Investigation of mechanisms of action of monoterpenoid insecticides on insect gamma-aminobutyric acid receptors and nicotinic acetylcholine receptors

by

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CHAPTER 1. GENERAL INTRODUCTION

LITERATURE REVIEW

Monoterpenoids for pest control

Synthetic pesticides play an important role in protecting against pests in agriculture, medicine, industry and the household in the 20th and 21st century. The use of these conventional pesticides is believed to be one of the major reasons for the increase in agricultural productivity in the 20th century. However, there are more and more public concerns all over the world about long-term health and environmental effects concerning the use of synthetic pesticides. Many laboratory studies and clinical cases showed that almost all the conventional synthetic pesticides have the potential to significantly alter ecosystems; and a large number of them have acute or chronic toxicity to humans or other non-target organisms. On the basis of these problems, there is an urgent demand to reduce the use of the conventional pesticides and develop alternatives with fewer harmful effects on the environment and lower toxicity to non-target organisms.

For centuries, natural products (such as nicotine from tobacco, pyrethrum from chrysanthemums, rotenone from Derris root, sabadilla from lilies, ryania from the ryania shrub, limonene from citrus peel, and neem from the tropical neem tree) have been used to protect crops from pest invasions in many countries. In recent

years, many natural products and their derivatives have been developed and used in commercial pest management to protect crops and human health as alternatives to conventional pesticides. Some of these pesticides have been developed from microbes, such as BT toxins, spinosads, and avermectins [1-3]; some are originally from plants, such as pyrethrins [4], and plant essential oils [5-8].

Among the natural products used for pest control, one of the most successful botanical pesticide groups are monoterpenoids [9], which are mostly found in plant essential oils [5; 10-13]. Monoterpenoids are related to or derived from monoterpenes, a class of terpenes, containing two units of isoprene, and are 10-carbon molecules. These compounds are plant secondary metabolites and play a key role in defending the plants against herbivores or pathogens. For hundreds of years, monoterpenoids have been widely used as food additives, fragrances, decongestants, external analgesics, and antimicrobials [9; 12].

In the past decades, some monoterpenoids have been considered good alternatives for conventional synthetic pesticides for the following reasons. First, these compounds were identified to be good insecticides, acaricides, and insect repellents [5; 7; 8; 10-15]. Many monoterpenoids have shown broad-spectrum insecticidal and acaricidal activities. Table 1 lists some lethal dose 50% (LD_{50}) values of monoterpenoids to different pests. Secondly, monoterpenoids are relatively safe to mammals, aquatic organisms and other non-target organisms [5].

In Table 2, some LD_{50} values to mammals are listed. More importantly, most monoterpenoids are biodegradable, and are very friendly to the environment [16-18]. They can be degraded very fast and do not accumulate in the food chain. In Table 3, some environmental fate data of monoterpenoids are shown. In addition, the U.S. Environmental Protection Agency has ruled that many essential oils extracted from plants are exempt from regulation, under special provisions of the Food Quality Protection Act.

Possible modes of action of monoterpenoid insecticides

Although monoterpenoid pesticides have been developed and studied for many years, their mode of action is not yet very clear. Current evidence indicates that monoterpenoids may act on various targets in insects and mammals, especially on the nervous system, including γ-aminobutyric acid (GABA)-gated chloride channels, octopamine receptors, tyramine receptors, acetylcholine esterase, nicotinic acetylcholine receptors (nAChR), sodium channels, and possibly other targets.

Several different monoterpenoids were reported, in the recent research, to bind to ionotropic GABA receptors in human, rodents, and insects. Thymol, linalool, menthol, camphor, carvone, borneol, and other monoterpenoids were suggested to be positive allosteric modulators of mammalian $GABA_A$ receptors [19-25]. They all could increase the chloride current induced by GABA in recombinant GABA

receptors in different models. Moreover, thymol also showed a positive modulatory effect on *Drosophila melanogaster* homomeric RDLac GABA receptor [26]. A whole-cell patch clamp technique and [^{3}H]-EBOB binding assays were used to show that α-thujone was a non-competitive antagonist at the $GABA_A$ receptor in mouse, which inhibited the chloride current induced by GABA, and bound to the receptor at the typical non-competitive antagonist binding site, which is the same binding site for many chloride channel inhibitors, such as picrotoxin, lindane, dieldrin, endosulfan, and fipronil [27; 28].

G-protein-coupled receptors in insects, including octopamine receptors and tyramine receptors, are also important candidate targets for monoterpenoid insecticides. α-Terpineol, carvacrol, pulegone, eugenol, and about 20 other monoterpenoids were demonstrated to have binding activities at an octopamine receptor from American cockroach with high sensitivities. They were either antagonists or agonists of the octopamine receptor [29; 30]. *p*-Cymene, thymol, carvone, terpineol, and carvacrol were identified to target insect tyramine receptors to either increase (thymol) or decrease (*p*-cymene, carvone, terpineol, and carvacrol) the cAMP levels and change the intracellular calcium levels in S2 cells expressing *Drosophila melanogaster* tyramine receptors [31].

In addition to these two receptors as possible targets, some studies suggested that monoterpenoids also act on other receptors or enzymes in the nervous system.

However, these cases were mostly demonstrated in vertebrates. Pulegone and 16 other monoterpenoids with *p*-menthane skeletons were indicated to be inhibitors of acetylcholinesterase (AChE) from bovine erythrocytes with an IC_{50} of 0.89 mM for pulegone [32]. Borneol and camphor have also been demonstrated to be non-competitive inhibitors of mammalian nAChRs (in bovine adrenal cells) by increasing intracellular calcium and sodium levels induced by a nAChR agonist, 1,1-dimethyl-4-phenylpiperazinium iodide (DMPP) [33; 34]. Thymol and menthol were reported to block sodium currents in HEK293 cells that expressed rat neuronal (rat type IIA) and human skeletal muscle (hSkM1) sodium channels [35].

Among these possible targets of monoterpenoid insecticides, ion channels (GABA-gated chloride channels or acetylcholine-gated cation channels) may contribute to the fast knock-down effects due to their presence in the peripheral nervous system in insects, especially in the neuromuscular junction. G-protein-coupled receptors, such as octopamine receptors and tyramine receptors, on the other hand, may induce slower toxicity to insects because of their delayed effects and complicated pathways.

Insect GABA receptors and nicotinic acetylcholine receptors

Most of the reports on the effects of monoterpenoids on GABA receptors and nicotinic acetylcholine receptors were in mammals. It is not yet known if these compounds kill insects by interfering with the functions of GABA receptors or

nicotinic acetylcholine receptors in insects. In this dissertation, I will focus on the mode of action of monoterpenoid insecticides on GABA-gated chloride channels and nicotinic acetylcholine receptors in insects.

GABA (γ-aminobutyric acid) is the major inhibitory neurotransmitter in the insect central and peripheral nervous systems [36; 37]. GABA molecules act by binding to specific transmembrane receptors in the plasma membrane of neurons. This binding causes the opening of Cl^- channels to allow the flow of chloride ions into the neurons. This typically results in enhancement of the negative charge in the transmembrane potential, usually causing hyperpolarization [36; 37].

GABA receptors are transmembrane proteins on post-synaptic membranes responding to GABA molecules. There are two types of GABA receptors found in mammals, ionotropic GABA receptors ($GABA_A$ receptors), which are linked with chloride channels, and metabotropic GABA receptors ($GABA_B$ receptors), which are G-protein-coupled receptors [36-38]. In insects, both ionotropic GABA receptors and metabotropic GABA receptors have been found [39; 40]. However, the ionotropic GABA receptors are the major targets of many insecticides. Ionotropic GABA receptors belong to the Cys-loop ligand-gated ion channel superfamily, which also includes the nicotinic acetylcholine receptor, glycine receptor, and 5-HT_3 receptor. Ionotropic GABA receptors are composed of five protein subunits around a central pore to form an ion channel. They possess a

characteristic loop formed by a disulfide bond between two cysteine residues, which is the reason why it is called the Cys-loop superfamily. The GABA molecules bind at the interface between subunits in the extracellular domain. Each subunit of the receptor contains four membrane-spanning alpha helixes (M1, M2, M3, and M4). M2 helix is thought to line the channel pore, and the M3-M4 linker is the intracellular domain that binds the cytoskeleton [41; 42].

Insect ionotropic GABA receptors are different from mammalian GABA receptors in three ways. Structurally, ionotropic GABA receptors in mammals are composed of α, β, γ, δ, ε, π, θ, and ρ subunits, whereas, in insects different subunits were identified in different insect species, for example, in *Drosophila melanogaster* GABA receptors, RDL, GRD, and LCCH3 are the major subunits [39; 40]. Functionally, insect ionotropic GABA receptors can mediate fast inhibitory synaptic transmission in both the central and peripheral nervous system [43], but in mammals, they are only found in the central nervous system [36; 37]. Pharmacologically, insect and mammalian ionotropic GABA receptors have different sensitivities to various chemicals. For instance, bicuculline, which is a competitive antagonist for mammalian $GABA_A$ receptors, shows no effects on most insect GABA receptors [40; 44]. Some benzodiazepines, such as diazepam, and flunitrazepam, have much higher affinity for mammalian $GABA_A$ receptors than to insect GABA receptors [40].

Based on these differences mentioned above, insect GABA receptors are a very important target for many insecticides, such as lindane, dieldrin, endosulfan, avermectins, and fipronil. Lindane, dieldrin, endosulfan, and fipronil act on insect GABA receptors as non-competitive inhibitors, which block the chloride channels opened by the endogenous neurotransmitter, GABA, and kill insects by causing an over excitation of the insect nervous system [43; 45-48]. Avermectins can bind to GABA receptors at a binding site distinct from those non-competitive channel blockers, enhancing the chloride uptake induced by GABA, and killing insects by causing inhibition of the insect nervous system [1; 43; 49].

The other possible target we focused on are the insect nicotinic acetylcholine receptors. The nicotinic acetylcholine receptor (nAChR) is a cholinergic receptor that forms ligand-gated ion channels in the plasma membranes of certain neurons in the insect central nervous system. The binding of the acetylcholine, an excitatory neurotransmitter, activates the nicotinic acetylcholine receptor, and opens the cation channels on the post-synaptic membrane. The diffusion of Na^+ and K^+ across the receptor causes depolarization that opens voltage-gated sodium channels on the post-synaptic membrane, which results in an action potential [50; 51].

The nicotinic acetylcholine receptor is also a member of the Cys-loop superfamily [52]. The nAChR, like ionotropic GABA receptors, is composed of five

subunits to form a central channel. In humans, 17 types of subunits have been identified, including α1-10, β1-4, γ, δ, and ε subunits [52]. In insects, distinct α and β-type subunits were also identified in different insect species [53; 54].

In insects, the nAChR is a major target for some widely used insecticides. Neonicotinoid insecticides, such as imidacloprid, clothianidin, and acetamiprid, act as receptor agonists, which mimic the function of acetylcholine. The binding of neonicotinoids to the nAChR can cause excitatory effects on the insect nervous system [54-58]. Another type of insecticide, spinosads, binds to the nAChR and enhances the sensitivity of the receptor to the endogenous neurotransmitter, acetylcholine [49; 57].

