) Four different types of sensillum: trichoid, basiconic, coeloconic, and clavate sensilla (Tangtrakulwanich *et al*., 2011).

2.6. Data analysis

Statistical significance analysis of behavioral index and EAG data was analyzed using ANOVA, Student's *t* test, and Bonferroni-Dunn's test for multiple comparisons (SAS® 9.3, SAS Institute, Cary, NC).

Results

2.1. Behavioral patterns of the stable fly in release-in-cage

The stable flies exhibited avoidance behaviors to the repellent chemicals after 5–15 min of exposure to the treatments depending on compound and concentration tested. Most of the flies were randomly distributed throughout the cages and showed normal flight patterns in the R and C cages. However, the flies were less robust in flight and mostly landed on the bottom of the cage when entering the T cage, showing that the repellent vapors might inhibit the locomotion and flight patterns of the flies (data not show). In contrast, when the test attractant chemical, BA or Oct, was treated in the T cage, the flies showed vivid flight behaviors similar to host finding behaviors compared to those in the C cage for 5–15 min after release.

2.2. Behavioral responses of the stable fly to test materials in release-in-cage

The responses of female stable flies to ZP-SD and ZA-SO at 5 and 10 mg/filter paper were evaluated using a release-in-cage test at 5 and 15 min time points after fly release (Table 13). The distribution of the flies was significantly low in the T cages containing ZP-SD- or ZA-SO-treated filter papers compared to that in the R and C cages. Values of repellent behaviors of both EOs at 5 mg/filter paper were significantly lower compared to those at 10 mg ($P < 0.05$, *t* test). At 10 mg/filter paper, the repellency caused by ZP-SD and ZA-SO was 73 and 87% and 70 and 86% at 5 and 15 min, respectively. Both ZP-SD and ZA-SO induced similar repellent effects to the flies at the test concentration.

Table 13. Behavioral index (BI) of female stable flies to two *Zanthoxylum* essential oils, two attractant compounds, and air mixtures of repellent and attractant using the release-in-cage bioassay

Treatment[a]	Conc., mg/cage	% BI (mean ± SE[b]) at min after treatment		*P*-value[c]
		5	15	
ZP-SD	10	73 ± 2.9 a	87 ± 2.6 a	0.0256
	5	58 ± 4.3 abc	75 ± 2.5 ab	0.0248
ZA-SO	10	70 ± 4.2 ab	86 ± 2.9 a	0.0405
	5	54 ± 3.0 bc	71 ± 3.7 ab	0.0246
BA	2	61 ± 4.8 abc	77 ± 3.6 ab	0.0492
	0.2	45 ± 2.8 cd	64 ± 3.3 b	0.0128
Oct	2	43 ± 2.0 cd	59 ± 4.8 bc	0.0448
	0.2	30 ± 2.1 d	42 ± 3.4 c	0.0483
ZP-SD + BA	10 + 2.0	50 ± 3.9 c	65 ± 3.5 b	0.0467
	10 + 0.2	61 ± 2.2 abc	73 ± 3.0 ab	0.0370
ZP-SD + Oct	10 + 2.0	62 ± 2.4 abc	75 ± 1.8 ab	0.0133
	10 + 0.2	71 ± 3.7 ab	86 ± 3.0 a	0.0462

[a] ZP-SD, *Z. piperitum* steam distillate; ZA-SO, *Z. armatum* seed oil; BA, butyric acid; Oct, 1-octen-3-ol.

[b] Means within a column followed by the same letter are not significantly different (P = 0.05, Bonferroni-Dunn's test).

[c] Student's t test.

When the attractant compounds BA and Oct were treated at 0.2 and 2.0 mg/filter paper in the release-in-cage, the stable flies were attracted to T cage at 5 and 10 min point times (Table 13). Both compounds showed greater attractant at 2.0 mg than that at 0.2 mg/filter paper. At 2.0 mg/filter paper, the test flies showed stronger attractant behaviors to BA (61 and 77% at 5 and 15 min, respectively) than Oct (43 and 59% at 5 and 15 min, respectively) after fly release.

In addition, when the flies were exposed to the air mixtures of repellent (10 mg) and attractant (0.2 or 2.0 mg/filter paper), the repellent behaviors were observed at 5 and 15 min after fly release (Table 13). However, the repellency of the EOs decreased due to the interference of attractants. The mixture of ZP-SD (10 mg) and BA (2.0 mg) caused 50 and 65% repellency in comparison with 73 and 87% of ZP-SD (10 mg) alone at 5 and 15 min after treatments, respectively. Repellency caused by the mixture of ZP-SD (10 mg) and BA (0.2 mg) slightly reduced (61 and 73%) compared with ZP-SD (10 mg) alone at 5 and 15 min after treatments, respectively. In contrast, ZP-SD (10 mg) + Oct (0.2 mg) caused 71 and 86% repellency similar to that of ZP-SD alone (10 mg) at 5 and 15 min after treatments, respectively. Repellency of the air mixture of ZP-SD (10mg) and Oct (2.0 mg) was 62 and 75% compared with ZP-SD (10 mg) alone after 5 and 15 min of treatments, respectively.

2.3. Behavioral responses of stable fly to test compounds

According to the repellent behavior results from the test EOs, the test EOs at 10 mg/filter paper produced significantly a stronger repellent effect than that of the 5 mg dose. Therefore, the effects of each EO component on the fly's behaviors in the release-in-cage at 10 mg/filter paper were examined (Table 14). At this concentration, most test compounds induced repellent behaviors at 15 min, which were significantly higher than that at 5 min after treatment. Based on the percentage of behavioral index at 15 min after treatment, cuminaldehyde was turned out to be a most repellent compound (94%), followed slightly lower by citronellal, neral, linalool oxide, piperitone, 1,8-cineole, linalool, and terpinen-4-ol (83–86%). Moderate repellent activities against the flies were obtained from *p*-cymene, *α*-phellabdrene, *γ*-terpinene, *α*-terpinene, geraniol, and *α*-terpineol (56–64%). Low repellent effects (43–15%) were observed with other terpenoid compounds such as limonene, cuminyl alcohol, citronellol, nerol, carveol, methyl cinnamate, geranyl acetate, citronellyl acetate, *β*-caryophyllene, and *β*-caryophyllene oxide, while *β*-myrcene, *α*-, *β*-pinene, *α*-humulene, *α*-copaene did not show any repellent effects to the flies at the test concentrations during 15 min of treatments. In present behavioral experiments, DEET did not affect the flight behaviors of the stable fly at the same concentration, indicating that the volatility of compounds appeared to be an important factor in this test.

Table 14. Behavioral index (BI) of female stable flies to 14 test compounds using the release-in-cage bioassay

Compound (10 mg/cage)		% BI (mean ± SE[a]) at min after treatment		*P*-value[b]
		5	15	
Aldehydes	Cuminaldehyde	76 ± 4.1 a	94 ± 1.5 a	0.0086
	Citronellal	72 ± 4.6 ab	86 ± 2.6 ab	0.0454
	Neral	72 ± 2.7 ab	85 ± 3.6 ab	0.0499
Ketone and	Linalool oxide	70 ± 3.4 ab	84 ± 3.3 ab	0.0398
	1,8-Cineole	70 ± 3.9 ab	84 ± 3.0 abc	0.0424
	Piperitone	69 ± 4.7 abc	83 ± 2.5 abc	0.0480
Hydrocarbons	*p*-Cymene	50 ± 2.6 bcd	64 ± 4.3 bcd	0.0453
	γ-Terpinene	46 ± 2.8 cd	62 ± 3.9 cd	0.0299
	α-Phellandrene	44 ± 4.5 d	61 ± 3.8 d	0.0492
	α-Terpinene	44 ± 3.0 d	59 ± 4.4 d	0.0489
Alcohols	Linalool	72 ± 3.5 ab	85 ± 3.1 ab	0.0486
	Terpinen-4-ol	70 ± 3.4 ab	84 ± 1.5 abc	0.0180
	Geraniol	43 ± 2.3 d	60 ± 4.9 d	0.0332
	α-Terpineol	40 ± 4.8 d	56 ± 3.2 d	0.0491

[a] Means within a column followed by the same letter are not significantly different (P = 0.05, Bonferroni-Dunn's test).

[b] Student's t test.

2.4. Antennal olfactory responses to single attractive chemicals

The antennal olfactory responses of stable fly to two attractant odorants, Oct and BA were investigated using an EAG technique (Fig. 8). BA elicited upward responses whereas Oct induced normal downward responses in the EAG recordings at all test concentrations (Fig. 8A). Acidic compounds typically showed dose-dependent upward EAG responses. Upward EAG responses by BA, which were not present in non-chemosensory body parts of the stable fly (data not shown), were observed. Based on the absolute values of EAG amplitude to BA and Oct, olfactory sensitivity in the fly antennae to these compounds was significantly different (Fig. 8B). BA induced an EAG amplitude significantly higher than Oct at concentrations of 10^{0}, 10^{-1}, and 10^{-2} (Fig. 8B), whereas both of them elicited no significantly different EAG amplitudes at low concentrations.

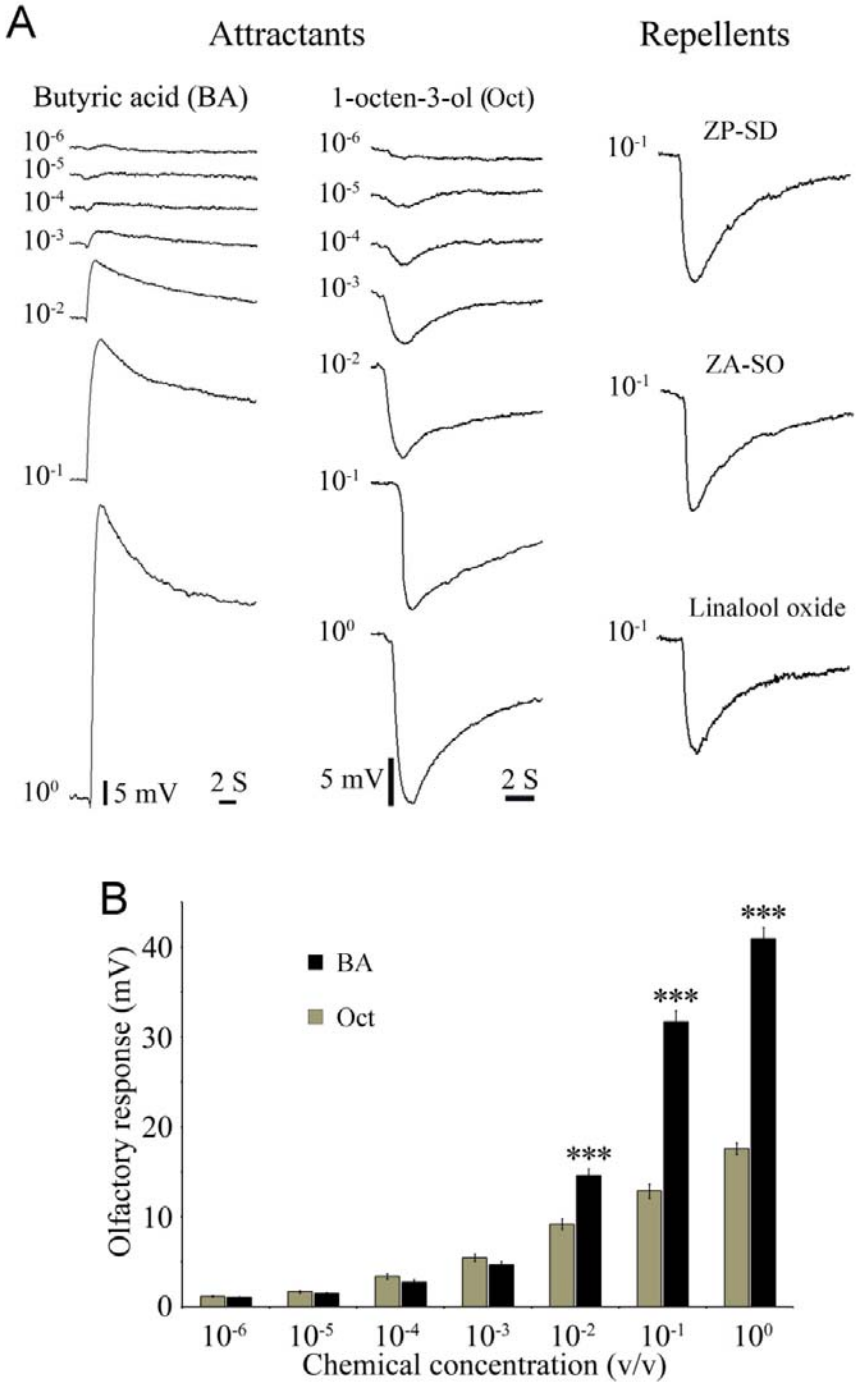
A
Attractants
Repellents
Butyric acid (BA)
1-octen-3-ol (Oct)
ZP-SD
ZA-SO
Linalool oxide
5 mV
2 S
B
BA
Oct
Olfactory response (mV)
Chemical concentration (v/v)
***

Fig. 8. Dose-responses of attractants and repellents to female stable fly antennae. (A) Examples of EAG traces of the two attractants, BA and Oct at 10^{-6}–10^{0} and the three repellents (ZP-SD, ZA-SO, and linalool oxide) at 10^{-1}. (B) The EAG amplitudes (mean ± SE) of the flies (n = 10) to BA and Oct indicate EAG increases as concentrations increased from 10^{-6} to 10^{0}. The amplitude value of BA is significantly different from that of Oct at each concentration of 10^{-2}, 10^{-1}, and 10^{0} ('***' indicates $P < 0.001$, *t* test), whereas no significantly different at each concentration of 10^{-6}, 10^{-5}, 10^{-4}, and 10^{-3} ('no label' indicates $P > 0.05$, *t* test).