In addition to the nAChR, there is another type of acetylcholine receptor responding to acetylcholine molecules, the muscarinic acetylcholine receptor, found in both mammals and insects [59-61]. The muscarinic acetylcholine receptor is a metabotropic receptor, which is coupled to G-protein, and mediates G-protein pathways. However, so far, this receptor is poorly developed as an insecticide target [60].

Research Interest

The purpose of this dissertation is to further study the mode of action of monoterpenoid insecticides on insect GABA receptors and nicotinic acetylcholine receptors. The major objectives of this research include: (1) insect GABA receptor

binding activities of monoterpenoid insecticides, (2) effects of monoterpenoids on Cl^- uptake in insect ventral nerve cords, (3) relationship between monoterpenoid structures and GABA binding activities. (4) monoterpenoid insecticides binding activity with insect nicotinic acetylcholine receptors.

DISSERTATION ORGNIZATION

This dissertation is organized into five chapters, including a general introduction (Chapter 1), three chapters of studies related to the research objectives (Chapter 2, Chapter 3, and Chapter 4), and a general conclusion (Chapter 5). Chapters 2, 3, and 4 are written as manuscripts in journal format to be published.

Chapter 1 includes the introduction and review of the uses of monoterpenoids as pesticides, possible mechanisms of action of monoterpenoid insecticides, and two important possible protein targets.

Chapter 2 addresses Objectives 1 and 2. The [^{3}H]-TBOB binding assay and $^{36}Cl^-$ uptake assay were used to examine the modulations of insect GABA receptors by monoterpenoid insecticides. The results indicate that some monoterpenoids are positive modulators for insect GABA receptors, and they can enhance the Cl^- uptake induced by GABA, which causes inhibitory effects in the insect nervous system.

Chapter 3 addresses Objective 3. Quantitative structure-activity relationship

(QSAR) studies and [^{3}H]-TBOB binding assays for 22 monoterpenoids were used to illustrate the relationship between monoterpenoid structures and GABA-binding activities.

Chapter 4 addresses Objective 4. Data from the [^{14}C]-nicotine binding assay of a monoterpenoid insecticide, carvacrol, suggested that carvacrol can bind to the house fly nicotinic acetylcholine receptor, and binding inhibited nicotine binding non-competitively. This finding showed that carvacrol may also inhibit the binding of acetylcholine, and cause inhibitory effects on the insect nervous system.

Chapter 5 describes the conclusions obtained from results in all the research work in this dissertation.

TABLES

Table 1. Monoterpenoids' toxicity to different pests

LD_{50} (µg/ pest) / LC_{50} (w/v)	Monoterpenoids		
	Carvacrol	Eugenol	Thymol
M. domestica LD_{50}	92	77	29
B. germanica LD_{50}	101	109	70
I. scapularis LC_{50}	0.0068	No data	No data
D. virgifera LD_{50}	No data	12	No data

Table2. Mammalian toxicity of some monoterpenoids

Monoterpenoids	Rats oral LD_{50} (mg/kg)
Carvacrol	810
Carvone	1640
1,8-Cineole	2480
Eugenol	2680
Limonene	4600
Linalool	2790
Menthol	3180
Thymol	980

Table 3. Environmental fate data of some monoterpenoids

Monoterpenoids	Half-life in water (day)	Half-life in soil (day)
Thymol	16	5
Phenethyl propionate(PEP)	5	4

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CHAPTER 2. EFFECTS OF SOME MONOTERPENOID INSECTICIDES ON [^{3}H]-TBOB BINDING IN HOUSE FLY GABA RECEPTOR AND $^{36}CL^{-}$ UPTAKE IN AMERICAN COCKROACH VENTRAL NERVE CORD

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ABSTRACT

Monoterpenoids and their derivatives from plant essential oils showed good insecticidal activities in previous studies, but the mechanisms of their action as natural insecticides are not known yet. In the present work, we evaluated the pharmacological action of five monoterpenoids (α-terpineol, carvacrol, linalool, pulegone, and thymol) on native insect GABA receptors from house flies and American cockroaches using radiotracer methods. In the [^{3}H]-TBOB binding assay, carvacrol, pulegone, and thymol all enhanced the [^{3}H]-TBOB binding to membrane preparation of house fly heads with EC_{50} values of 48μM, 432μM, and 6mM, respectively. Moreover, these three monoterpenoids at concentrations of 500 μM and 1mM also significantly increased the $^{36}Cl^{-}$ uptake induced by GABA in membrane microsacs prepared from American cockroach ventral nerve cords. These results revealed that carvacrol, pulegone, and thymol are all positive allosteric modulators at insect GABA receptors. The other two monoterpenoids

that were tested, α-terpineol and linalool, showed little or no effect in both the [^{3}H]-TBOB binding and $^{36}Cl^{-}$ uptake assays.

Key words: Monoterpenoid; Insecticide; GABA receptor; House fly; American cockroach; [^{3}H]-TBOB binding; Cl^{-} uptake

1. INTRODUCTION

GABA (Gamma-aminobutyric acid) is the major inhibitory neurotransmitter in both the insect's central and peripheral nervous systems. GABA acts by binding to specific transmembrane receptors in the plasma membrane of neurons. This binding causes the opening of Cl^{-} channels to allow the flow of chloride ions into the neurons. This typically results in a negative charge in the transmembrane potential, usually causing hyperpolarization. Many other ligands besides GABA interact with the insect's GABA receptor to increase chloride uptake (agonists), to prevent the binding of endogenous GABA or agonists (antagonists), or to modulate the binding activity between GABA receptor and agonists (modulators) [1; 2]. Insect's GABA receptor coupled with Cl^{-} channel is an important target for a lot of insecticides, such as dieldrin, lindane, fipronil, etc. [3-6]. These insecticides bind to the insect's GABA receptors, either decrease or increase the Cl^{-} influx into the neurons, and kill insects by causing too much excitation or inhibition of the nervous system [7].

Monoterpenoids are naturally occurring compounds and widespread in plants,

especially in their essential oils. These compounds are secondary metabolites for plants, and play a key role to defend against pathogens or herbivores. In previous research from our lab, some monoterpenoids from plants' essential oils possess biological activity against insects. Some have acute toxicity, while others have repellent or inhibitory effects [8-11]. Most of these botanical insecticides showed low toxicity to non-target organisms, and rapid biodegradation [12; 13]. Besides being used as insecticides, monoterpenoids are also used widely as natural flavor additives for food, as fragrances in perfumery, in aromatherapy, and in traditional and alternative medicines. Most are considered generally recognized as safe (GRAS) by US Food and Drug Administration.

Although toxicity tests of many monoterpenoids have been done previously, and some compounds are currently being used commercially as insecticides or repellents, the insecticidal mode of action of monoterpenoids is not well understood. Several different monoterpenoids were reported, in recent research, to bind to ionotropic GABA receptors in human, rodents, and insects. Thymol, linalool, and borneol are all positive allosteric modulators of mammalian $GABA_A$ receptors, and thymol also showed a positive modulatory effect on *Drosophila melanogaster* homomeric RDLac GABA receptors [14-17]. α-thujone was found to be a non-competitive antagonist for the $GABA_A$ receptor in mouse [18]. However, the effects of most monoterpenoid insecticides on insect's native GABA receptors

are still poorly studied.

We conducted a study to examine the mechanism of action of monoterpenoid insecticides: to determine the binding activities of monoterpenoids to insect GABA receptors, and to find out how the monoterpenoids can affect the chloride influx in insect's GABA system.

2. MARERIALS AND METHODS

2.1 Materials

The five monoterpenoids (α-terpineol, carvacrol, linalool, pulegone, and thymol), the antagonist convulsant picrotoxin (PTX), and the agonist muscimol were purchased from Sigma-Aldrich Chemical Co.. The [^{3}H]-*t*-butylbicycloorthobenzoate (TBOB) and [^{36}Cl]-HCl were purchased from GE Healthcare. The structures of monoterpenoids are shown in Fig. 1.

2.2 [^{3}H]-TBOB Binding Assay

House fly heads (0.5g) were homogenized in 10 mM tris-HCL buffer (pH 7.5) containing 0.25M sucrose (buffer A) with a glass homogenizer. The homogenate was centrifuged at 1,000*xg* for 5 minutes. The supernatant was filtered through four layers of cheesecloth and centrifuged at 25,000*xg*, 4 ^{o}C for 30 minutes. The supernatant was discarded, and the pellet was homogenized and resuspended in ice cold buffer A for 30 minutes. The suspension was centrifuged at 25,000*xg*, 4 ^{o}C for 30 minutes. The final pellet was suspended in 10 mM phosphate buffer (pH 7.5)

containing 300 mM NaCL (buffer B) and used directly for the assays. Lowry protein assay was used to determine a final concentration of protein [19].

Membrane preparation containing 20 µg of protein was incubated for 70 minutes at room temperature (20 ^{o}C) with 4 nM [^{3}H]-TBOB (specific activity 22Ci mmol^{-l}), different amounts of candidate chemicals (monoterpenoids or positive controls) and buffer B. The total assay buffer volume was 500 µL. After incubation, samples were filtered on glass fiber filter papers (Whatman GF/B) and washed with 10 mL ice-cold buffer B three times. Radioactivity was measured by a Beckman liquid scintillation counter LS5000 CE. Specific binding was used to estimate the binding activities of candidate chemicals and calculated as the difference between the total ^{3}H bound and nonspecific ^{3}H bound with 100 µM PTX. The specific binding was 60-70% of total binding at 4 nM [^{3}H]-TBOB. Each experiment was repeat at least three times using different membrane homogenates. [20; 21]

2.3 GABA-activated $^{36}Cl^{-}$ Uptake Assay

Thoracic and abdominal ganglia with ventral nerve cords were removed from 7 American cockroaches for each replicate and homogenized in 2.0 mL ice-cold buffer of the following composition (in millimoles per liter): NaBr, 145; KBr, 5.0; $MgSO_4$, 1.0; $Ca(NO_3)_2$, 1.0; D-glucose, 10.0; N-2-hydroxyethylpiperazine-N'-2-ethanesulphonicacid (HEPES), 10.0 (adjusted to pH 7.5). The homogenate was centrifuged in 1.0 mL aliquots at 10,000*xg*, 4 ^{o}C for 20

min, and the pellets were re-suspended in buffer, centrifuged at 10,000*xg*, 4 ^{o}C for another 20 min. The final pellets were suspended in buffer to facilitate the formation of microsacs for assaying GABA-activated $^{36}Cl^{-}$ uptake.

Aliquots (200 µl) of this microsac preparation were incubated at 30 ^{o}C for 3 min after which 150 µL of a solution of $^{36}Cl^{-}$ (0.2 µCi ml^{-l}; specific activity 580 µCi mmol^{-l}) containing 1 µM GABA was added. Candidate monoterpenoids or positive controls were incubated with the tissue sample for 3 min before addition of the solution containing $^{36}Cl^{-}$ and GABA. To stop the influx of $^{36}Cl^{-}$, 2.0 mL of ice-cold 10 mM phosphate buffer (pH 7.5) was added after 10 s and the solution was rapidly filtered over a glass fiber filter (Whatman GF/B) followed by washing with 10.0 ml ice-cold 10 mM phosphate buffer twice. Radioactivity was measured by a Beckman liquid scintillation counter LS5000 CE. Each experiment was repeat at least three times using different ventral nerve cord microsacs. [2; 4; 22; 23]

2.4 Data Analysis

Results are shown as mean ± standard error of mean (S.E.M.), and were analyzed by using GraphPad Prism software 5.0 (GraphPad Software, Inc., San Diego, CA, U.S.A.). The EC_{50} and IC_{50} values are the concentrations for half maximal enhancement and inhibition, respectively. E_{max} values are the percentage for maximal enhancement. EC_{50}, IC_{50} and E_{max} values were also calculated by GraphPad Prism software 5.0. Two-tailed Student's *t*-test and one-way ANOVA

were used to compare data. A *p*-value of less than 0.05 was considered to be statistically significant.

3. RESULTS

3.1 [^{3}H]-TBOB Saturation Binding Assay in Membrane Preparation from House Fly Heads

[^{3}H]-TBOB can bind to house fly GABA receptor at the PTX binding site, which is the same binding site for a number of radioligands, such as [^{3}H]-EBOB, and [^{35}S]-TBPS [20]. It is also an important binding site for many insecticides, such as dieldrin, lindane, and fipronil. Fig. 2 illustrated the [^{3}H]-TBOB saturation binding to membrane preparation from house fly heads containing GABA receptor with Kd of 10.2±2.9 nM, and B_{max} of 8.5±1.1pmol/mg protein. The specific binding was 60-70% of total binding.

3.2 Inhibitory Binding of [^{3}H]-TBOB by Lindane and Dieldrin

Lindane and dieldrin both have been shown to be noncompetitive antagonists of the insect GABA receptor [4; 24]. They can bind to the same binding site as PTX, which is the typical antagonist of the insect GABA receptor [24; 25]. We assessed the effect of the binding of lindane and dieldrin on [^{3}H]-TBOB binding to house fly GABA receptor in the membrane preparation in order to compare and contrast the effects caused by the monoterpenoids. Competition curves for lindane and dieldrin are shown in Fig. 3. The binding of lindane and dieldrin both activated

a concentration-dependent inhibition of the [^{3}H]-TBOB binding to house fly membrane homogenates, with the IC_{50}, for lindane, of 22 ± 1 nM, and, for dieldrin, of 61 ± 5 nM.

3.3 The Modulation of Monoterpenoids on [^{3}H]-TBOB Binding in Membrane Preparation of House Fly Heads

Five monoterpenoids (α-terpineol, carvacrol, linalool, pulegone, and thymol) were used in this study to estimate their effects on [^{3}H]-TBOB binding to membrane preparation from house fly heads containing GABA receptors. Fig. 4 demonstrated that carvacrol, pulegone, and thymol had similar effects on [^{3}H]-TBOB binding to the membrane homogenates, which were different from lindane and dieldrin. Rather than inhibition of [^{3}H]-TBOB binding, carvacrol, pulegone, and thymol induced a concentration-dependent increase of the amount of [^{3}H]-TBOB binding to the membrane preparation. Linalool also showed a tendency to increase the [^{3}H]-TBOB binding, but the there was no statistical significance for linalool's effect on the [^{3}H]-TBOB binding (Fig. 5A). The potentiation of the binding indicated that carvacrol, pulegone, and thymol may bind to house fly GABA receptors in the membrane homogenates from house fly heads, but at different binding sites than the PTX binding site (TBOB binding site). Another monoterpenoid, α-terpineol, showed neither enhanced nor inhibitory effects on the [^{3}H]-TBOB binding to the membrane preparation of house fly heads

(Fig. 5B). The values of EC_{50}, E_{max} and LD_{50} of adult house fly 24h after topical application [9; 10] of these five monoterpenoids were summarized in Table 1. Table 1 also showed a correlated relationship between the TBOB binding efficiency (maximum binding) and the mortality of monoterpenoids to house fly, which indicated that the efficiency of the binding of monoterpenoids to house fly GABA receptors may be an important reason of the toxicity to house fly. However, from the [^{3}H]-TBOB binding assay alone, we cannot tell if these monoterpenoids have positive or negative effects on the GABA system; therefore the $^{36}Cl^-$ uptake assay was used to answer this question.

3.4 Inhibition of $^{36}Cl^-$ Uptake by Antagonists of the Insect GABA Receptor

GABA at a concentration of 1 μM was found to increase $^{36}Cl^-$ uptake in cockroach microsacs to 267 ± 15% (mean ± S.E.M., n= 9, $p<0.01$) of the control level, which was consistent with previous experiments done by Lummis *et al* [26]. PTX, dieldrin, and fipronil at 10 μM all showed an inhibitory effect on this increase of $^{36}Cl^-$ uptake induced by GABA. They reduced the $^{36}Cl^-$ influx to 114 ± 4%, 106 ± 6%, and 168 ± 11% (mean ± S.E.M., n= 3, $p<0.01$), respectively, of the control level in the presence of 1 μM GABA (Fig. 6).

3.5 $^{36}Cl^-$ Uptake Induced by the Agonist of the Insect GABA Receptor

Muscimol is an agonist of the insect GABA receptor [27]. Muscimol at a concentration of 10 μM directly induced $^{36}Cl^-$ uptake in the cockroach nerve cord

membrane microsacs to 183 ± 15% (mean ± S.E.M., n= 3, $p<0.01$) of the control level, in absence of GABA (Fig. 7).

3.6 Effects of Monoterpenoids on $^{36}Cl^-$ Uptake Induced by GABA

In the absence of GABA in the assay buffer, these five monoterpenoids showed no significant effects on the $^{36}Cl^-$ uptake of the control level (Fig. 8). These results suggested that these compounds didn't affect the GABA receptor directly, and none of these monoterpenoids was an agonist for American cockroach's GABA receptor.

Fig. 9 illustrated that, in the presence of 1 μM GABA, carvacrol, pulegone and thymol at the concentrations of 500 μM, and 1 mM all elicited an increase of the $^{36}Cl^-$ influx induced by GABA. Carvacrol enhanced the $^{36}Cl^-$ influx induced by GABA to 191± 30%, and 291± 30% at 500 μM and 1 mM, respectively; pulegone potentiated the $^{36}Cl^-$ influx induced by GABA to 116± 26% at 500 μM, and 162± 1% at 1 mM; thymol increased the $^{36}Cl^-$ influx induced by GABA to 132± 11% at 500 μM, and 196± 12% at 1 mM. The enhancement of the $^{36}Cl^-$ uptake by carvacrol, pulegone, and thymol can all be inhibited by adding 10 μM PTX, which is a specific inhibitor for insect's GABA receptors. The inhibition of the $^{36}Cl^-$ uptake by PTX indicated that the enhancement of the $^{36}Cl^-$ uptake was due to the effects of monoterpenoids on the GABA gated chloride channels, not on other chloride channels or breaking the membrane of neurons to introduce the $^{36}Cl^-$ influx. The

Carvacrol showed the highest efficiency to enhance the chloride uptake at both concentrations followed by thymol and pulegone, which was not consistent with the order of efficiency we found in house fly GABA receptor binding assay (Table 1). This inconsistency may have resulted from the species specificity of insect GABA receptors. GABA receptors in various insect species differ a lot in their subunit combinations and sensitivities to different ligands [2; 28]. These data, together with the results that they could not open the chloride channel directly at concentration of 1 mM, indicated that carvacrol, pulegone, and thymol were all positive allosteric modulators for the American cockroach's GABA receptor.

The other two monoterpenoids, α-terpineol and linalool, showed only minor effects on the $^{36}Cl^-$ uptake activated by 1 μM of GABA (Fig. 10).

4. DISCUSSION

Based on the [^{3}H]-TBOB binding assay and $^{36}Cl^-$ uptake assay, this study provided evidence that some monoterpenoids can affect the functioning of insect's GABA system by binding to the GABA receptor and increasing the chloride uptake activated by GABA. The potentiation of [^{3}H]-TBOB binding to house fly GABA receptor by carvacrol, pulegone, and thymol indicates that these compounds can bind to house fly GABA receptor, but not to the PTX binding site, like dieldrin, lindane, and fipronil. Linalool also had a tendency to potentiate the binding of TBOB, although there's no statistical significance for the increase. There's no

significant changing of [^{3}H]-TBOB binding for α-terpineol. The binding affinities for these monoterpenoids are much lower than those traditional insecticides targeting GABA receptors, as we showed in Fig. 3 and 4. The increased binding of [^{3}H]-TBOB precludes any antagonistic effect on GABA receptor; however, from the [^{3}H]-TBOB binding assay alone, we cannot identify the binding site at the GABA receptor or any effect that could cause the toxicity of these compounds to insects, so the $^{36}Cl^{-}$ uptake assay was used to determine whether these monoterpenoids enhanced chloride uptake at the insect GABA receptor. The $^{36}Cl^{-}$ uptake assay revealed that, in American cockroach's ventral nerve cord, carvacrol, pulegone, and thymol all significantly increased the $^{36}Cl^{-}$ uptake stimulated by GABA, and that they didn't increase the $^{36}Cl^{-}$ uptake in the absence of GABA in the assay system. These findings supported that carvacrol, pulegone, and thymol were all positive allosteric modulators for insect's GABA receptor. The inhibition of the enhancement of $^{36}Cl^{-}$ uptake by PTX, a potent insect GABA receptor antagonist, provides further evidence of this conclusion. The fact that monoterpenoids didn't increase $^{36}Cl^{-}$ uptake in the absence of GABA precludes any agonistic effect on GABA receptor. The other two monoterpenoids, linalool and α-terpineol had no effects on the $^{36}Cl^{-}$ uptake in American cockroach's ventral nerve cords. These findings indicated that insect's GABA receptor is an important site of action for carvacrol, pulegone, and thymol. Linalool and α-terpineol may have mechanisms

of action other than targeting the GABA receptor.

Our findings on thymol's effects were also consistent with previous results that thymol is a positive allosteric modulator of the mammalian $GABA_A$ receptor and the homo-oligomeric RDLac GABA receptor from *Drosophila melanogaster* [14; 15]. In the studies in mammalian nervous system, thymol increased the [^{3}H]-flunitrazepam and [^{3}H]-muscimol binding to the mouse $GABA_A$ receptor, and increased the chloride uptake or chloride current elicited by GABA at concentrations of 1-100μM. Thymol also potentiated the chloride current in response to GABA at *Drosophila melanogaster* RDLac GABA receptor at concentration of 1-100μM. Besides positive allosteric modulation to GABA receptors, thymol also showed a tendency to be an agonist for both mammalian $GABA_A$ receptor and *Drosophila melanogaster* RDLac GABA receptor at concentration of 200μM. The study done by Waliwitiya *et al*. also suggested that thymol, in blowfly, can interfere with the function of flight muscle as well as the central nervous system by mimicking GABA's action [29]. However our study did not support this conclusion in the American cockroach's nervous system.