2.5. Olfactory responses to two *Zanthoxylum* oils and volatile compounds

In order to characterize the antennal olfactory responses to ZP-SD and ZA-SO that showed repellent behavioral activities in the release-in-cage test, the vapors of these EOs and their constituents were stimulated to the antennae of test flies with various concentrations from 10^0 to 10^{-3} (Fig. 9). DEET evoked low EAG responses at 10^0 and showed no responses at 10^{-1}–10^{-3}.

At 10^0 concentration, strong EAG responses were elicited by linalool oxide (LO), which was similar to that of Oct, followed by ZP-SD, ZA-SO, and citronellal whose EAG responses were slightly lower than Oct. 1,8-cineole, limonene, *β*-myrcene, *α*-phellanderene, *γ*-terpinene, terpinen-4-ol, linalool, and *α*-terpineol induced EAG responses significantly lower than of Oct. Moderate EAG responses were evoked by neral, cuminaldehyde, piperitone, *β*-caryophyllene oxide, *α*- and *β*-pinene, *α*-terpinene, *p*-cymene, citronellol, nerol, geraniol, carveol, geranyl acetate, citronellyl acetate, and methyl cinnamate compared with that of Oct. Low EAG responses were observed from cuminyl alcohol, *β*-caryophyllene, *α*-humulene, and *α*-copaene (Fig. 9).

Similar results were observed at 10^{-1} concentration. Oct, ZP-SD, ZA-SO, citronellal, LO, terpinene-4-ol, and linalool elicited similar EAG responses, followed by slightly reducing EAG responses to limonene, *β*-myrcene, *α*-phellanderene, *α*-terpineol and *γ*-terpinene. At low concentrations of 10^{-2} and 10^{-3}, the EAG responses were significantly lower than those at 10^0 and 10^{-1}. However ZP-SD, ZA-SO, and only six compounds such as citronellal, *β*-myrcene, terpinen-4-ol, linalool, LO and *α*-terpineol elicited EAG responses similar or slightly lower than Oct at the low concentrations.

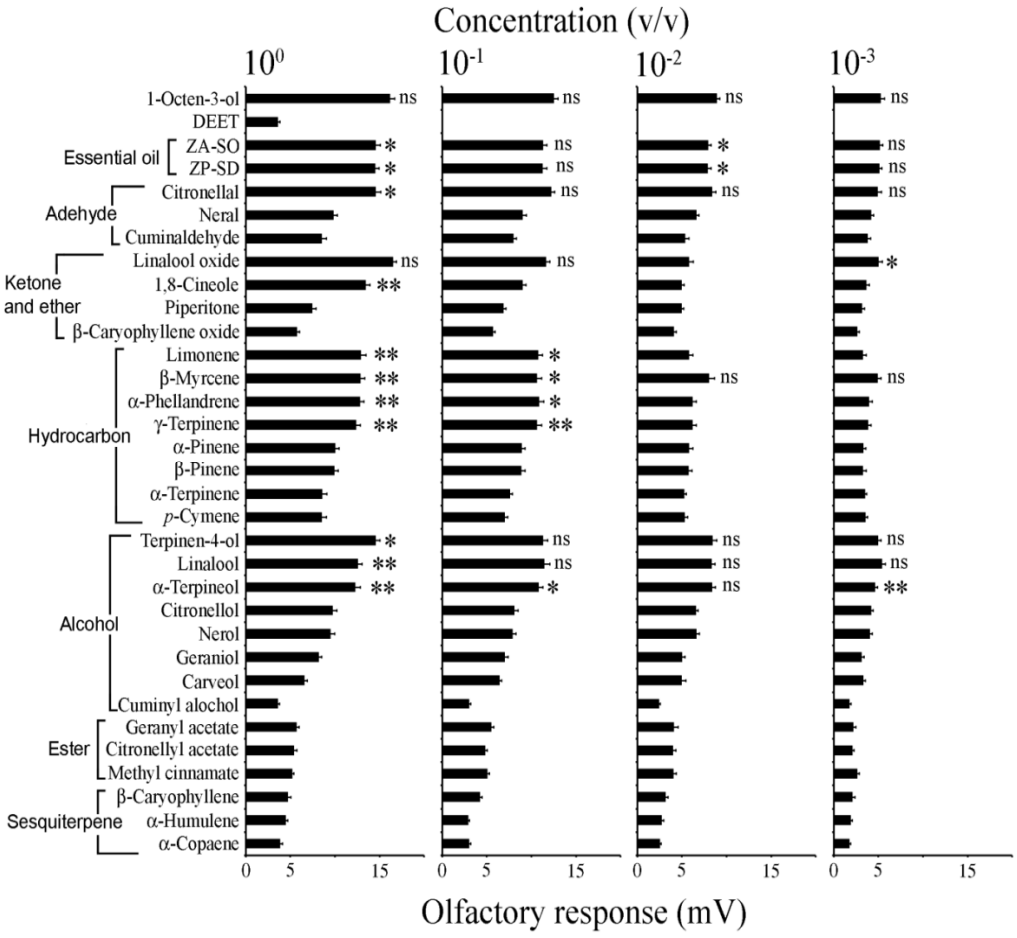

Fig. 9. The EAG comparisons of the attractant (Oct) with the given repellent EOs (ZP-SD and ZA-SO), twenty-nine compounds, and DEET at 10^0 (A), 10^{-1} (B), 10^{-2} (C), and 10^{-3} (D). EAG responses of female fly antennae to the EOs and volatiles indicated the repellents elicited EAG patterns from ORNs of the fly antennae similar to that of Oct. 'ns' depicts no significantly different at $P > 0.05$, '*' indicates significantly different at $P < 0.05$, '**' indicates significantly different at $P < 0.01$ and no labeled bars indicate significantly different at $P < 0.001$ compared to EAG responses by Oct (n = 10).

2.6. Antennal olfactory responses to binary mixtures

Behavioral patterns of the stable fly in the release-in-cage to EOs mixed with attractant compounds showed that this air blend were likely to affect olfactory processing at the peripheral level. The alternation of antennal olfactory responses by air mixtures of EO (ZP-SD or ZA-SO) and Oct compared with those of Oct, ZP-SD, and ZA-SO alone at 10^{0}–10^{-6} was evaluated (Fig. 10).

Dose-dependent EAG responses were observed with both ZP-SD and ZA-SO, which was not different from that of Oct at the same concentration. Notably, ZP-SD + Oct and ZA-SO + Oct mixtures elicited dose-dependent EAG patterns and their EAG amplitudes were significantly higher than those of Oct, ZP-SD and ZA-SO alone at the same concentrations (Fig. 10).

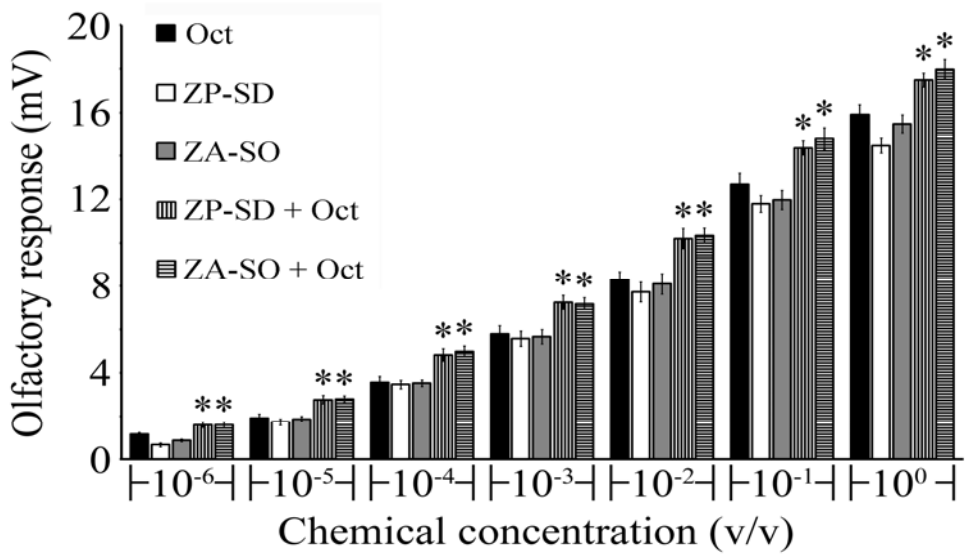

Fig. 10. The EAG comparisons of ZP-SD + Oct and ZA-SO + Oct mixtures with those of Oct, ZP-SD, and ZA-SO alone. Dose-dependent EAGs (mean ± SE) of the fly antennae (n = 10) were observed on the attractant (Oct), the repellents (ZP-SD and ZA-SO), and the mixtures at 10^{-6}–10^{0}. The EAGs of the mixtures are significantly higher than those of Oct, ZP-SD, ZA-SO alone at each concentration compared ('*' indicates $P < 0.05$). EAGs of Oct, ZP-SD, and ZA-SO alone at each concentration are not significantly different ($P > 0.05$).

ZP-SD and ZA-SO produced strong repellency to stable flies in the behavioral test. However, when the oils were blended with butyric acid (BA), behavioral pattern was significantly changed (Table 13). Here, antennal olfactory responses to the three mixtures of repellent + attractant (ZP-SD + BA, ZA-SO + BA, and LO + BA) were examined and compared with those of an attractant + attractant mixture (Oct + BA). Linalool oxide (LO) was employed as a single compound because LO alone elicited strong repellent behavior

in stable fly and evoked similar EAG response to that of Oct (Fig. 9). Since BA produced upward negative EAG amplitudes compared to EOs and LO, this experiment was carried out to investigate how these binary mixtures alter olfactory responses. When the test flies' antennae were stimulated by the mixtures of repellent + attractant such as ZP-SD (10^{-1}) + BA (10^{-1}), ZP-SD (10^{-1}) + BA (10^{-2}), and the mixtures of attractant + attractant such as Oct (10^{-1}) + BA (10^{-1}) and Oct (10^{-1}) + BA (10^{-2}), similar upward EAG patterns was observed compared to BA (10^{-1}) alone (Fig. 11A). In contrast, the two mixtures, ZP-SD (10^{-1}) + BA (10^{-3}) and Oct (10^{-1}) + BA (10^{-3}) elicited downward EAG patterns similar to ZP-SD (10^{-1}) and Oct (10^{-1}) alone (Fig. 11A). BA (10^{-1}) alone elicited an upward EAG responses significantly higher than those of ZP-SD (10^{-1}) + BA (10^{-1}), ZA-SO (10^{-1}) + BA (10^{-1}), and LO (10^{-1}) + BA (10^{-1}) (Fig. 11B, C, and D). Interestingly, the Oct (10^{-1}) + BA (10^{-1}) mixture, however, elicited upward EAG responses similar to BA (10^{-1}) alone (Fig. 11E).

Similar results were obtained from ZP-SD (10^{-1}) + BA (10^{-2}), ZA-SO (10^{-1}) + BA (10^{-2}), and LO (10^{-1}) + BA (10^{-2}) mixtures. These binary mixtures elicited negative upward EAG responses, which were significantly lower than that of BA (10^{-2}) alone (Fig. 11B, C, and D). However, Oct (10^{-1}) + BA (10^{-2}) elicited an upward EAG amplitude similar to BA (10^{-2}) alone (Fig. 11E). The binary mixtures, ZP-SD (10^{-1}) + BA (10^{-3}), ZA-SO (10^{-1}) + BA (10^{-3}), LO (10^{-1}) + BA (10^{-3}), and Oct (10^{-1}) + BA (10^{-3}) elicited downward positive EAG amplitudes (Fig. 11B, C, D, and E). However, these positive EAG responses were significantly lower than those of ZP-SD, ZA-SO, and LO (10^{-1}) alone (Fig. 11B, C, D), except for the Oct (10^{-1}) + BA (10^{-3}) mixture, which showed similar EAG values with that of Oct (10^{-1}) alone (Fig. 11E).