Thymol and carvacrol are both phenols, and have structures similar to propofol (2,6-diisopropylphenol), which is a strong positive allosteric modulator of mammalian $GABA_A$ receptor. According to the study of Krasowaski *et al*., an aromatic ring and a hydroxyl group bonded to the phenyl ring are both key

properties to interact with $GABA_A$ receptors for propofol analogs [30]. In our study, thymol and carvacrol also potentiated the GABA-related activity in insects. In insects, the phenol group is also probably one of the major structural features for the positive modulation of insect GABA receptors. Thymol and carvacrol are isomers, and the only difference in structure is the position of the hydroxyl group on the aromatic ring. Thymol had the higher toxicity to house fly, but it had less potency (higher EC_{50}) and higher efficiency (higher E_{max}) in [^{3}H]-TBOB binding to house fly head membrane preparation. Thymol also had less potentiation of $^{36}Cl^-$ uptake induced by GABA in American cockroach, compared to carvacrol. Although linalool and α-terpineol both have a hydroxyl group, and linalool also enhanced the GABA-elicited response in mammalian $GABA_A$ receptors expressed in *Xenopus oocytes* [16], in the current study they did not show significant increases in TBOB binding in house fly GABA receptor or $^{36}Cl^-$ uptake in American cockroach's ventral nerve cord, probably due to the absence of an aromatic ring.

Pulegone is a ketone, with the carbonyl group in a six-carbon ring. In mammalian models, a number of monoterpenoids with a ketone group have been reported to have interactions with mammalian $GABA_A$ receptors. Based on the study of Hall *et al*., at concentration of 100μM, menthone, camphor, and carvone all slightly potentiated the GABA currents in recombinant human $GABA_A$ receptor; however this was much lower than the enhancement caused by related

monoterpenoids with an alcohol group (menthol, and borneol) [31]. Another monoterpenoid with a ketone group, α-thujone, was reported to be an inhibitor for mammalian $GABA_A$ receptor. It bound to the mouse $GABA_A$ receptor at the PTX binding site and inhibited [^{3}H]-EBOB binding [18]. α-Thujone also suppressed the chloride current induced by GABA in rat dorsal root ganglion neurons [18]. Pulegone's structure is more like menthone, and carvone than α-thujone, which is a bicyclohexanone (Fig. 11). That may be one of the reasons why pulegone showed positive rather than negative effects on the American cockroach GABA receptor. Although pulegone had similar effects to thymol and carvacrol on both TBOB binding in house fly GABA receptor and chloride uptake in American cockroach ventral nerve cords, they may not share the same binding site due to pulegone's absence of a hydroxyl group as a hydrogen bond donor, as well as its lack of an aromatic ring [30].

Besides binding to GABA receptors, a number of monoterpenoids also showed activities on other protein targets in mammalian and insect nervous system. Haeseler *et al*. reported that thymol blocked sodium currents in HEK293 cell-expressed rat neuronal (rat type IIA) and human skeletal muscle (hSkM1) sodium channels [32]. The blockage of voltage-gated sodium channels together with the potentiation of the GABA function contributed to the inhibitory properties of thymol on the nervous system. Pulegone and 16 other monoterpenoids with

p-menthane skeletons were suggested to be inhibitors of acetylchonlinesterase (AChE) from bovine erythrocytes with IC_{50} of 0.89 mM [33]. Enan reported that α-terpineol, carvacrol, and pulegone all demonstrated binding activities with octopamine receptor in American cockroach with high sensitivities [34; 35]. Based on the effects on cAMP production, heartbeat, and [^{3}H]-octopamine binding, α-terpineol and pulegone were found to be an antagonist of American cockroach's octopamine receptor, and carvacrol was an agonist of the receptor. Thymol, in Enan's study, had no effects on the octopamine receptor [34]. Accordingly, the octopamine receptor is also considered to be a major target for some monoterpenoid insecticides. It is possible that the toxic action of monoterpenoids is mediated by both octopamine and GABA receptors. Modulation of octopamine receptors by pulegone and carvacrol may contribute to the mortality of insects due to their octopamine receptor effect, while effects of these two compounds on GABA system may cause fast knock-down of insects, based on the numerous GABA receptors in the neuromuscular junction in insects [2].

In summary, our data provided strong evidence that the insect GABA receptor is a site of action for carvacrol, pulegone, and thymol. They modulated the insect GABA system by binding at the receptor and increasing chloride anion influx into the neurons. Results from [^{3}H]-TBOB binding assay demonstrated their binding activity at the GABA receptor, which was quite different from the binding pattern of

traditional insecticides targeting GABA receptors (dieldrin, lindane, and fipronil). The $^{36}Cl^-$ uptake assay by American cockroach's ventral nerve cords illustrated that carvacrol, pulegone, and thymol were all positive allosteric modulators for the GABA receptor, by showing the enhancement of $^{36}Cl^-$ uptake stimulated by GABA.

We still have a number of questions to answer, including: what are the binding sites for carvacrol, pulegone, and thymol in insect GABA receptor? What is the reason for the selectivity in favor of mammals for these insecticides? What are the other possible targets for monoterpenoids, such as octopamine receptors, sodium channels, nicotinic acetylcholine receptors, glutamate-gated chloride channels? How are the structures of monoterpenoids correlated to the binding activity or neurotoxicity? Further research will be focused on these issues.

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TABLES

Table 1 Binding activities of monoterpenoids and LD_{50} of monoterpenoids on adult house fly

	EC_{50}	E_{max}	LD_{50} (μg/insect) [9, 10]
α-terpineol	>10 mM	112%	173
Carvacrol	48μM	162%	92
Linalool	>10mM	145%	116
Pulegone	432μM	270%	39
Thymol	6.2mM	475%	29

FIGURES

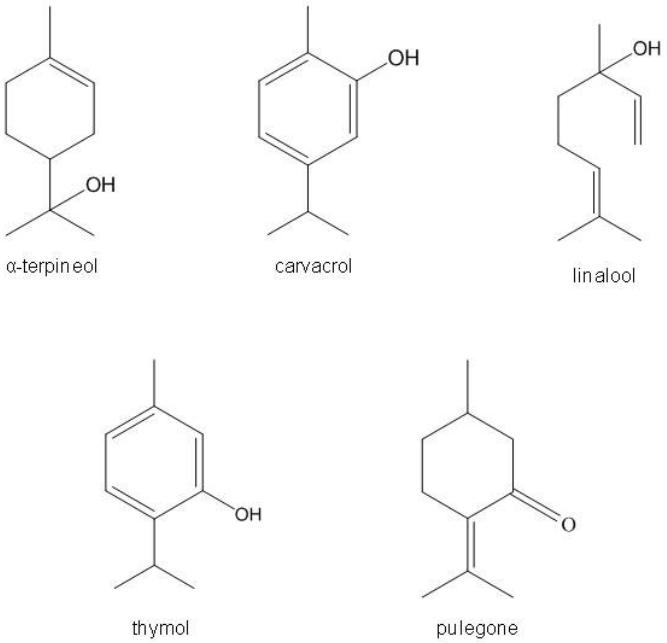

Fig. 1 Structures of the five monoterpenoids tested in this study

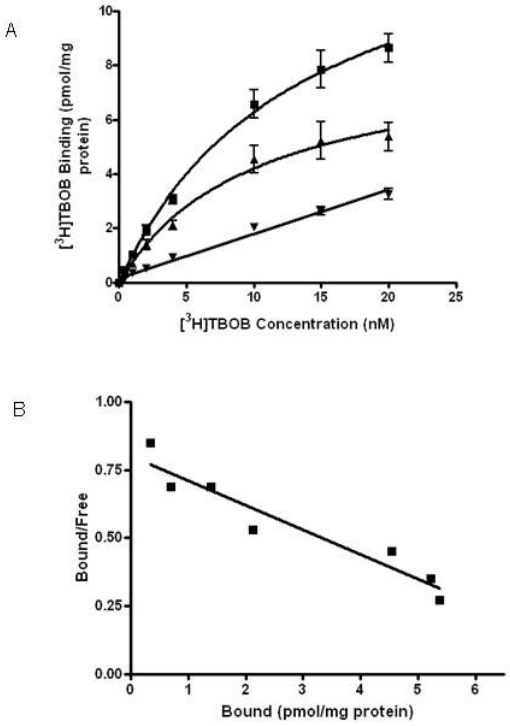

Fig. 2 Saturation binding curves (A) and Scatchard plots (B) for [^{3}H]-TBOB binding to the house fly GABA receptor. Values represent means S.E.M., n=3 (3 separated membrane preparations). In Fig. 2A, the symbols correspond to total binding (■), specific binding (▲), and non-specific binding (▼) of [^{3}H]-TBOB.

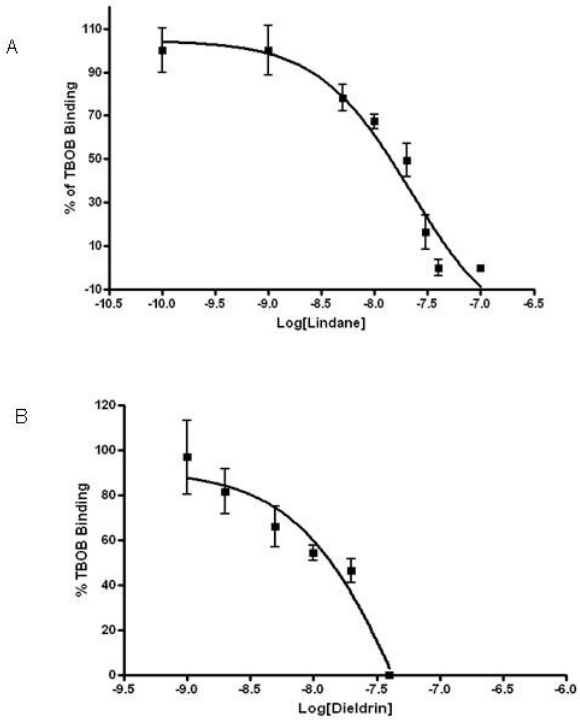

Fig. 3 Inhibitory effects on [^{3}H]-TBOB binding by typical insect GABA receptor antagonists, lindane (A) and dieldrin (B), to house fly GABA receptor. Results are the percentage of [^{3}H]-TBOB binding in the absence of the antagonists. Each point represents the mean ± S.E.M., n=3.