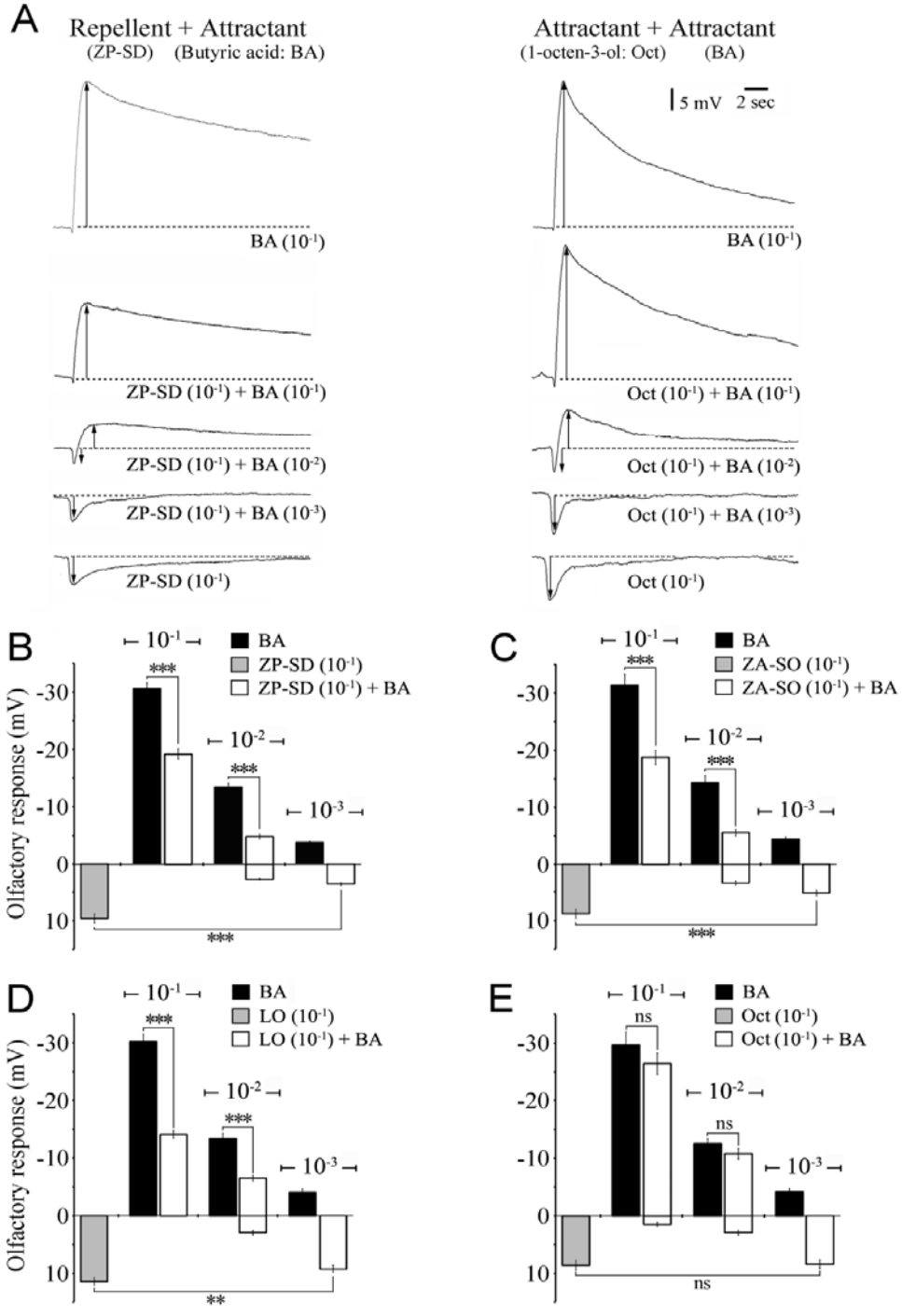
A
Repellent + Attractant
(ZP-SD) (Butyric acid: BA)
Attractant + Attractant
(1-octen-3-ol: Oct) (BA)
5 mV 2 sec
BA (10-1)
ZP-SD (10-1) + BA (10-1)
ZP-SD (10-1) + BA (10-2)
ZP-SD (10-1) + BA (10-3)
ZP-SD (10-1)
Oct (10-1) + BA (10-1)
Oct (10-1) + BA (10-2)
Oct (10-1) + BA (10-3)
Oct (10-1)
B
C
D
E
Olfactory response (mV)
-30
-20
-10
0
10
10-1
10-2
10-3
BA
ZP-SD (10-1)
ZP-SD (10-1) + BA
ZA-SO (10-1)
ZA-SO (10-1) + BA
LO (10-1)
LO (10-1) + BA
Oct (10-1)
Oct (10-1) + BA
***
**
ns

Fig. 11. The EAG comparisons of repellent + attractant mixture to attractant + attractant mixture. (A) Examples of EAG traces, illustrating the EAG patterns elicited by ZP-SD + BA and Oct + BA compared with those of ZP-SD, Oct, and BA alone. EAG responses (mean ± SE) of female fly antennae (n = 5) to the binary mixtures, ZP-SD + BA (B), ZA-SO + BA (C), and LO + BA (D) illustrate that the effects of repellents in the binary mixtures on the BA perception of the flies' antennae, whereas the presence of Oct in the mixture Oct + BA (E) indicate no significant (ns) effect on the BA perception of the flies' antennae ('***' indicates $P < 0.001$, '**' indicates $P < 0.01$, and 'ns' indicates $P > 0.05$, *t* test).

Discussion

The behavioral study clearly indicates that the EOs of *Zanthoxylum* plants possess repellency against the stable fly. *Z. piperitum* and *Z. armatum* and other species such as *Z. bugeanum*, *Z. limonella*, *Z. alatum*, and *Z. schinifolium* have been used as insect repellents, and feeding deterrents (Bowers *et al*., 1993; Hieu *et al*., 2010b; Kwon *et al*., 2011). However, the mechanisms of behavioral repellency of the stable fly to these botanical volatiles in the presence of attractants such as BA and Oct are still poorly understood. In the present study, BA is significantly stronger than Oct in alteration of behavioral patterns to repellent EOs. Odors from animal feces mainly results from low molecular weight fatty acids such as BA and indicated that this acid compound plays an important role in host identification and searching in the close range from a host animal as reported previously (Kwon *et al*., 2006). Therefore, BA might play as a much stronger olfactory cue than Oct. It has been reported that Oct alone failed to trap the stable fly (Alzogaray and Carlson, 2000) and to lure the fly to blue cylindrical cloth treated with insecticide (Mullens *et al*., 1995). However, blends of Oct, CO_2, acetone, and mixture with phenolic compounds were reported to evoke attraction behaviors of the stable fly to sticky panels (Cilek, 1999). This indicates that repellent behaviors of the stable fly to EOs would not be changed by Oct itself in comparison with that of BA at the test concentrations.

Several types of functional groups and vapor pressure parameter play a significant role in determining repellencies of the test MTs to stable flies. Among 29 test compounds identified in the previous study (Hieu *et al*., 2010b), the aromatic aldehyde

cuminaldehyde caused higher repellency than analogous hydrocarbons and alcohols such as *p*-cymene and cuminyl alcohol. Also, cuminaldehyde induced slightly higher repellent effects than acyclic aldehyde forms such as citronellal and neral. This result supports the fact that functional groups and the skeletal structure of monterpenoids plays a significant role in the repellency to the stable fly, which is also reported in the structure-activity relationships of MTs in fumigant toxicity against stable fly (Hieu *et al*., 2012) and German cockroach (Jang *et al*., 2005). Among the alcohol constituents, linalool and terpinen-4-ol showed similar repellency to citronellal and neral, and stronger repellency than geraniol and α-terpineol, while other alcohols such as cuminyl alcohol, citronellol, nerol, and carveol gave average and low repellency to the stable fly. It was also reported that geraniol demonstrated a significant repellency to first-instar nymphs of *Rhodnius prolixus* Stål (Sfara *et al*., 2009) and to mosquitoes in both indoor and outdoor settings (Muller *et al*., 2009), while linalool and limonene showed low repellency to *R. prolixus* nymphs (Sfara *et al*., 2009).

In hydrocarbons, aromatic and cyclic hydrocarbons such as *p*-cymene, α-terpinene, γ-terpinene, and α-phellandrene produced repellency similar to the stable flies, whereas bicyclic hydrocarbons, α-, β-pinene and acyclic hydrocarbon, β-myrcene exhibited exhibited low to very low repellency to stable fly. However, α- and β-pinenes were reported to act as an insecticide against *Aedes aegypti* L. (Morais *et al*., 2006), *Culex pipinens* L. (Traboulsi *et al*., 2002), *Pediculus humanis capitis* De Geer (Yang *et al*., 2004a), and cockroaches (Jang *et al*., 2005; Jung *et al*., 2007). This indicates that even same hydrocarbon constituents may have different modes of action in repellency and

toxicity to different insect species. DEET is a suitable repellent against many insect species with contact action (Qiu *et al*., 1998; Pickett *et al*., 2008). In the present study, DEET did not hamper the flight behaviors of the stable flies due to its extremely low volatility. In contrast, volatile ZP-SD and ZA-SO oil constituents repelled the stable flies at a certain distance from the applied skin or applied space, indicating that vapor phase insecticides may be more effective fumigant and repellent activities and moreover safer to humans (Lee *et al*., 2003).

Most olfactory-related structures of the insect are located on antennae and have been functionally adapted to perceive airborne volatiles (Visser *et al*., 1986). The olfactory responses of the stable fly to two major attractant odors, BA and Oct have demonstrated that the fly's olfactory systems are sensitive to detect the presence of BA and Oct at low concentrations. EAG responses to Oct are known to be bigger than octan-1-ol, octanal, 2-octanone, octanoic acid and nonane (Schofield *et al*., 1995). Notably, EAG amplitudes to BA were significantly higher than Oct at 10^{0}–10^{-2}, implying that olfactory systems of the stable fly are extremely sensitive to BA. BA elicited upward (or inverted) EAG response curves in a dose dependent manner. Upward EAG amplitudes have been reported in both houseflies (Kelling, 2001) and the stable fly (Warnes and Finlayson, 1986) at high doses. Several acid volatile compounds such as butyric, isovaleric, oxovaleric and acetic acid have been reported to elicit upward responses from the proboscis of the mosquitoes (Kwon *et al*., 2006). At various test concentrations, several repellent volatiles from ZP-SD and ZA-SO evoked olfactory responses. This suggests that stable fly possesses ORNs responding to repellent chemicals. Therefore, stable fly's sensory systems may encode unwanted or dangerous factors at the peripheral sensory levels. The relationships between

EAG responses and behavioral repellency of stable flies are not significantly correlated. For instance, although cuminaldehyde, neral, piperitone, *α*-terpinene and *p*-cymene induced a moderate EAG, these compounds exhibited strong repellent effects to the flies. Limonene, citronellol, and nerol elicited strong and moderate EAG responses, but they produced low repellency to the flies. Interestingly, chemicals with aldehyde moiety produced bigger EAG responses than alcohol and ether moieties. Also, oxide forms elicited higher EAG amplitudes than non-oxide forms. This indicates that structural characteristics such as carbon skeleton, degrees of saturation, and types of functional groups, and vapor pressure parameter appear to play a role in determining repellency of the test MTs to stable flies.

The olfactory responses to the mixtures of two *Zanthoxylum* oils and Oct (ZP-SD + Oct and ZA-SO + Oct) have demonstrated that olfactory systems of the fly antennae detect and discriminate the mixture of repellent and attractant at the peripheral level of the sensory system. EAG outputs primarily present summed information on electrical responses of recorded area on the olfactory organs. Along with behavioral results of present study, olfactory sensitivity of ORNs in the antennae to the repellent-attractant mixtures, however, could explain and help to identify biologically active constituents in the interaction with attractant agents available in environment. This implies that olfactory modulation by mixture effects may be attributed to complicated peripheral olfactory systems. The fluctuation of olfactory responses by the blend of odorants has been reported in several insects (Riffell *et al*., 2009; Hiller and Vickers, 2011). This implies that olfactory neurons of the fly antennae possess various odorant receptor neurons responding to EO and Oct. In behavioral responses, the ZP-SD + Oct mixture elicited

repellency against stable flies similar or slightly lower than that of ZP-SD alone, whereas the repellency of ZP-SD was significantly reduced by the presence of BA. EAG amplitudes elicited by ZP-SD + BA, ZA-SO + BA, and LO + BA were significantly lower than that of BA alone, indicating that the presence of repellent chemicals such as ZP-SD, ZA-SO, and LO modulate the local encodings of host odorant cue BA. The results from the attractant mixture BA + Oct, however, demonstrated here that the presence of Oct did not significantly affect on the active of BA sensitive olfactory neurons, implying that there might be different local interaction mechanisms underlying the detection of host olfactory cues versus repellent cues in olfactory systems in the periphery.