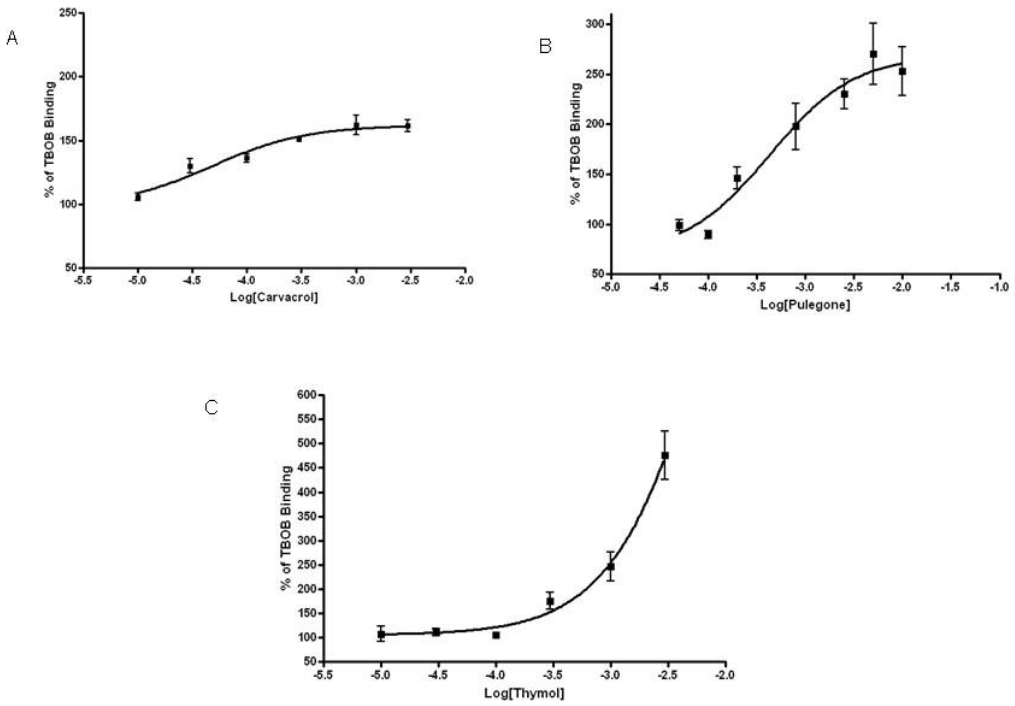

Fig. 4 Modulation of [^{3}H]-TBOB binding by carvacrol (A), pulegone (B), and thymol (C) to house fly GABA receptor. The [^{3}H]-TBOB binding in the absence of the candidate monoterpenoids is expressed as 100%. Each point represents the mean ± S.E.M., n=3.

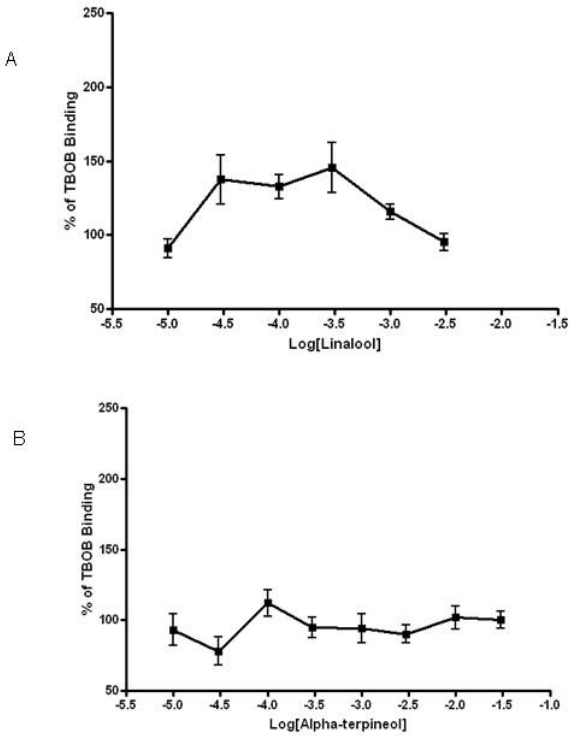

Fig. 5 Modulation of [^{3}H]-TBOB binding by linalool (A) and α-terpineol (B) to house fly GABA receptor. The [^{3}H]-TBOB binding in the absence of the candidate monoterpenoids is expressed as 100%. Each point represents the mean ± S.E.M., n=3.

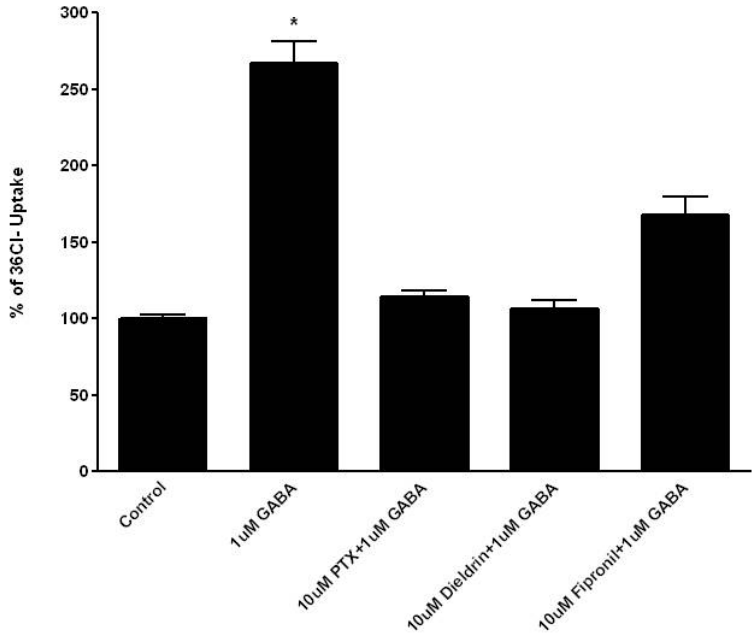

Fig. 6 Effects of GABA and the GABA antagonists, PTX, dieldrin, and fipronil, on $^{36}Cl^-$ uptake by American cockroach ventral nerve cord membrane microsacs. The control level was defined as the amount of $^{36}Cl^-$ uptake in the absence of any drugs and was measured as 100%. Values represent means ± S.E.M., n=9 (9 unique nerve cord microsacs) for GABA, n=3 for antagonists. * indicates $p < 0.01$.

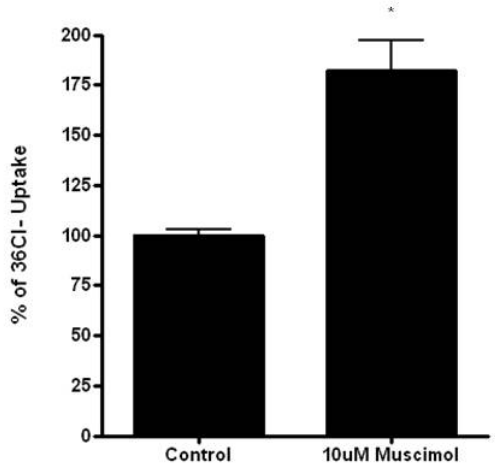

Fig. 7 Effects of the GABA agonist, muscimol, on $^{36}Cl^-$ uptake by American cockroach ventral nerve cord membrane microsacs. The control level was defined as the amount of $^{36}Cl^-$ uptake in the absence of any drugs and was measured as 100%. Values represent means ± S.E.M., n=3. * indicates $p < 0.01$.

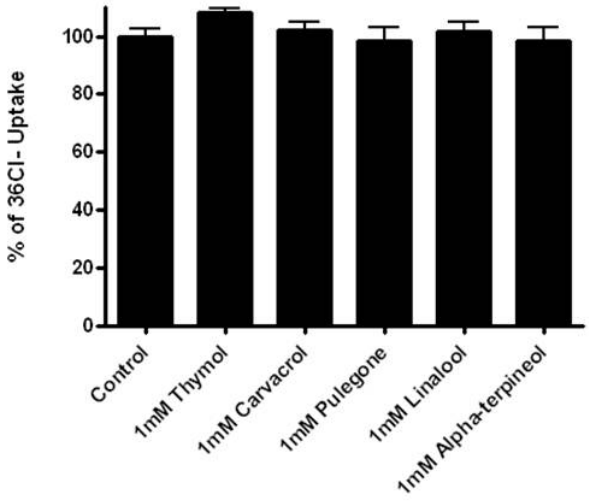

Fig. 8 Effects of the five monoterpenoids, at concentration of 1mM, on $^{36}Cl^-$ uptake by American cockroach ventral nerve cord membrane microsacs in the absence of GABA. The control level was defined as the amount of $^{36}Cl^-$ uptake in the absence of any drugs and was measured as 100%. Values represent means ± S.E.M., n=3.

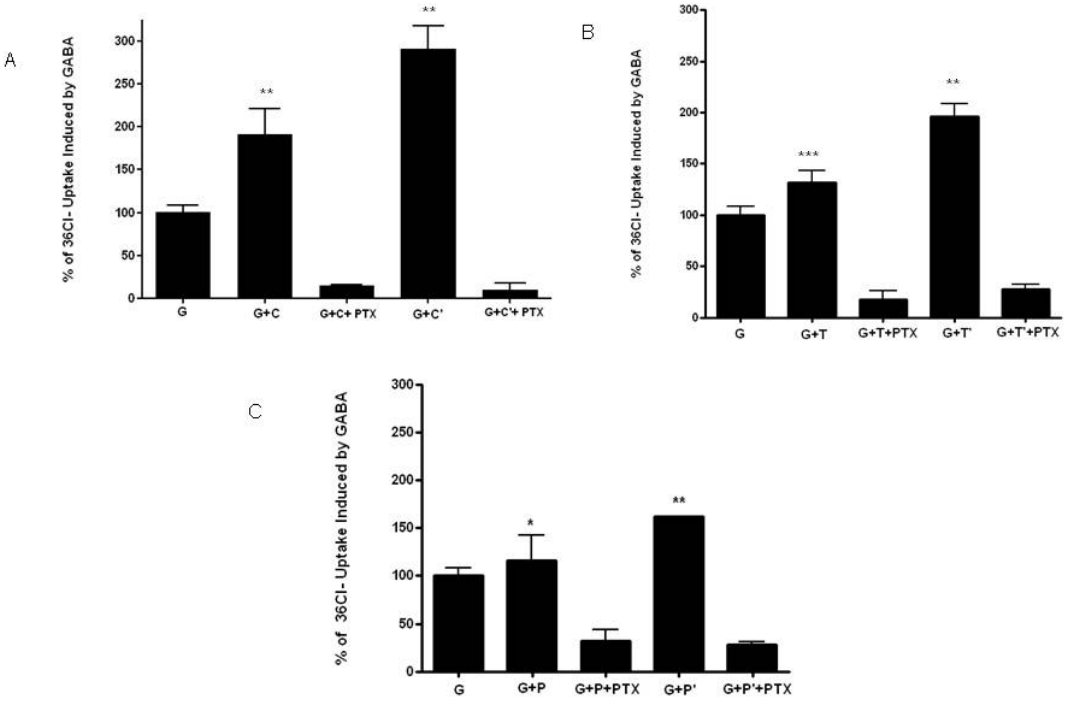

Fig. 9 Effects of carvacrol (A), thymol (B), and pulegone (C) at concentrations of 500 μM and 1 mM on $^{36}Cl^-$ uptake by American cockroach ventral nerve cord membrane microsacs induced by 1 μM of GABA. The amount of $^{36}Cl^-$ uptake induced by GABA was measured as 100%. G, 1 μM GABA; C, 500 μM carvacrol; PTX, 10 μM picrotoxin; C', 1 mM carvacrol; T, 500 μM thymol; T', 1 mM thymol; P, 500 μM pulegone; P', 1 mM pulegone. Values represent means ± S.E.M., n=3. *, **, and *** indicate that $p > 0.05$, $p < 0.01$, and $p < 0.05$, respectively.