Chapter 3

Insecticidal activity and mode of actions of *Zanthoxylum* oil constituents and related compounds to *Stomoxys calcitrans*

Introduction

Biocides from plant essential oils (EOs) have been suggested as an alternative to conventional synthetic insecticides, in part, because certain EO preparations meet the criteria of minimum-risk pesticides (Isman, 2008). They often act at multiple and novel target sites (Kostyukovsky *et al*., 2002; Priestley *et al*., 2003; Isman, 2006), thereby reducing the potential for resistance (Ahn *et al*., 1997; Yang *et al*., 2009). They can be applied to stable fly breeding places in the same manner as conventional insecticides. Recently, *Zanthoxylum* plants (Rutaceae) have drawn attention because they contain insecticidal principles against the cowpea aphid, *Aphis craccivora* Koch (Nissanka *et al*., 2001), and the maize weevil, *Sitophilus zeamais* Motschulsky (Wang *et al*., 2011). No information is available concerning the potential of *Z. piperitum* and *Z. armatum* for controlling stable flies, although the repellency of ZP-SD and ZA-SO and their constituents to stable flies has been reported previously (Hieu *et al*. 2010a and 2010b).

In the present study, the potential of ZP-SD and ZA-SO, their 28 constituents (Tiwary *et al*., 2007; Hieu *et al*., 2010b), and eight structurally related compounds was assessed for use as future commercial insecticides. The toxicities of these materials to female stable flies were assessed by contact and vapor-phase mortality bioassays and compared with those of two conventional OPs with fumigant action, chlorpyrifos and dichlorvos. The insecticide mode of action and structure–activity relationships of the test compounds are also discussed.

Materials and methods

3.1. Stable fly

The stock cultures of stable flies, originally collected at the Seoul National University animal farm in early October 2008, have been maintained in the laboratory without exposure to any known insecticide (Hieu *et al*., 2010b). Adult flies were reared as stated in Chapter I. The female stable flies (3–5 days old) were used for insecticidal activity assay.

3.2. Essential oils and test chemicals

ZP-SD was prepared as reported previously (Hieu *et al*., 2010b). ZA-SO was purchased from Seema International (Delhi, India). The thirty-six commercially available organic compounds examined in this study are listed in Table 15, along with their sources. For the structure–activity relationship analysis, values of molecular weight (MW) and vapor pressure (VP) for the 31 monoterpenoids (MTs) were obtained from ACD/ChemSketch (ACD/LAB 12.0 for Microsoft Window, Advanced Chemistry Development, Inc., Montreal, Canada) (Table 15). Dichlorvos (98.9% purity) and chlorpyrifos (99.5% purity) were supplied by Chem Service (West Chester, PA, USA). Acetylthiocholine iodide (ATChI), 5,5'-dithio-bis-(2-nitrobenzoate) (DTNB), octopamine, dopamine, theophyline, trizma maleate, adenosine 5′-triphosphate disodium salt hydrate (ATP disodium salt hydrate), ethylene glycol-bis-(2-aminoethylether)-*N,N,N′,N′*-tetraacetic acid (EGTA), and bovine serum albumin (BSA) were purchased from Sigma-Aldrich. All of the other chemicals used in this study were analytical grade and commercially available.

3.3. Vapor-phase mortality bioassay

A vapor-phase mortality bioassay was used to evaluate the toxicity of ZP-SD, ZA-SO, and all compounds to female stable flies. Groups of 50 female flies (3–5 days old) were placed separately in acrylic cages (20 × 30 × 40 cm). Based on the preliminary test results, four to six concentrations of each test compound in 100 μl of ethanol were separately pipetted to 55 mm diameter Whatman no. 2 filter papers (Maidstone, UK) placed onto the bottom section of glass petri dishes (55 × 12 mm). After air-drying for 1 min, each dish was sealed with gauze no. 3. Each petri dish was placed onto the bottom section of the cage, which prevented direct contact of stable flies with the test compound. The door of each cage was then closed and sealed with parafilm. Chlorpyrifos and dichlorvos served as positive controls and were similarly prepared. Negative control filter papers received 100 μl of ethanol only.

Table 15. Molecular weight (MW) and vapor pressure (VP) values of 36 compounds examined for structure-activity relationship in the stable fly

Compound	MW	VP [a]	Source
Carvacrol [b]	150.22	0.0296	S-A [e]
Carveol [c,d]	152.23	0.0117	S-A
β-Caryophyllene [d]	204.35	–	S-A
β-Caryophyllene oxide [c]	220.35	–	S-A
1,8-Cineole [c,d]	154.25	1.65	S-A
Citronellal [c]	154.25	0.215	FA [f]
Citronellol [c]	156.27	0.0183	FA
Citronellyl acetate [c]	198.30	0.0137	TCI [g]
α-Copaene [c]	204.35	–	S-A
Cuminaldehyde [c,d]	148.20	0.0482	FA
Cuminyl alcohol [c]	150.22	0.0124	TCI
p-Cymene [c]	134.22	1.65	S-A
Geraniol [d]	154.25	0.013	S-A
Geranyl acetate [c]	196.29	0.0256	S-A
α-Humulene [d]	204.35	–	TCI
Limonene [c,d]	136.23	1.541	S-A
Linalool [c,d]	154.25	0.091	S-A
Linalool oxide [c,d]	170.25	0.0205	TCI
Methyl cinnamate [d]	162.19	–	FA
β-Myrcene [c,d]	136.23	2.29	S-A
(–)-(*Z*)-Myrtanol [b]	154.25	0.0249	S-A

(–)-Myrtenal[b]	150.22	0.145	S-A
(–)-Myrtenol[b]	152.23	0.0179	SC[h]
Neral[b]	152.23	0.0712	KI[i]
Nerol[d]	154.25	0.0133	TCI
α-Phellandrene[d]	136.23	1.86	TCI
α-Pinenec[c,d]	136.23	3.489	S-A
β-Pinene[c,d]	136.23	2.4	S-A
Piperitone[c,d]	152.23	0.0572	TCI
Terpinen-4-ol[d]	154.25	0.048	FA
α-Terpinene[d]	136.23	1.638	S-A
γ-Terpinene[d]	136.23	1.08	S-A
α-Terpineol[c,d]	154.25	0.0283	S-A
Thymol[b]	150.22	0.0376	S-A
(*S*)-(*Z*)-Verbenol[b]	152.23	0.0332	S-A
(1*S*)-(–)-Verbenone[b]	150.22	0.0773	FA

[a] Vapor pressure at 25°C.

[b] Compounds used in this study for structure–activity relationship.

[c] Constituents of *Z. piperitum* pericarp steam distillate reported by Hieu *et al*. (2010b).

[d] Constituents of *Z. armatum* seed oil reported by Tiwary *et al*. (2007).

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

[f] Purchased from Fluka (Buchs, Switzerland).

[g] Purchased from Tokyo Chemical Industry (Tokyo, Japan).

[h] Purchased from SAFC Supply Solutions (St. Louis, MO, USA).

[i] Obtained from the Korea Forest Research Institute (Seoul, Republic of Korea).

Treated and control (ethanol only) females were held under the same conditions as those used for colony maintenance. At 24 h post-treatment, a female was considered dead if its body and appendages did not move when prodded with a fine wooden dowel. If recovery from the treatment was observed, the recovered fly was counted as alive. Because not all bioassays could be conducted at the same time, treatments were blocked over time with a separate control treatment included in each block. Freshly prepared compound solutions were used for each block of bioassays (Robertson and Preisler, 1992). All treatments were replicated three times using 50 females per replicate.

3.4. Topical application method

Four to six concentrations of the test materials in 0.5 µl of acetone were topically applied to the dorsal thoracic dorsa of CO_2-anesthetized females (3–5 days old). Control flies received 0.5 µl of acetone. Treated and control females were held under the same conditions as those used for colony maintenance. Mortalities were recorded as stated above. Each assay was replicate three times using 30 females per replicate.

3.5. Acetylcholinesterase activity assay

3.5.1. AChE preparation from stable fly heads

Adult females (3–5 days old) frozen at –80°C were decapitated by shaking and 1,400 heads (1.85 g) were homogenized in 15 ml of ice-cold 0.1 M sodium phosphate buffer (pH 8.0) using a Teflon glass tissue homogenizer. The homogenate was centrifuged at 10,000 × *g* at 4°C for 20 min. The supernatant was filtered by a 0.22 µm-Millex-GV filter (Millipore, Cork, Ireland) and was used as the AChE preparation. Protein concentrations

were determined using the Bradford protein assay kit. Bovine serum albumin (BSA) was used as a protein standard.

3.5.2. Hydrolytic preparations of acetylthiocholine iodide

The hydrolysis of ATChI was carried out following the method of Parvari *et al*. (1983). TChI was obtained from ATChI by complete enzymatic hydrolysis of the AChE preparation. Amount of 50 μg enzyme preparation in 5 ml of 0.1 M sodium phosphate buffer (pH 8.0) containing 0.1 M KCl was incubated with 0.01 M of ATChI at 30°C for 3 h on a platform SH30 orbital shaker (FINEPCR, Gyennggi-do, Korea) at 30 rpm. The complete reaction was verified by measuring the concentration of the TChI produced using the Ellman technique (Ellman *et al*., 1961). Following the hydrolysis, the TChI product was diluted into phosphate buffer (pH 8.0) containing 0.1 M KCl, aliquoted, and stored at –80°C until used. Aliquots of the TChI product were thawed only once and used immediately for calibration curve measurements. Based on the standard curve of the TChI product and the OD values of the AChE activity after 15 min, units of the enzyme activity per mg protein per min at pH 8.0 and 30°C were determined.

3.5.3. Kinetics of AChE preparation

Kinetics of AChE was implemented to determine an enzyme unit per well for the AChE inhibition assay following the method of Ellman *et al*. (1961). Serial mixtures consisting of 180 μl of the AChE preparation at five different concentrations (1.4, 0.7, 0.37, 0.175, and 0.0875 μg) and 10 μl of 7.5 mM DTNB in phosphate buffer (pH 7.0) was added to each well of 96-well plate. The enzymatic reaction was initiated with the addition of 10 μl of 6.25 mM ATChI to each well. The reaction mixtures were

immediately incubated at 30°C with gentle shaking for 5 seconds before the values of the enzymatic reactions were read at 412 nm every min for a period of 35 min using a VersaMax microplate reader (Molecular Devices, Sunnyvale, CA, USA).

3.5.4. AChE inhibition assay

A microplate AChE assay was carried out following the method of Moores *et al*. (1988) adapted from Ellman *et al*. (1961). Based on the kinetics of the enzymatic reaction (Fig. 14), the enzyme preparation (0.7 μg protein per well) were used for the inhibition assay. The reaction mixture consisted of 80 μl of the enzyme preparation (0.7 μg protein), 10 μl of 7.5 mM DTNB in phosphate buffer (pH 7.0), and 100 μl of various concentrations of the test compounds in 2.5% acetone. The reaction mixture was incubated at 30°C for 5 min with gentle shaking at 30 rpm in a platform SH30 orbital shaker (FINEPCR, Gyennggi-do, Korea) and 10 μl of 6.25 mM ATChI was then added to the mixture. Chlorpyrifos and dichlorvos served as positive controls and were similarly prepared. Control wells contained without test materials. Blank wells contained no enzyme, DNTB and test materials, whereas background wells contained test materials and DNTB with no enzyme added. The absorbance was recorded at 412 nm using a VersaMax microplate reader. All bioassays were repeated three times in triplicates.

3.6. Effect of monoterpenoids on cyclic AMP levels in the fly head homogenate

Adult females (3–5 days old) frozen at –80°C were decapitated by shaking. Fourteen heads (~52.45 mg) in each 1.5 ml-tip were homogenized in 500 μl of 2 mM Tris-maleate buffer (pH 7.4) containing 0.2 mM EGTA. The head homogenate was used for cAMP *in vitro* assay. Adenylate cyclase activity was measured according to the method of Stefano

et al. (1981). The assay was conducted in a total volume of 100 µl containing 80 mM tris-maleate buffer (pH 7.4), 5 mM theophylline (to inhibit phosphodiesterase activity), 2 mM $MgSO_4$, 0.5 mM ATP, 0.2 mM EGTA, 30 µl of fly head homogenate (1.5 µg protein), and the test compounds (in 2mM 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 2.5 min and then the assay tube was cooled and centrifuged for 10 min at 8,000 × *g*. Then, 100 µl aliquots of the supernatant were assayed in triplicate for level of cAMP.

Levels of cAMP in each test head homogenates was measured using the cAMP immunoassay (R&D systems Inc., Minneapolis, MN, USA) following the manufacturer's instruction. This assay is based on the competitive binding technique. A monoclonal antibody specific for cAMP binds to the goat anti-mouse antibody coated onto the microplate. Following a wash to remove excess monoclonal antibody, cAMP present in a sample competes with a fixed amount of horseradish peroxidase (HRP)-labeled cAMP for sites on the monoclonal antibody. This is followed by another wash to remove excess conjugate and unbound sample. A substrate solution is added to the wells to determine the bound enzyme activity. The color development is stopped and the absorbance is read at 450 nm.