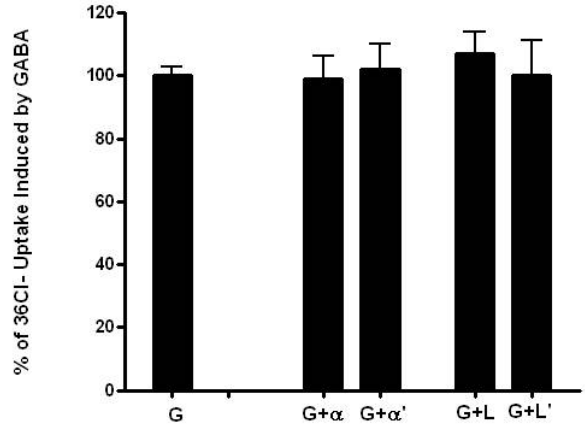

Fig. 10 Effects of α-terpineol (A), and linalool (B) at concentrations of 500 μM and 1 mM on $^{36}Cl^-$ uptake by American cockroach ventral nerve cord membrane microsacs induced by 1 μM of GABA. The amount of $^{36}Cl^-$ uptake induced by GABA was measured as 100%. G, 1 μM GABA; α, 500 μM α-terpineol; α', 1 mM α-terpineol; L, 500 μM linalool; L', 1 mM linalool. Values represent means ± S.E.M., n=3.

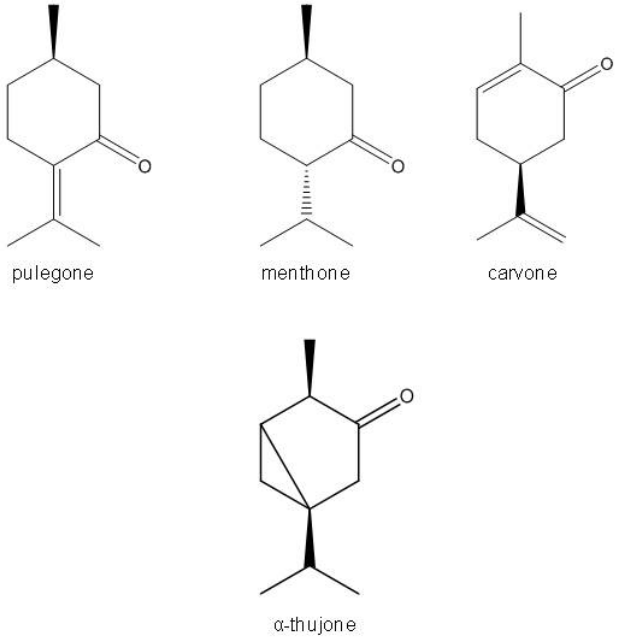

Fig. 11 Structures of monoterpenoids with ketone group.

CHAPTER 3. QUANTITATIVE STRUCTURE-ACTIVITY RELATIONSHIP EVALUATION OF MONOTERPENOID BINDING ACTIVITIES TO HOUSE FLY GABA RECEPTOR

Fan Tong, and Joel R. Coats

A paper to be submitted to *Journal of Agricultural and Food Chemistry*

ABSTRACT

Monoterpenoids are a large group of plant secondary metabolites. Many of these naturally occurring compounds showed good insecticidal potency on pest insects. Previous studies in this laboratory have indicated that some monoterpenoids have positive modulatory effects on insect GABA receptors. In this study, we determined the key properties of monoterpenoids involved in the monoterpenoids' binding activity at the GABA receptor. A [^{3}H]-TBOB-binding assay in house fly head membrane homogenates was used to screen 22 monoterpenoids' binding activities at a concentration of 500 μM to develop quantitative structure-activity relationship (QSAR) models. Multiple linear regression models were determined for nine monoterpenoids that showed significantly strong effects on [^{3}H]-TBOB binding, as well as in nine p-menthane analogs with at least one oxygen atom attached to the ring. In the first model for nine monoterpenoids with strong effects on TBOB binding, the Mulliken charge on carbon 4 and the log P

value were strongly involved in the multiple regression, and in the second model, for the nine p-menthane analogs, the total energy and the Mulliken charge on carbon 6 showed a significant relationship with [^{3}H]-TBOB binding activities.

Keywords: Monoterpenoid; insecticide; quantitative structure-activity relationship (QSAR); GABA receptor; [^{3}H]-TBOB

INTRODUCTION

Monoterpenoids are derived from or structurally related to monoterpenes, which are terpenes containing two isoprene units. Monoterpenoids are mostly found in plant essential oils. These natural products are secondary metabolites in higher-order plants. Unlike primary metabolites of plants, which are necessary in growth, development, and reproduction of plants, monoterpenoids are often involved in plant defense against herbivores and pathogens (*1-3*).

For hundreds of years, monoterpenoids have been used in the production of food additives, cosmetics, perfumes, shampoos and other personal care products, due to their pleasant, natural flavors and fragrances, and/or their antimicrobial properties. In the past 20 years, some monoterpenoids have shown very good insecticidal or insect-repellent activities (*1, 4-12*). These compounds have been considered as good alternatives for conventional synthetic insecticides, based on their wide-spectrum insecticidal activities, their low toxicities to mammals and other non-target organisms, and biodegradability in the environment (*3, 13-15*).

Although some monoterpenoid insecticides are used commercially, the mechanisms of action of these botanical insecticides have not been fully elucidated. Previous studies on modes of action of some monoterpenoids revealed several possible protein targets in the insect nervous system, including ionotropic γ-aminobutyric acid (GABA) receptors (*16-18*), octopamine receptors (*19, 20*), tyramine receptors (*21, 22*), acetylcholinesterase (AChE) (*23*)and nicotinic acetylcholine receptors (nAChR) (*24, 25*). Among these targets, the ionotropic GABA receptor may be involved in the fast response to monoterpenoids in both central and peripheral nervous system in insects. From earlier studies of monoterpenoids' effects on insect GABA receptors, some monoterpenoids were indicated to bind to the insect GABA receptor, and interfere with the chloride movement mediated by GABA. Thymol was reported to be a positive allosteric modulator of a homo-oligomeric GABA receptor from *Drosophila melanogaster (16)*. Thymol, carvacrol, and pulegone were also indicated to increase the binding of [^{3}H]-TBOB, which is a non-competitive antagonist for insect GABA receptors, in house fly head membrane preparations, and potentiate $^{36}Cl^{-}$ uptake induced by GABA in ventral nerve cords of American cockroach (*17*). However, the quantitative-structure activity relationship (QSAR) between monoterpenoid molecules and their binding activities to insect GABA receptor has not been evaluated yet, so the specific physicochemical properties of monoterpenoids that

determine the binding of a monoterpenoid to the GABA receptor are unknown.

In this paper, the TBOB-binding activities of 22 monoterpenoids to house fly GABA receptors were determined using radioligand binding assays, and the binding data were used to build QSARs with a variety of descriptors, which can describe physical, chemical, structural, and electronic properties of monoterpenoids tested in this binding assay; they also help to explain ligand-receptor relationships. The QSAR models will be helpful to illustrate and predict the interactions between monoterpenoids and insect GABA receptors and provide guidance for searches for more potent analogs.

MATERIALS AND METHODS

Chemicals. Monoterpenoids (eugenol, thymol, carvacrol, linalool, α-terpineol, menthol, vanillin, citronellal, citronellic acid, cinnamic acid, 1,8-cineole, 1,4-cineole, limonene epoxide, limonene, p-cymene, methyl salicylate, phenethyl propionate (PEP), piperonal, safrole, camphor, menthol, pulegone) and the GABA receptor antagonist convulsant picrotoxin (PTX) were purchased from Sigma-Aldrich Chemical Co., St. Louis, MO. The [^{3}H]- *t*-butylbicycloorthobenzoate (TBOB) was purchased from GE Healthcare Life Sciences, Piscataway, NJ.

[^{3}H]-TBOB Binding Assay. House fly heads (0.8g) were homogenized in 10 mM tris-HCl buffer (pH 7.5) containing 0.25M sucrose (buffer A) with a glass homogenizer. The homogenate was centrifuged at 1,000*xg* for 5 minutes. The

supernatant was filtered through four layers of cheesecloth and centrifuged at 25,000*xg*, and 4 ^{o}C for 40 minutes. The supernatant was discarded, and the pellet was homogenized and resuspended in ice-cold buffer A for 30 minutes. The suspension was centrifuged at 25,000*xg*, and 4 ^{o}C for 40 minutes. The final pellet was suspended in 2 mL of 10 mM phosphate buffer (pH 7.5) containing 300 mM NaCl (buffer B) and used directly for the assays. Lowry protein assay was used to determine a final concentration of protein (*26*).

Membrane preparation containing 20 µg of protein was incubated for 90 minutes at room temperature (20 ^{o}C) with 4 nM [^{3}H]-TBOB (specific activity 22Ci mmol^{-l}), 500 µM of candidate monoterpenoids and buffer B. The total assay buffer volume was 200 µL. After incubation, samples were filtered on glass fiber filter papers (Whatman GF/B) and washed with 10 mL ice-cold buffer B three times. Radioactivity was measured by a Beckman liquid scintillation counter LS5000 CE. Specific binding was used to estimate the binding activities of candidate chemicals and was calculated as the difference between the total ^{3}H-bound and nonspecific ^{3}H-bound with 100 µM PTX. The specific binding was 60-70% of total binding at 4 nM [^{3}H]-TBOB. Each experiment was repeated at least three times using different membrane homogenates. (*17, 27, 28*)

The specific [^{3}H]-TBOB binding value in the absence of the any candidate chemicals was expressed as 100%. The percentage of a monoterpenoid's effect at

a concentration of 500 μM on the [^{3}H]-TBOB binding to house fly head membrane preparations was calculated using the following formula:

percentage monoterpenoid's effect = (specific [^{3}H]-TBOB binding with 500 μM monoterpenoid/ specific [^{3}H]-TBOB binding w/o monoterpenoid) * 100

The difference between the effect of a monoterpenoid and 100 was used to develop QSAR models in the next step.

QSAR Analysis. Descriptors related to receptor-ligand interactions, including log P (octanol-water partition coefficient), Mulliken charge for each carbon of the ring, dipole moment, total energy, highest occupied molecular orbital (HOMO), lowest unoccupied molecular orbital (LUMO), and electrotopological state (E-state), were selected to describe chemical, physical, molecular, and topological properties of the monoterpenoids. Mulliken charge, dipole moment, total energy, HOMO, and LUMO were calculated in GAMESS, using an interface with ChemBio3D Ultra 12.0 (CambridgeSoft Corp., Cambridge, MA). The energy and geometry of all candidate monoterpenoids were analyzed with a split valence basis set and a polarization function (6-31*d) calculation using GAMESS. Log P values were calculated in a free on-line cheminformatics services provided by www.molinspiration.com.

Electrotopological state descriptors (E-state) were calculated in E-Calc (SciVision, Inc., Burlington, MA).