Fifty microliters of primary antibody solution was added to each well except the blank wells (or the non-specific binding (NSB) wells). These wells covered with the adhesive strip were incubated for 1 h at 25°C in a shaking incubator (~150 rpm) and followed by four times washing steps, each with 400 µl of wash buffer. The 100 µl of the

test samples for cAMP determination and cAMP standard were added to appropriate wells. Control, blank (NBS), and zero standard wells were added with 100 µl of the diluent RD5-55 buffer. Fifty microliters of cAMP conjugate (HRP-labeled cAMP) was then added to all wells. The plate covered with a new adhesive strip was incubated for 2 h at 25°C on the shaker and followed by the four times washing steps again. Then, 200 µl of substrate solution was added to each well and the test plate was incubated for 30 min at 25°C on the benchtop and protected from light. Finally, 100 µl of 2N sulfuric acid was added to stop the enzyme reaction. Optical densities (ODs) at 450 and 540 nm were measured using a Molecular Devices VersaMax microplate reader. The readings at 540 nm were subtracted from the readings at 450 nm. All assays were done in duplicate. Based on the standard curve and B/B_o values (see the section below) of test samples, cAMP levels were calculated and presented in mean ± SE of at least three separate experiments.

Seven two-fold serial dilutions of standard cAMP (240, 120, 60, 30, 15, 7.50, and 3.75 pmol/ml) were made in calibrator diluent RD5-55 buffer and the buffer only serves as the zero standard (B_o) (0 pmol/ml). A standard curve was constructed by plotting the mean OD values on a linear y-axis against the cAMP concentration on a logarithmic x-axis. The % B/B_o was calculated according to the formula:

$$\%B/B_o = [(OD_{sample} - OD_{NSB})/(OD_{zero\ standard} - OD_{NSB})] \times 100$$

where B = OD of sample at 450 nm and B_0 = OD of cAMP standard at 0 pmol/ml. The cAMP (nmol) in unknown samples is calculated from the standard curve and the size of the aliquot measured. The cAMP concentrations are expressed as nmol/µg protein.

3.7. Data analysis

The Bonferroni multiple-comparison method was used to test for significant differences among the treatments using SAS 9.1 software program. Student's *t* test was used to test for significant differences between two treatments. Means ± standard error (SE) of untransformed data are reported. Concentration– or dose–mortality data were subjected to probit analysis using SAS 9.1 software program. The LC_{50} or LD_{50} values of the treatments were considered to be significantly different from one another when their 95% confidence intervals failed to overlap. The IC_{50} (the concentration required to inhibit the AChE activity by 50%) value for each test compound was determined using GraphPad Software GraphPad Prism 5 (San Diego, CA, USA). Multiple regression and correlation analyses of the toxicities of compounds to female stable flies were determined using their LC_{50} values and physical parameter (MW and VP) values for the test MTs.

Results

3.1. Fumigant toxicity

The fumigant toxicity of ZP-SD, ZA-SO, 36 compounds, and two OPs to female stable flies was evaluated using a vapor-phase mortality bioassay (Table 16). Based on 24 h LC_{50} values, cuminaldehyde (0.075 μg/cm^3) was the most toxic compound, followed by thymol, (1*S*)-(–)-verbenone, (–)-myrtenal, and carvacrol (0.104–0.124 μg/cm^3). Strong fumigant toxicity was also obtained from (*S*)-(*Z*)-verbenol, ZP-SD, cuminyl alcohol, ZA-SO, piperitone, (–)-(*Z*)-myrtanol, and citronellal (LC_{50}, 0.242–0.456 μg/cm^3). However, these compounds were five orders of magnitude less toxic than either chlorpyrifos or dichlorvos. Moderate toxicity was obtained from neral, (–)-myrtenol, terpinen-4-ol, *p*-cymene, α-terpineol, γ-terpinene, linalool, methyl cinnamate, and citronellyl acetate (LC_{50}, 0.529–1.003 μg/cm^3). Low (LC_{50}, 1.267–3.842 μg/cm^3) or no toxicity (LC_{50}, > 5 μg/cm^3) was observed with the other 15 and two compounds, respectively. There was no mortality in the ethanol-treated controls.

Table 16. Fumigant toxicity of *Z. piperitum* pericarp steam distillate (ZP-SD), *Z. armatum* seed oil (ZA-SO), 36 compounds, and two insecticides to female stable flies during a 24 h exposure

Material	n^a	LC_{50} (95% CI[b]), μg/cm^3	Slope ± SE	χ^{2c}	*P*-value
ZP-SD	750	0.264 (0.248 – 0.278)	5.7 ± 0.50	7.64	0.66
ZA-SO	600	0.347 (0.326 – 0.368)	4.5 ± 0.34	8.71	0.56
Cuminaldehyde	900	0.075 (0.071 – 0.078)	6.0 ± 0.49	7.37	0.69
Thymol	600	0.104 (0.099 – 0.109)	6.8 ± 0.43	8.45	0.58
(1*S*)-(–)-Verbenone	750	0.116 (0.111 – 0.121)	6.6 ± 0.44	6.38	0.78
(–)-Myrtenal	600	0.120 (0.115 – 0.125)	6.9 ± 0.57	9.82	0.45
Carvacrol	600	0.124 (0.116 – 0.130)	6.2 ± 0.54	7.63	0.66
(*S*)-(*Z*)-Verbenol	600	0.242 (0.226 – 0.257)	5.4 ± 0.35	8.40	0.59
Cuminyl alcohol	600	0.324 (0.303 – 0.346)	4.7 ± 0.31	11.24	0.34
Piperitone	750	0.399 (0.376 – 0.422)	5.4 ± 0.36	8.79	0.55
(–)-(*Z*)-Myrtanol	600	0.443 (0.412 – 0.475)	3.7 ± 0.28	8.18	0.61
Citronellal	600	0.456 (0.425 – 0.489)	4.5 ± 0.29	12.17	0.27
Neral	750	0.529 (0.489 – 0.572)	3.6 ± 0.24	10.65	0.39
(–)-Myrtenol	600	0.577 (0.525 – 0.626)	3.2 ± 0.30	6.96	0.73
Terpinen-4-ol	600	0.647 (0.598 – 0.699)	3.6 ± 0.25	6.29	0.79
p-Cymene	750	0.734 (0.688 – 0.776)	5.3 ± 0.45	11.65	0.31
α-Terpineol	750	0.760 (0.701 – 0.829)	3.4 ± 0.25	7.04	0.72
γ-Terpinene	600	0.860 (0.814 – 0.906)	5.9 ± 0.40	9.46	0.49
Linalool	600	0.882 (0.807 – 0.974)	3.2 ± 0.26	10.15	0.43
Methyl cinnamate	750	0.945 (0.898 – 0.991)	6.8 ± 0.50	10.74	0.38

Citronellyl acetate	600	1.003 (0.937 – 1.074)	3.9 ± 0.30	8.88	0.54
Citronellol	600	1.267 (1.194 – 1.352)	4.7 ± 0.40	8.33	0.60
Geranyl acetate	750	1.421 (1.342 – 1.506)	4.4 ± 0.40	8.22	0.61
Linalool oxide	600	1.570 (1.477 – 1.667)	5.3 ± 0.40	9.65	0.47
α-Terpinene	600	1.657 (1.526 – 1.811)	3.4 ± 0.27	6.61	0.76
1,8-Cineole	600	1.705 (1.587 – 1.836)	4.5 ± 0.31	6.26	0.79
α-Phellandrene	750	1.837 (1.739 – 1.937)	4.7 ± 0.43	9.09	0.52
Geraniol	600	1.973 (1.847 – 2.107)	4.0 ± 0.32	11.10	0.35
β-Caryophyllene oxide	600	2.009 (1.895 – 2.127)	4.9 ± 0.36	9.00	0.54
Limonene	750	2.014 (1.875 – 2.125)	4.9 ± 0.61	5.34	0.87
Nerol	600	2.025 (1.874 – 2.155)	4.1 ± 0.47	9.05	0.53
Carveol	600	2.179 (2.008 – 2.370)	3.1 ± 0.27	12.61	0.25
β-Caryophyllene	600	2.243 (2.105 – 2.367)	4.7 ± 0.49	10.85	0.37
α-Copaene	600	2.631 (2.409 – 2.836)	3.2 ± 0.38	8.04	0.63
β-Myrcene	600	3.033 (2.850 – 3.255)	3.9 ± 0.42	9.83	0.46
α-Humulene	600	3.842 (3.525 – 4.299)	3.0 ± 0.37	6.95	0.73
α-Pinene	450	> 5			
β-Pinene	450	> 5			
Dichlorvos	750	2.8×10^{-6} ($2.6 – 3.0 \times 10^{-6}$)	3.2 ± 0.20	8.91	0.78
Chlopyrifos	900	5.0×10^{-6} ($4.5 – 5.5 \times 10^{-6}$)	2.6 ± 0.15	12.76	0.47

[a] The number of female stable flies used.

[b] CI denotes confidence interval.

[c] Pearson χ^2, goodness-of-fit test.

3.2. Topical toxicity

The contact toxicity of ZP-SD, ZA-SO, and all compounds to female stable flies was examined using topical application (Table 17). As judged by 24 h LD_{50} values, thymol (8.025 μg/female) and carvacrol (8.375 μg/female) were the most toxic compounds, followed by cuminyl alcohol, ZP-SD, and cuminaldehyde (10.288–14.557 μg/female). These compounds were three orders of magnitude less toxic than either chlorpyrifos or dichlorvos. Moderate toxicity was produced by citronellol, (1*S*)-(–)-verbenone, neral, citronellal, and ZA-SO (LD_{50}, 20.107–25.599 μg/female). Low (LD_{50}, 29.136–43.685 μg/female) or no toxicity (LD_{50}, > 50 μg/female) was observed with the other 13 and 15 compounds, respectively.

Table 17. Topical toxicity of *Z. piperitum* pericarp steam distillate (ZP-SD), *Z. armatum* seed oil (ZA-SO), 21 compounds, and two insecticides to female stable flies during a 24 h exposure

Material	n^a	LD_{50} (95% CI[b]), μg/♀[c]	Slope ± SE	χ^{2d}	*P*-value
ZP-SD	540	11.058 (10.162 – 11.915)	3.7 ± 0.49	13.55	0.41
ZA-SO	450	26.981 (22.820 – 36.215)	2.2 ± 0.38	6.55	0.92
Thymol	450	8.025 (7.487 – 8.625)	4.7 ± 0.38	12.10	0.52
Carvacrol	450	8.375 (7.782 – 9.049)	4.4 ± 0.36	15.07	0.30
Cuminyl alcohol	360	10.288 (9.150 – 11.474)	3.0 ± 0.33	8.94	0.54
Cuminaldehyde	450	14.557 (12.594 – 16.324)	2.7 ± 0.46	6.77	0.75
Citronellol	360	20.107 (17.807 – 22.487)	2.9 ± 0.33	10.17	0.43
(1*S*)-(–)-Verbenone	360	20.722 (18.883 – 22.500)	3.8 ± 0.62	3.86	0.95
Neral	540	21.659 (19.305 – 24.328)	2.5 ± 0.19	20.62	0.19
Citronellal	360	25.599 (22.115 – 29.070)	2.6 ± 0.29	9.25	0.51
(*S*)-(*Z*)-Verbenol	450	29.136 (25.751 – 32.875)	2.6 ± 0.23	10.06	0.69
Nerol	360	29.370 (25.724 – 33.178)	2.7 ± 0.28	9.29	0.50
Piperitone	450	30.216 (26.579 – 33.973)	2.8 ± 0.22	11.85	0.54
Terpinen-4-ol	360	31.154 (27.861 – 34.732)	3.1 ± 0.34	12.17	0.27
Geraniol	450	32.407 (28.423 – 36.487)	2.5 ± 0.23	10.08	0.69
α-Terpineol	450	32.524 (28.028 – 37.156)	2.2 ± 0.21	12.080	0.52

Linalool oxide	450	35.412 (30.262 – 41.049)	2.0 ± 0.19	17.41	0.18
Limonene	450	37.581 (33.449 – 41.903)	2.9 ± 0.24	12.37	0.50
Linalool	450	38.105 (33.526 – 42.483)	2.8 ± 0.28	16.57	0.22
1,8-Cineole	360	39.631 (33.493 – 45.269)	2.5 ± 0.32	13.08	0.22
Methyl cinnamate	360	40.782 (36.761 – 45.482)	3.1 ± 0.36	13.85	0.18
Carveol	450	42.370 (35.938 – 48.379)	2.4 ± 0.32	6.82	0.74
p-Cymene	450	43.685 (36.873 – 49.932)	2.3 ± 0.27	14.29	0.35
Dichlorvos	540	0.0026 (0.0023 – 0.0029)	2.9 ± 0.29	15.33	0.29
Chlopyrifos	450	0.0106 (0.0088 – 0.0123)	2.3 ± 0.25	7.14	0.71

[a] The number of female stable flies used.

[b] CI denotes confidence interval.

[c] µg/female

[d] Pearson χ^2, goodness-of-fit test.

3.3. Structure–activity relationship

Comparisons were made to determine fumigant toxicity differences involving the skeletal structure, configuration, saturation, and functional groups of the 31 MTs (Table 18 and Fig. 12). The acyclic terpenoids geraniol and nerol (geometric isomers) did not differ significantly in toxicity against female stable flies. Geraniol and nerol (primary alcohols) and linalool (a tertiary alcohol) exhibited significant differences in their toxicity. The aldehyde citronellal was more toxic than the less saturated geraniol, nerol, and linalool. Citronellal and neral exhibited greater activity than the corresponding alcohols citronellol and nerol. The oxygenated bicylic terpenoid myrtanol exhibited significantly higher toxicity than myrtenol, which is less saturated than myrtanol. Myrtenal was more toxic than myrtanol. Verbenol was significantly less toxic than the corresponding ketone verbenone. In the case of *p*-menthane terpenoids, terpinen-4-ol and α-terpineol (tertiary alcohols), whose only structural difference is the position of the alcohol functional group, showed significant changes in activity patterns: the former is significantly more toxic than the latter against female flies. The terpene phenol thymol was more toxic than alcohols such as geraniol and nerol. Cuminaldehyde was more active than its corresponding alcohol cuminyl alcohol.

Multiple linear regression analysis showed significant relationship between the fumigant toxicity (LC_{50} values) of the compounds to female stable flies and the physical parameters (MW and VP values) for the 29 MTs (except for ineffective α- and β-pinene; Table 16) ($R^2 = 0.39$). Correlation analysis showed that VP is positively correlated with LC_{50} ($r = 0.552$), while MW is not clearly correlated with LC_{50} ($r = -0.066$).

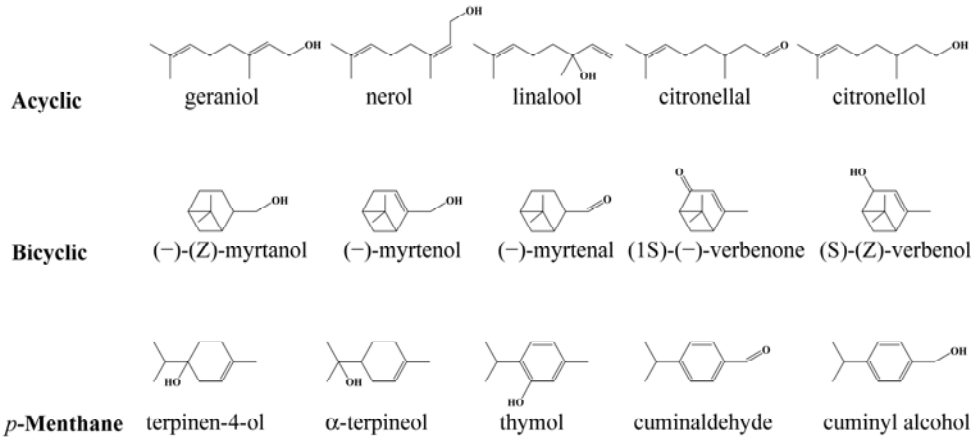

Fig. 12. Structures of the acyclic, bicyclic, and *p*-menthane terpenoids discussed for their structure-activity relationship to stable flies.

Table 18. Structure–activity relationship of 31 monoterpenoids in the stable fly

	Fumigant toxicity category (LC_{50}, μg/cm^3)							
MT	I (< 0.2)	II (0.2–0.5)	III (0.6–1.0)	IV (1.1–1.5)	V (1.6–2.0)	VI (2.1–2.5)	VII (2.6–5.0)	VIII (> 5.0)
Acyclic		Citronellal	Neral	Citronellol	Geranyl acetate	Nerol	*β*-Myrcene	
			Linalool		Linalool oxide			
			Citronellyl acetate		Geraniol			
Bicyclic	(1*S*)-(–)-Verbenone	(*S*)-(*Z*)-Verbenol	(–)-Myrtenol					*α*-Pinene
	(–)-Myrtenal	(–)-(*Z*)-Myrtanol						*β*-Pinene
p-Menthane	Cuminaldehyde	Cuminyl alcohol	Terpinen-4-ol		*α*-Terpinene	Limonene		
	Thymol	Piperitone	*p*-Cymene		1,8-Cineole	Carveol		
	Carvacrol		*α*-Terpineol		*α*-Phellandrene			
			γ-Terpinene					

3.4. Acetylcholinesterase activity

The ATChI hydrolysis by the AChE preparation was examined (Fig. 13). The enzyme unit was determined based on the standard curve of TChI produced by the complete hydrolysis and was expressed as μmoles of substrate hydrolyzed per min. Therefore, one unit of the enzyme is defined as the amount of the enzyme that catalyzes the production of 1 μmole of TChI per min at pH 8.0 and 30°C. In the present study, an amount (1 mg) of the AChE preparation was determined to be equivalent to 4.3 units.

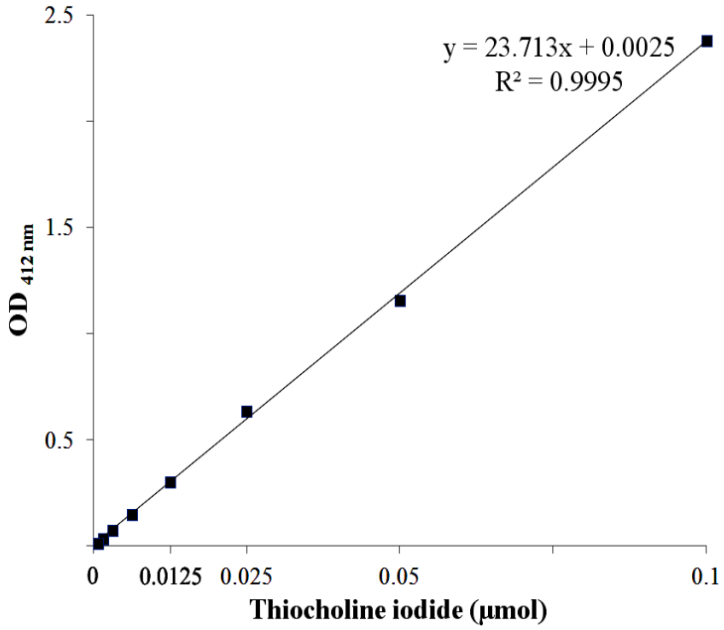

Fig. 13. Calibration curve of thiocholine iodide

The kinetics of the AChE preparation is shown in Fig. 14. The AChE-catalyzed hydrolysis of ATChI (6.25 mM) in the presence of DTNB (7.5 mM) increased as the higher concentrations of the AChE preparation (0.0875, 0.175, 0.37, 0.7, and 1.4 μg/well)

were used. These concentrations of the AChE preparation are equivalent to 0.000375, 0.00075, 0.0015, 0.003, and 0.006 units per well, respectively. The OD values at 412 nm of the AChE activity at 0.000375, 0.00075, and 0.0015 units were significantly low during 10–30 min of the enzymatic reaction at pH 8.0 and 30°C. The enzyme activity at 0.003 and 0.006 units per well showed remarkably increase in OD values during the period of 10–20 min. However, the OD values of the enzymatic reaction almost unchanged at 0.006 units per well during 20–30 min. Therefore, the AChE preparation (0.7 µg, ~0.003 units per well), which gave OD value 0.9–1.2 at 15 min of the AChE reaction, was used for the AChE inhibitory assay.

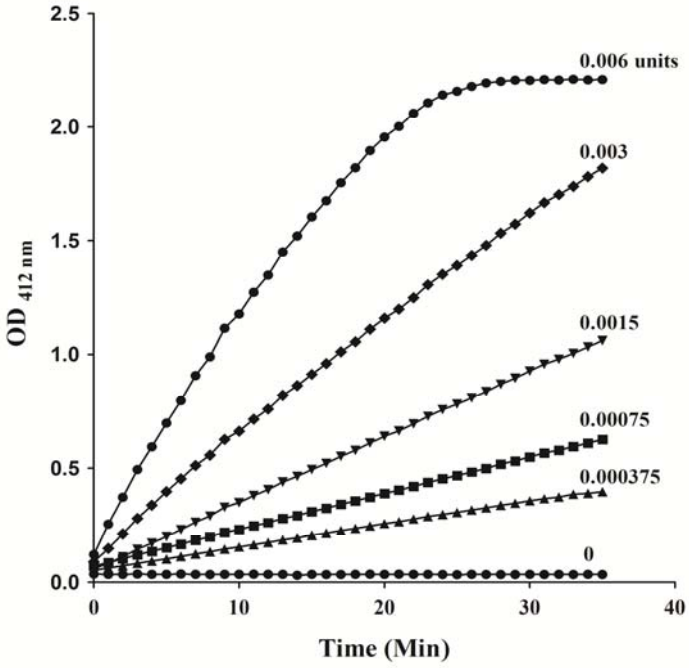

Fig. 14. Kinetics of the AChE reaction 0–0.006 units per well, each 200 µl

3.5. Acetylcholinesterase inhibition

The AChE inhibitory activity of all compounds is recorded in Table 19. Based on IC_{50} values, strong AChE inhibition was produced by citronellyl acetate, α-pinene, thymol, carvacrol, and α-terpineol (1.20–2.73 mM). These compounds were significantly less inhibitory than either chlorpyrifos or dichlorvos. Moderate AChE inhibition was obtained from cuminaldehyde, α-copaene, (1*S*)-(–)-verbenone, β-pinene, and α-terpinene (IC_{50}, 4.32–8.60 mM). Low (IC_{50}, 11.06–35.64 mM) and no inhibition (IC_{50}, > 50 mM) were observed with the other seven and twenty compounds, respectively.

Table 19. *In vitro* inhibition of the AChE extracted from stable fly heads by 22 compounds along with fumigant toxicity category

Compound	IC_{50}, mM	Fumigant toxicity category[a]
Citronellyl acetate	1.20	III
α-Pinene	1.38	VIII
Thymol	1.48	I
Carvacrol	1.85	I
α-Terpineol	2.73	III
Cuminaldehyde	4.32	I
α-Copaene	4.58	VII
(1*S*)-(–)-Verbenone	5.95	I
β-Pinene	8.07	VIII
α-Terpinene	8.60	V
Terpinen-4-ol	11.06	III
Linalool	11.70	III
(–)-Myrtenal	13.27	I
Piperitone	15.85	II
(–)-(*Z*)-Myrtanol	18.03	II
Neral	23.67	III
Citronellal	> 50	II
(*S*)-(*Z*)-Verbenol	> 50	II

Cuninyl alcohol	> 50	II
(–)-Myrtenol	> 50	III
p-Cymene	> 50	III
γ-Terpinene	> 50	III
Chlorpyrifos	1.61×10^{-6}	
Dichlorvos	1.33×10^{-6}	

[a] Fumigant toxicity category was given in Table 18

3.6. *In vitro* effect of monoterpenoids 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. 15).

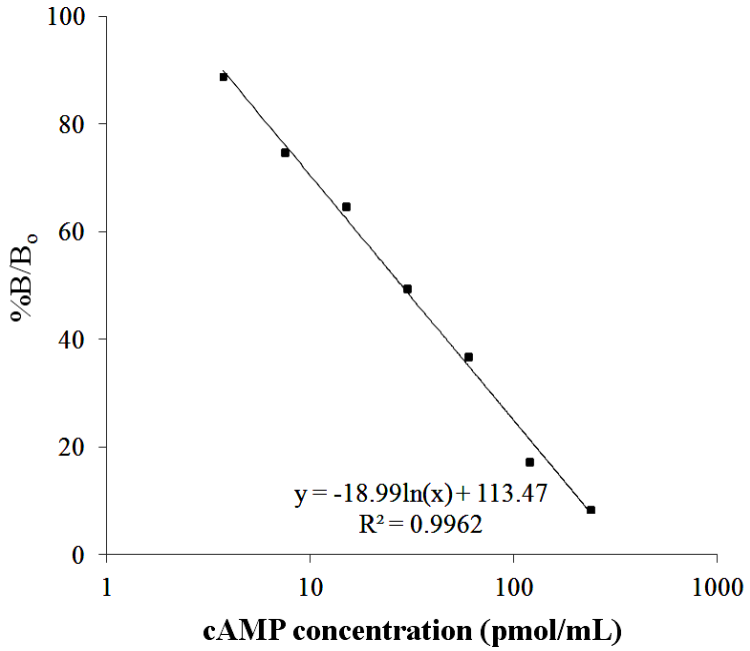

Fig. 15. cAMP standard curve. Assays were performed with the cAMP standard as described in Section 3.6.