Descriptors and the [^{3}H]-TBOB binding data were analyzed by simple linear and multiple linear regressions for evidence of correlation. The [^{3}H]-TBOB binding data were shown as log (TB), which expresses the log value of the difference between the percentage of effect of a monoterpenoid on [^{3}H]-TBOB binding and 100. The square of the correlation coefficient (R^2) and cross-validation (Q^2) were used to evaluate the fitness of regression models. All linear and multiple regressions were analyzed using SAS 9.1. Regression models with $R^2 > 0.8$ were selected first, and then validation of these models were examined by using the leave-one-out method using the following equations:

$$\text{Cross-validation } Q^2 = 1 - (\text{PRESS/SSTO})$$

where

$$\text{PRESS} = \Sigma_y (Y_{\text{predicted}} - Y_{\text{actual}})^2$$

and SSTO is the sum of squares total. Any models with cross validation (Q^2) values >0.6 were suggested to be a nonrandom relationship (*29*).

RESULTS

Effects of monoterpenoids on [^{3}H]-TBOB binding to house fly GABA receptors. The 22 monoterpenoids (Fig. 1) were selected to test their efficacies to modulate the [^{3}H]-TBOB binding in house fly head membrane preparations at a

concentration of 500 μM. Among these candidates, only nine of them resulted in statistically significant ($p<0.05$) changes of [^{3}H]-TBOB binding in the house fly head membrane homogenates. In these nine compounds, 1,8-cineole, carvacrol, citronellic acid, pulegone, and thymol potentiated the [^{3}H]-TBOB binding in house fly head membrane preparations, which indicated that these monoterpenoids could bind to the house fly GABA receptor at a different binding site from the TBOB binding site; the others, including camphor, menthol, safrole, and vanillin, inhibited the [^{3}H]-TBOB binding significantly, suggesting that these compounds could also bind at the house fly GABA receptor, either at the TBOB binding site to inhibit the TBOB binding competitively, or at an allosteric binding site to inhibit TBOB binding non-competitively (Table 1). The binding data for these nine monoterpenoids were used to develop the QSAR model in the following step. Furthermore, we found that six monoterpenoids out of these nine compounds showed structural similarity to p-menthane, so we also selected a subset of nine p-menthane analogs, each with at least one oxygen atom bonded to the ring from the 22-monoterpenoid list to develop another QSAR model.

Numbering of carbon atoms for monoterpenoids. The structures of the selected 22 monoterpenoids show some similarities. Most of them are cyclic monoterpenoids (cyclohexane or aromatic ring), except for citronellic acid, which can exist in a conformation that can resemble an aliphatic ring, and all of them

have at least one carbon atom in the ring bonded with an oxygen atom. Based on these structural details, we numbered the carbon connected to an oxygen atom on the ring as carbon 1. (Fig. 2, and Fig. 3)

QSAR models. The log values of differences between the percentage effects of monoterpenoids on TBOB binding and 100 were used to develop QSAR models (log (TB)). For the nine monoterpenoids which showed significant differences for [^{3}H]-TBOB binding, we found an excellent multiple regression model which contained log P, and the Mulliken charge on carbon atom 4 (MULC-C4): log (TB) = 2.02 (±0.27) + 0.13 (±0.05) [log P] + 2.81(±0.69) [MULC-C4]. The fitness of the model and the cross-validation provided good evidence of this multiple regression model (N = 9, F = 13.73, R^2 = 0.82, Q^2 = 0.78). Observed and calculated log (TB) values are compared in Table 2, and a good correlation between them is shown in Fig. 4. This model indicates the monoterpenoid's binding efficiency to house fly GABA receptor is positively related to the partition coefficient of this molecule as well as the charge on the carbon atom 4.

The second QSAR model was developed from nine monoterpenoids which are analogs of p-menthane with at least one oxygen atom connected to the ring. The structures and the numbering of carbon atoms are shown in Fig. 3. The Mulliken charges on the carbon atom 6 (MULC-C6) and total energy (TE) were illustrated to be involved in this model, which is described: log (TB) = 67.3 (±21.3) + 227*10^{-6}

($\pm 74*10^{-6}$) [TE] + 0.65 (±0.15) [MULC-C6]. Strong evidence based on the fitness of the model and the cross-validation showed a multiple linear relationship of this model (N = 9, F = 11.34, R^2 = 0.80, Q^2 = 0.74). Observed and calculated log (TB) values are compared in Table 3, and a good correlation between them is shown in Fig. 5. This relationship suggests that as the charge on the carbon atom 6 or the total energy increases, the monoterpenoid's binding efficiency also increases.

DISCUSSION

Of the 22 monoterpenoids tested in this study, two subsets of them were selected to develop QSAR models, due to their significant effects on the [^{3}H]-TBOB binding in house fly head membrane preparations, to evaluate the relationships between chemical properties of these monoterpenoids and their binding activities at insect GABA receptors. We found that moieties apparently needed to contain an oxygen atom for these monoterpenoids to interact strongly with the house fly GABA receptor, because all nine compounds with strong effects on the house fly GABA receptor have at least one carbon in the ring bonded to oxygen to form an alcohol, phenol, ketone, ether, or carboxyl group. Moreover, six of these monoterpenoids are analogs of p-menthane, indicating that this type of monoterpenoids skeleton may also play an important role in the binding between monoterpenoids and insect GABA receptors. Based on these structural features, two QSAR models were developed from two subsets of nine compounds to

illustrate relationships between monoterpenoid structures and their GABA receptor binding activities.

For both models, the electronic properties of the monoterpenoids were important to the binding of these compounds to the GABA receptor. As the charge on certain carbon atoms increased, the binding efficiency of monoterpenoids to the receptor also increased. This relationship indicated that the carbon atom 4 (for significant effects on TBOB-binding model) and carbon atom 6 (for p-menthane analogs model) might be directly involved in the binding of these compunds to the house fly GABA receptor. The log P is also a crucial factor in the first QSAR model (significant effects on TBOB binding model). The enhanced log P value improved the binding efficiency of these monoterpenoids to house fly GABA receptors, which suggested the lipophilic interaction might play a key role in the binding between this set of monoterpenoids and the GABA receptor, and lipophilic moieties might be included in the binding site on the GABA receptor. Total energy is another key parameter in the p-menthane analogs model. Total energy has been used in other QSAR studies to show stability of a molecule (*30*). The relationship with total energy indicated that the binding efficiency is related to the stability of monoterpenoids with p-menthane skeleton.

Although some of monoterpenoids showed significant effects on the binding of [^{3}H]-TBOB, which is a non-competitive inhibitor binding to the pictotoxin binding

site at insect GABA receptors, the binding sites of these compounds may not be the same due to their different patterns of [^{3}H]-TBOB binding, as well as their variation in structural features. 1,8-Cineole, carvacrol, citronellic acid, pulegone, and thymol enhanced the [^{3}H]-TBOB binding to house fly head membrane preparations. Among these five chemicals, carvacrol and thymol, which are phenols, may have the same binding site on house fly GABA receptor, based on their similar structures and similar modulatory effects on insect GABA receptor from our previous study (*17*). 1,8-Cineole, citronellic acid, and pulegone, which are a bicyclic ether, acyclic acid, and cyclic ketone, respectively, may not share the same binding site with carvacrol and thymol due to the lack of electron donors, which is important for the binding of phenols to the GABA receptor (*31*), although the previous study from our laboratory demonstrated that pulegone showed similar effects with thymol and carvacrol on [^{3}H]-TBOB binding and $^{36}Cl^{-}$ uptake in insect nervous system (*17*). The other four monoterpenoids, camphor, menthol, safrole, and vanillin significantly inhibited the [^{3}H]-TBOB binding to house fly head membrane preparations, but we could not determine if this inhibition was competitive (binding to the same site as GABA-receptor inhibitors TBOB and picrotoxin to inhibit GABA responses) or non-competitive (binding to an allosteric site, not the TBOB binding site) from this assay. According to A.C. Hall *et al.(32)*, camphor and menthol, analogs of p-menthane, showed positive modulatory effects

on recombinant human $GABA_A$ receptor expressed in *Xenopus* oocytes, which indicated they did not bind to the non-competitive antagonist (TBOB/picrotoxin) binding site on human $GABA_A$ receptor. H. Aoshima *et al.(33)* found that vanillin inhibited GABA-induced Cl^- current non-competitively in the rat $GABA_A$ receptor expressed in *Xenopus* oocytes at a high concentration (10mM), which illustrated that vanillin might also bind to the non-competitive antagonist binding site on mammalian GABA receptors. However, in insects, we have yet to find evidence for the binding sites of these four monoterpenoids for modulation of insect GABA receptors.

Research on the mechanism of action of monoterpenoids on insects faces numerous challenges. Monoterpenoids are a large group of compounds with diverse structural skeletons and functional groups. They may have various targets in insects and mammals. Many researchers have shown mechanisms of action of monoterpenoids on targets other than GABA in either insects or mammals, such as octopamine receptors (*19, 20*), tyramine receptors (*21, 22*), nicotinic acetylcholine receptors (*24, 25*), thermo-transient receptors (*34, 35*) and acetylcholinesterase (*23*). Furthermore, even when the monoterpenoids are targeting the same protein (receptors or enzymes), different categories of monoterpenoids may have different binding sites and different effects on the functions of these targets. Octopamine receptors in insects, as an example, were shown as targets for many

monoterpenoids, however, some monoterpenoids enhanced the binding of octopamine to the receptor, including pulegone, vanillin, and p-cymene, while others inhibited the octopamine binding to the receptor, including carvacrol, piperonal, etc. (*19, 20*) On the other hand, a single monoterpenoid may affect several targets and therefore have multiple modes of action contributing to the toxicity to insects. For example, carvacrol and pulegone were illustrated to have effects on both octopamine receptors and GABA receptors in insects (*17, 19, 20*).

In summary, we studied the interaction between the chemical and structural properties of monoterpenoids and their binding activities of house fly GABA receptors. Our two QSAR models provided evidence that (i) functional groups with oxygen atom(s) may be necessary for the binding, (ii) electronic properties on carbons 4 and 6 affect the binding activities, and (iii) the hydrophobicity and total energy level of monoterpenoid molecules play key roles for the binding to the receptor.

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TABLES

Table 1. Effects of monoterpenoids on [^{3}H]-TBOB-binding in house fly head membrane preparations

monoterpenoids	% of [^{3}H]-TBOB-binding (mean ± SEM)	Difference from 100%
1,4-cineole	117 ± 20	17
1,8-cineole	122 ± 8*	22*
α-terpineol	95 ± 10	5
camphor	70 ± 7*	30*
carvacrol	156 ± 2*	56*
cinnamic acid	101 ± 5	1
citronellal	92 ± 7	8
citronellic acid	138 ± 12*	38*
eugenol	88 ± 7	12
limonene	86 ± 7	14
limonene oxide	141 ± 20	41
linalool	124 ± 22	24
menthol	80 ± 10*	20*
menthone	86 ± 13	14
methyl salicylate	103 ± 8	3
p-cymene	90 ± 8	10
phenethyl propionate	95 ± 10	5
piperonal	93 ± 8	7
pulegone	132 ± 4*	32*
safrole	43 ± 3*	57*
thymol	180 ± 12*	80*
vanillin	82 ± 5*	18*

* indicates significant difference from 100% ($p<0.05$).