The effects of octopamine or dopamine on the cAMP production in the stable fly head brain preparation were investigated (Fig. 16). There was no significant difference between the cAMP production in the stable fly nervous system tested with octopamine and dopamine. Dose dependences in cAMP levels were observed as octopamine and dopamine concentrations increased. cAMP levels slightly increased 0.333–0.409 or 0.306–0.357 nmol/ml in the presences of octopamine or dopamine at 0.1–1 nmol/ml, respectively. However, significant increases in cAMP levels (0.627–1.004 or 0.676–0.880 nmol/ml) were found in the presences of octopamine or dopamine at 10–100 nmol/ml, respectively. There was no remarkable increase in the cAMP production (1.026 or 0.905 nmol/ml) in the presence of 250 nmol/ml octopamine or dopamine, respectively.

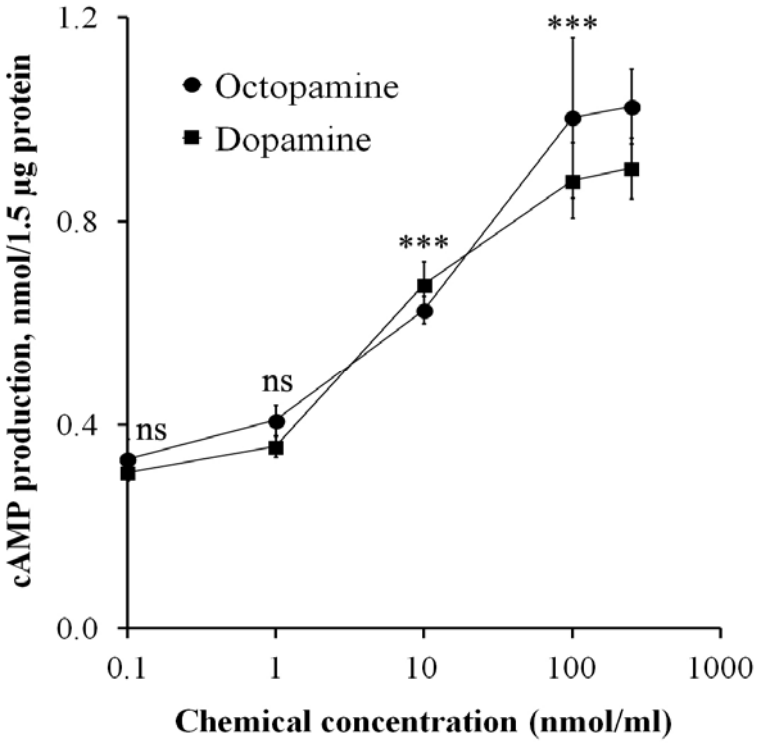

Fig. 16. Effects of octopamine and dopamine at 0.1, 1, 10, 100, and 250 nmol/ml on cAMP levels in the nervous system of the stable fly. '***' indicates significantly different at $P < 0.001$, compared to cAMP levels at both 0.1 and 1 nmol/ml of the test chemicals, which are no significantly different "ns" at $P > 0.05$. However, cAMP levels at 10 and 100 nmol/ml of the test chemicals are significantly different "*" at $P < 0.05$, *t* test (not shown). And there is no significant difference "ns" at $P > 0.05$, *t* test (not shown) in cAMP levels at 100 and 250 nmol/ml.

Because of the remarkable increase in cAMP production (0.985 nmol/ml) in the presence of 100 nmol/ml optopamine, compared to the cAMP (0.363 nmol/ml) of the control (0 nmol/ml octopamine), the effects of the test MTs at 10 and 100 nmol/ml on cAMP levels in the presence of 100 nmol/ml octopamine were examined (Fig. 17). Significant decreases in cAMP levels were observed in the presences of 100 nmol/ml MTs (citronellal, thymol, cuminyl alcohol, (1*S*)-(–)-verbenone, (–)-myrtenal, or cuminaldehyde) and 100 nmol/ml octopamine, compared to that of 100 nmol/ml octopamine alone. At 100 nmol/ml, citronellal and thymol were the strongest inhibitory compounds that significantly decreased cAMP concentrations (0.072 and 0.252 nmol/ml, respectively), followed by cuminyl alcohol, (1*S*)-(–)-verbenone, (–)-myrtenal, and cuminaldehyde (0.340–0.492 nmol/ml). At 10 nmol/ml, citronellal and thymol also induced significantly decreases in cAMP concentrations (0.167 and 0.357 nmol/ml, respectively), followed by cuminyl alcohol (0.490 nmol/ml) and (1*S*)-(–)-verbenone (0.540 nmol/ml). However, there were no significant changes in the cAMP concentrations at the treatments with 10 nmol/ml cuminaldehyde or (–)-myrtenal.

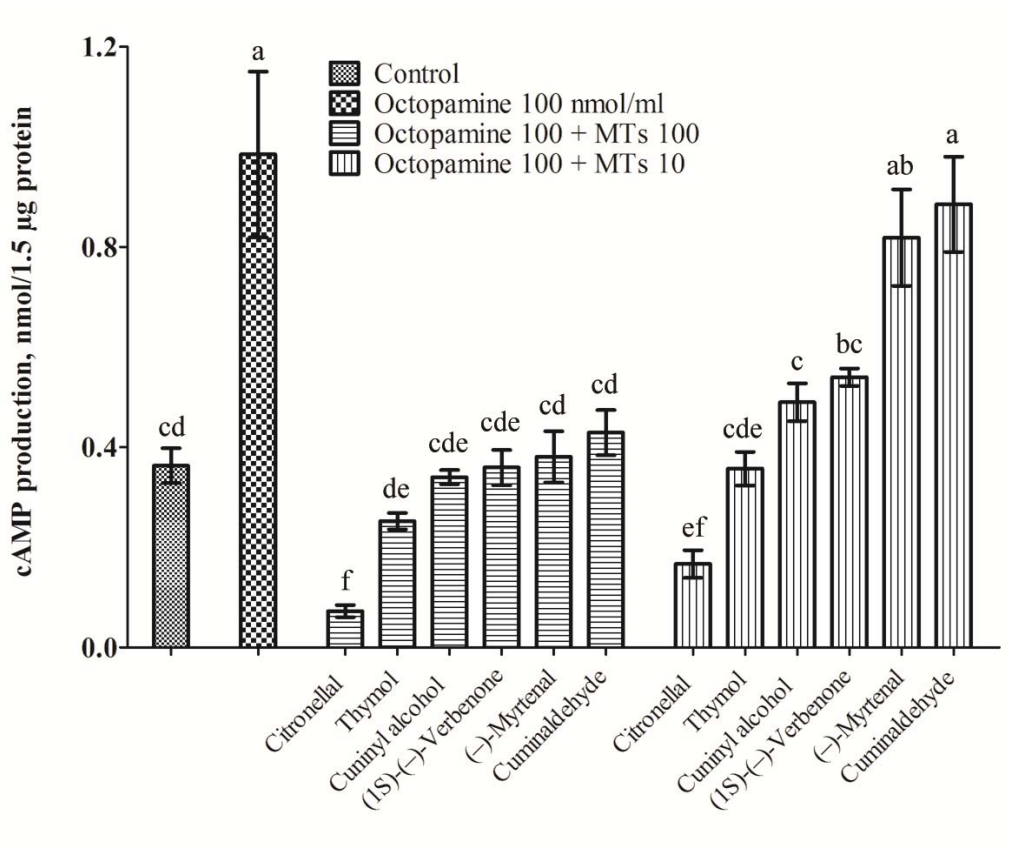

Fig. 17. Effects of 10 and 100 nmol/ml monoterpenoids (MTs) on cAMP levels in the presence of 100 nmol/ml octopamine using the stable fly brain preparation. Data of cAMP concentration in test samples were calculated as nmol per 1.5 µg protein based on the standard curve of cAMP production described above in Fig. 15. Column (mean ± SE) followed by the same letter(s) are not significantly different (P = 0.05, Bonferroni multiple-comparison test).

Discussion

It is well acknowledged that certain plant EOs can be developed into products suitable for integrated arthropod pest management because they can be selective, biodegrade to nontoxic products, and have few harmful effects on nontarget organisms and the environment (Ahn *et al*., 2006; Isman, 2006). They also can be used in conjunction with biological control (Isman, 2006). Certain plant EOs and their constituents manifest toxicity against multiple insect species of medical and veterinary importance, including stable fly (Mann *et al*., 2010; Kaufman *et al*., 2011), and have been proposed as possible alternatives to conventional synthetic insecticides. Elucidation of the insecticide modes of delivery provides important practical information for stable fly control, such as the most appropriate formulations and delivery means to be adopted for their future commercialization (Ahn *et al*., 2006). Volatile compounds of many plant EOs consist of alcohols, aldehydes, alkanes, and terpenoids, particularly monoterpenoids (MTs) (Lawless, 2002). They primarily act as fumigants with additional contact action. For example, the fumigant + contact toxicity to the German cockroach has been reported with linalool, α-terpineol, α-thujone, thymol, and verbenone (Jang *et al*., 2005); and borneol, camphor, citronellal, cumene, α-pinene, β-pinene, and terpinolene (Jung *et al*., 2007).

In the present study, potent fumigant toxicity was obtained from ZP-SD, ZA-SO, carvacrol, citronellal, cuminaldehyde, cuminyl alcohol, (–)-myrtenal, (–)-(*Z*)-myrtanol, piperitone, thymol, (*S*)-(*Z*)-verbenol, and (1*S*)-(–)-verbenone. This present finding indicates that their mode of delivery was, in part, a result of vapor action, although these compounds (except for (–)-myrtenal and (–)-(*Z*)-myrtanol) also exhibited contact toxicity.

The treated flies showed knockdown and paralysis 3 to 12 h after treatment. However, there was no mortality of recovered flies 24 h post-treatment. In addition, ZP-SD was significantly more toxic than ZA-SO. This might be attributable to differences in chemical composition between ZP-SD and ZA-SO, given the present data on toxicity of individual constituents to stable flies, or synergistic effects of the oil constituents. It has been demonstrated that (*E*)-anethole acts synergistically with thymol, citronellal, and *α*-terpineol against *Spodoptera litura* (Fab.) larvae (Hummelbrunner and Isman, 2001). Unlike the head louse (Yang *et al*., 2004a) and German cockroach (Jang *et al*., 2005), neither fumigant nor contact toxicity was observed with *α*- and *β*-pinene. The difference between the present and previous studies might be attributable to differences in one or more physiological or biochemical characteristics between stable fly and other insects: penetration, detoxifying enzyme activity, and the relative sensitivity to the toxicant at the target site (Terriere, 1984; Graham-Bryce, 1987). The fumigant action of ZP-SD- and ZA-SO-derived materials is of practical importance because volatile compounds can easily reach deep harborages in confinement systems such as dairies and feedlots resulting in good control (Yun *et al*., 2012). This system has advantages because exposure to volatile compounds can be easily controlled in confinement system using an appropriate automatic control facility. This original finding indicates that ZP-SD- and ZA-SO-derived preparations may hold promise for the development of novel and effective bioinsecticides against stable fly populations.

Structure–activity relationships of phytochemicals in many insect pests have been well noted. For example, Tsao *et al*. (1995) reported that enhanced potency of selected monoterpenes and phenols could be achieved through derivatization of the hydroxyl

groups, which increased VP (leading to greater fumigant action) and/or increased lipophilicity (leading to better penetration of the insect cuticle and bioavailability in the insect's body). Jang *et al*. (2005) studied the structure–activity relationship of MTs and suggest that structural characteristics, such as degrees of saturation and types of functional groups rather than VP parameter, appear to play a role in determining the MT toxicities to German cockroaches. In the present study, cyclic MTs were generally more toxic than acyclic ones. The terpene aldehydes and phenol were more toxic than alcohols, indicating the importance of subtle structural changes. In addition, the more saturated acyclic and bicyclic terpenoids were more toxic than the less saturated corresponding terpenoids. Structure–activity relationships indicate that structural characteristics, such as carbon skeleton, degrees of saturation, and types of functional groups, appear to play a role in determining toxicities of the MTs to stable flies. VP parameter of the MTs was positively correlated to the observed toxicities.

Investigations on the modes of action of botanical biocides may contribute to the development of selective fly control alternatives with novel target sites. Plant EOs have been suggested to exert their bioactivity by interacting with various molecular targets including tyramine and octopamine receptors (Kostyukovsky *et al*., 2002; Enan, 2001 and 2005), ionotropic GABA receptors (Priestley *et al*., 2003; Tong and Coats, 2012), and AChE (Grundy and Still, 1985; Lee *et al*., 2001; Abdelgaleil *et al*., 2009). Certain terpenoids inhibit AChE in the house fly and Madagascar roach (Grundy and Still, 1985), head louse (Picollo *et al*., 2008), rice weevil (Lee *et al*., 2001; Abdelgaleil *et al*., 2009), and rust red flour beetle (Abdelgaleil *et al*., 2009). Ryan and Byrne (1988) reported a relationship between insecticidal toxicity 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; Lee *et al*., 2001; Picollo *et al*., 2008; Abdelgaleil *et al*., 2009). In the present study, no correlation was found between fumigant toxicity and inhibition of AChE activity. Some of the terpenoids, such as carvacrol, cuminaldehyde, α-terpineol, thymol, and (1*S*)-(–)-verbenone, possessed potent AChE inhibitory and insecticidal activities. The most surprising result is that citronellyl acetate, α-copaene, and α-pinene were potent inhibitors of AChE but exhibited low to no toxicity. The other compounds were toxic to stable fly (except for β-pinene) but exhibited low to no AChE inhibitory activity, indicating that AChE was not the major site of action for these compounds.

Although not yet proven, the octopaminergic and γ-aminobutyric acid receptors have been also suggested as novel target sites for some EO constituents by Kostyukovsky *et al*. (2002) and Priestley *et al*. (2003), respectively. At 10 pmol/ml octopamine and EO constituents have been reported to cause a significant increase in intracellular cAMP concentration in *Helicoverpa armigera* (Kostyukovsky *et al*., 2002). A significant increase in the cAMP production was demonstrated at 1 nmol/ml of eugenol and a 3-blend of eugenol, α-tepineol, and cinnamic alcohol, while a significant decrease was found at 10-fold concentration in American cockroach nervous system (Enan, 2001). In the present study, some of the terpenoids, such as cuminaldehyde, thymol, and (1*S*)-(–)-verbenone were also found to have inhibitory activity to adenylate cyclase systems in the stable fly brain. Other three potent insecticidal compounds such as citronellal, (–)-myrtenal, and cuminyl alcohol showed low or no AChE inhibition, but significantly reduced cAMP level stimulated by octopamine. These results indicate that the

constituents described probably exert their bioactive effects by binding to octopamine receptors, thereby acting as potential insect pest control agents as suggested by Kostyukovsky *et al*. (2002) and Blenau *et al*. (2012).

CONCLUSION

The bioactive EO-derived products, including binary mixtures of each EO and with tamanu oil, could be useful as repellents for protecting humans and domestic animals from bites and nuisance caused by stable fly, provided that a carrier giving a slow release of active compounds can be selected or developed and sealing of the structure of barns or stables is maximized. The stable fly appears to be highly sensitive to direct contact via the chemoreceptors in the tarsi (Yeoman and Warren, 1968). However, most EO-based repellents are highly volatile and mainly act in the vapour phase via insect olfactory chemoreceptors. They are, therefore, far less effective than DEET. Tamanu nut oil that contains two major free fatty acids, oleic acid and linoleic acid, and the methyl esters of these fatty acids can provide a better maintenance on treated skin due to their low volatilities. Thus, these fatty acids and methyl esters are useful as active carriers that give a low release of bioactive constituents leading to enhanced repellency.

The stable fly possesses ORNs responding to given repellent EOs and its volatile constituents. Vapors of ZP-SD, ZA-SO, and several volatile compounds described could be practically used to repel the stable fly. Results from binary mixture of these EOs and Oct or BA suggest that olfactory neurons in the fly antennae may possess olfactory receptors to perceive both EO and Oct or BA. The fly antennal olfactory neurons which possess receptors for perception of BA were significantly hampered by the presence of the repellent EOs and their constituents, and vice versa. Different combination between of repellent and attractant ratios play a significant role in modulating host finding behaviors in the stable fly, which could elicit a repellent, attractant, or other type of behavioral

responses. The repellent molecules are known to interact with the insect olfactory receptors and block their sense of smell.

ZP-SD, ZA-SO, and compounds described could also be of practical use as fumigants with contact action for female stable fly. This original finding indicates that binary mixtures of ZP-SD, ZA-SO, or their bioactive constituents and CI-NO may hold promise for the development of novel and effective insecticides with repellency against stable fly. For practical use of the test materials as novel repellents and toxic fumigants to persist, further research is required to ensure the safety of human and livestock animals. However, EOs are used as fragrances and favoring agents for foods, beverages, and cosmetics (Sellar, 2001; Lawless, 2002). Although the OR3b coreceptor has been suggested as a novel target site for DEET (Ditzen *et al*., 2008), the present studies found that several MTs (terpenes and biogneically related phenols) were potent antagonists of the stable fly octopamine receptor. The octopamine-like toxic effects of the test MTs might act as biochemical blockades of octopamine reuptake in the biting insect. Thus, native octopamine receptors in insects are promising targets of EO constituents with no or low toxicity in vertebrates (Kostyukovsky *et al*., 2002; Blenau *et al*., 2012).

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산초나무속(*Zanthoxylum*) 식물 성분들의 침파리(*Stomoxy calcitrans* L.)에 대한 기피력 및 살충활성

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초록

침파리(stable fly, *Stomoxys calcitrans* L.)는 인간을 비롯한 소, 말, 돼지, 개와 같은 가축들을 흡혈하는 중요한 외부 기생성 축산해충이다. 본 연구에서는 22종 식물체 정유(精油), 이들의 조성물 그리고 이들 정유와 조성물의 타마뉴(tamanu) (*Calophyllum inophyllum*) nut 오일(CI-NO) 두 종 혼합물의 침파리 암컷 성충에 대한 기피효과 및 산초나무속(*Zanthoxylum*) 식물체 조성물의 침파리 안테나에 미치는 후각 반응을 조사하였다. 또한 이들 조성물의 침파리 암컷 성충에 대한 훈증독성 및 살충작용 기구를 조사하였다.

6명의 남성들이 참여한 기피력 시험에서 최초 흡혈한 시간을 기준으로 기피 효과(protection time, PT)를 평가하였을 때, 식물체 정유들 중 패촐리(patchouli), 정향(clove) 꽃봉우리, 정향 잎 및 로비지(lovage) 뿌리 정유들이 0.5 mg/cm^2 농도에서 3.20–3.62 시간 동안 완벽한 기피력을 나타냈다. 화학합성 기피제인 *N*,*N*-diethyl-*m*-toluamide (DEET)의 4.35 시간에 비해서는

약한 활성을 보였다. 또한 타임화이트 (thyme white) 정유의 활성이 우수했고(2.07 시간), 초피(*Zanthoxylum piperitum*) 과피 수증기 증류물(steam distillate) (ZP-SD), 제라늄(geranium), 타임레드(thyme red) 및 오레가노(oregano) 정유들도 1.01–1.12 시간의 기피력을 나타냈으나, *Zanthoxylum armatum* 종자 오일(seed oil) (ZA-SO) 및 버가못(bergamot) 정유는 약한 기피력을 나타내었다(각 0.60 및 0.57 시간). 0.25 mg/cm^2 농도에서 이들 모든 정유들의 기피력 (약 0.6–1 시간)은 DEET(약 2 시간) 보다 미흡했다. 비록 각 식물체 정유의 기피력은 DEET에 비해 약했지만, CI-NO와의 혼합물들의 기피력은 DEET에 버금가는 효과를 나타냈다. 특히 로비지 뿌리, 패출리, 정향 꽃봉우리 타임화이트 및 정향 잎 정유들과 CI-NO 혼합물들(0.25 + 2.0 mg/cm^2)의 기피력(PT, 2.17–2.64 시간)은 DEET 혼합물과 비슷했고(2.69 시간) 오히려 DEET(2.03 시간), 각 정유(0.54–1.07 시간) 또는 CI-NO(0.54 시간) 단독 처리보다 우수한 기피활성을 보였다. 더불어 사보리 정유를 제외한 나머지 식물체 정유, CI-NO 및 이들 혼합물들은 0.5 mg/cm^2 처리에서 시험자 들에게 어떤 부작용도 나타내지 않았다. ZP-SD 및 ZA-SO의 조성물들을 이용한 기피시험에서 cuminaldehyde, cuminyl alcohol, geraniol, limonene, linalool, methyl cinnamate, neral, peperitone 및 α-phelandrene 각 0.25 mg/cm^2 처리는 약 0.5 시간의 기피력을 보였으나 DEET(PT, ≈2.0 시간) 에 비해서는 약했다. 하지만 활성이 우수한 4종 화합물들(limonene, α-phellandrene, cuminyl alcohol, 및 cuminaldehyde)과 CI-NO의 혼합물(0.25 + 1.0 mg/cm^2)들의 기피력(PT, 2.36–2.57

시간)은 2.52 시간의 기피력을 나타낸 DEET 혼합물과 비슷했고, DEET 단독 처리에 비해서는 강한 활성을 보였다. Oleic acid와 linoleic acid와 같은 CI-NO에 함유된 주요 2종 지방산들과 methyl oleate와 methyl linoleate 같은 지방산 메틸 에스테르들은 CI-NO보다 기피 지속시간 이 길었다. 이들 활성이 우수한 산초나무속 식물체 정유들 및 타마뉴 오일의 실제 적용을 위해 이들을 유효성분으로 한 8종 에어로졸 제형을 조제하여 침파리 암컷 성충에 대한 기피력을 조사한 결과, 이들 에어로졸들은 DEET + CI-NO 및 DEET 단독 처리에 필적할만한 기피력 을 나타내었다.

산초나무속 식물체들에 함유된 1,8-cineole, citronellal, cuminaldehyde, linalool oxide, linalool, neral, piperitone 및 terpinen-4-ol 그리고 침파리의 유인물질들인 1-octen-3-ol과 butyric acid와의 조합물들에 대한 침파리의 행동 및 후각 반응 패턴을 침파리 촉각을 이용하여 조사하였다. 침파리 행동 및 후각 반응 패턴들은 식물체 정유들 이나 그 기피 성분들과 1-octen-3-ol과 butyric acid의 조합비에 따라 크게 변화됨을 알 수 있었다. 이와 같은 행동 및 전기신경생리학적인 결과로 판단할 때, 산초나무속 식물체 정유들은 기주 휘발성 물질들에 대한 침파리의 비행 행동 반응을 낮춰주는 것으로 보인다. 따라서 침파리는 안테나에 존재하는 어떤 후각 수용체를 이용해서 이들 식물 기피 물질들을 직접적으로 감지한다고 할 수 있다.

ZP-SD와 ZA-SO, 이들의 28종 조성물과 8종의 구조적으로 유사한 화합물들의 침파리 암컷 성충에 대한 훈증독성 및 침파리 암컷 머리의

아세틸콜린에스테라제 (acetylcholinesterase, AChE) 저해활성을 조사하였다. Cuminaldehyde, thymol, (1*S*)-(–)-verbenone, (–)-myrtenal, carvacrol, (*S*)-(*Z*)-verbenol, ZP-SD, cuminyl alcohol, ZA-SO, piperitone, (–)-(*Z*)-myrtanol 및 citronellal은 강한 살충력을 나타냈으나(LC_{50}, 0.075–0.456 μg/cm^3), 대조약제인 chlorpyrifos나 dichlorvos 살충력에는 미치지 못했다. 구조–활성 연구는 화합물들의 탄소골격, 작용기의 포화도 및 유형 그리고 증기압 등과 같은 요소들이 침파리에 대한 훈증력 발현에 중요함을 보여준다. 또한, citronellyl acetate, α-pinene, thymol, carvacrol 및 α-terpineol은 AChE 저해활성(IC_{50}, 1.20–2.73 mM)을 보였 으나, chlorpyrifos와 dichlorvos의 억제 활성에 크게 미치지 못하였다. 그러나, 옥토파민과 도파민은 침파리 뇌에서 adenylate cyclase system (ACS)를 자극하여 cAMP를 생산하는데, 100 nmol/ml 옥토파민 처리는 cAMP를 대조구 대비 2.7배나 더 증가 시켰다. 하지만 100 nmol/ml 수준에서 옥토파민의 이러한 증대효과는 citronellal과 thymol에 의해 13.7배와 3.9배로 감소되었고, cuminaldehyde, cuminyl alcohol, (–)-myrtenal 및 (1*S*)-(–)-verbenone에 의해서는 2.0–2.9배 감소되었다. 이들 결과는 식물체 정유들 및 살충 성분들의 신경독 작용점이 옥토파민 민감성 ACS 일 수 있다는 것을 암시한다.

결론적으로, 식물체 정유들과 산초나무속 화합물들은 침파리에 대한 기피제 및 훈증제로서 활용될 수 있을 것이며, 이로 인해 농업환경에서 독성이 높은 기피제나 살충제의 사용 수준을 낮출 수 있을 것으로 기대된다.

검색어: 침파리, 식물체 정유, 초피, 천연 기피제, 천연 훈증제, 아세틸콜린 에스터라제, cAMP 면역시험, 후각반응

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