Table 2. [^{3}H]-TBOB binding data, calculated values, and residual values for nine monoterpenoids with significant effects

monoterpenoids	observed TB[a]	observed log (TB)	calculated log (TB)	residual
1,8-cineole	22	1.33	1.37	0.04
camphor	30	1.47	1.51	0.04
carvacrol	56	1.75	1.85	0.1
citronellic acid	38	1.58	1.57	-0.01
menthol	20	1.31	1.4	0.09
pulegone	32	1.51	1.32	-0.19
safrole	57	1.75	1.72	-0.03
thymol	80	1.9	1.79	-0.11
vanillin	18	1.25	1.32	0.07

[a] TB means the difference between the percentage effect of a monoterpenoid on [^{3}H]-TBOB-binding and 100%.

Table 3. [^{3}H]-TBOB binding data, calculated values, and residual values for nine p-menthane analogs

monoterpenoids	observed TB[a]	observed log (TB)	calculated log (TB)	residual
1,4-cineole	17	1.24	1.37	0.13
1,8-cineole	22	1.33	1.33	0
camphor	30	1.47	1.56	0.09
carvacrol	56	1.75	1.84	0.09
limonene oxid	41	1.61	1.5	-0.11
menthol	20	1.31	1.16	-0.15
menthone	14	1.13	1.26	0.13
pulegone	32	1.51	1.46	-0.05
thymol	80	1.9	1.76	-0.14

[a] TB means the difference between the percentage effect of a monoterpenoid on [^{3}H]-TBOB-binding and 100%.

FIGURES

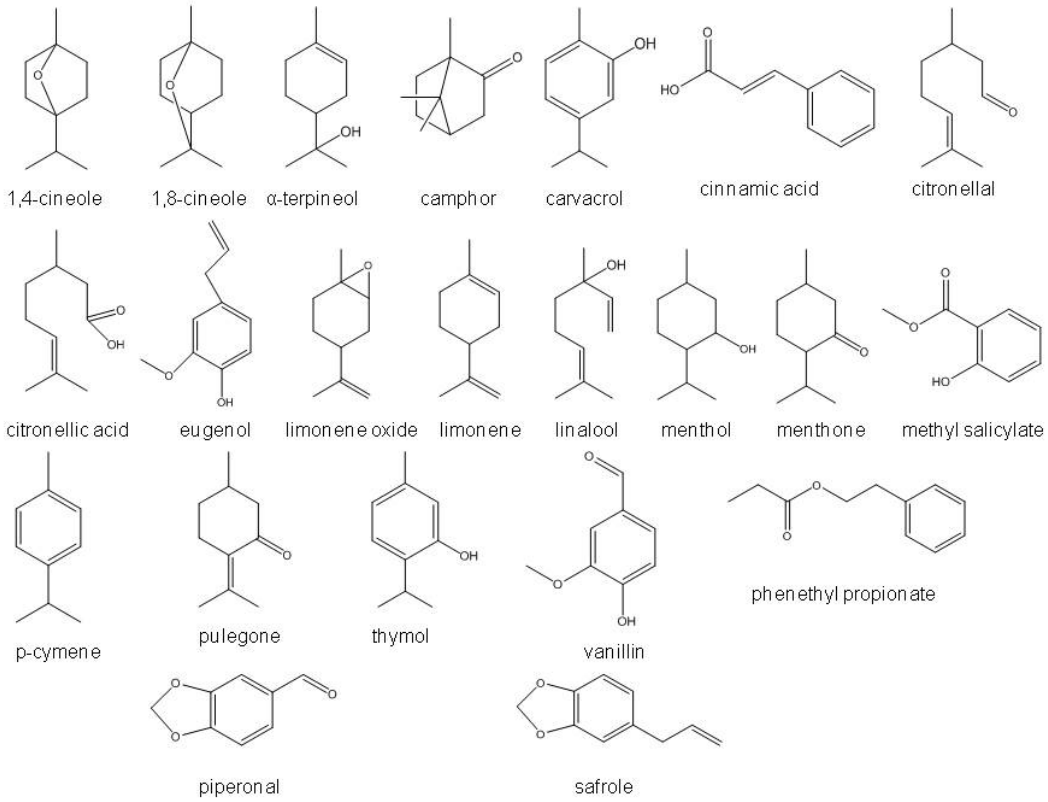

Figure 1. Structures of 22 monoterpenoids tested in this study.

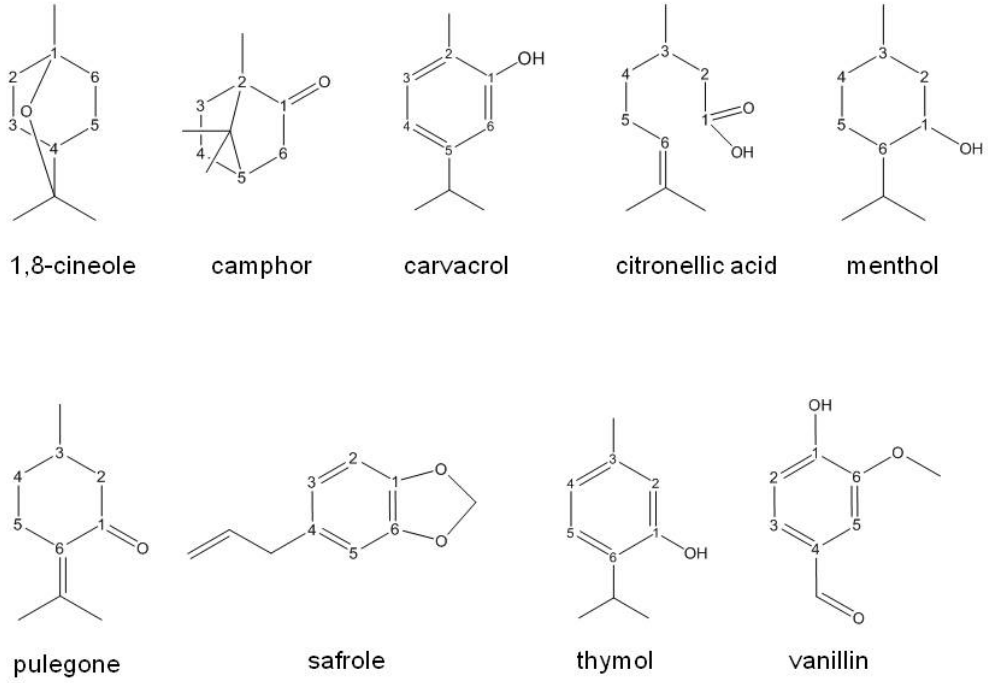

Figure 2. Numbering of the carbon atoms for monoterpenoids with significant effects on [^{3}H]-TBOB-binding to house fly head membrane preparations.

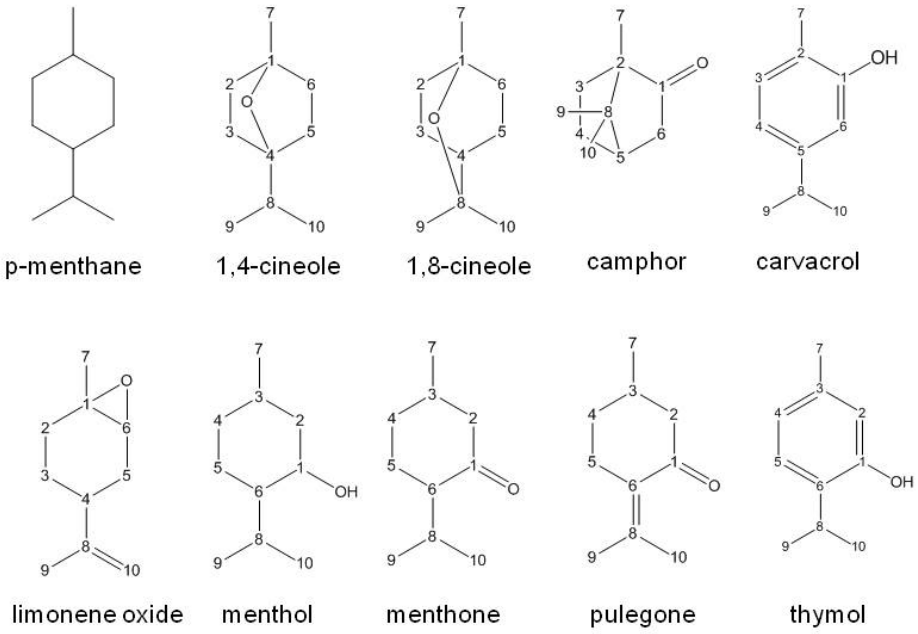

Figure 3. Structures of p-menthane and numbering of the carbon atoms for nine analogs with at least one oxygen atom connected with the ring.

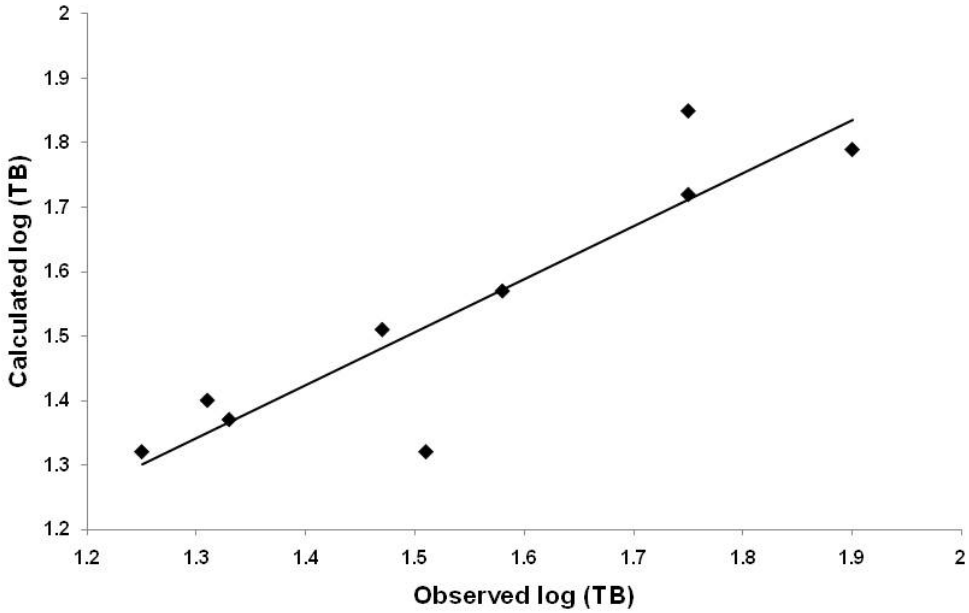

Figure 4. Plot of observed-versus-calculated [^{3}H]-TBOB-binding activities for nine monoterpenoids with significant effects on [^{3}H]-TBOB-binding. TB is the difference between the percentage effect of a monoterpenoid on [^{3}H]-TBOB-binding and 100.

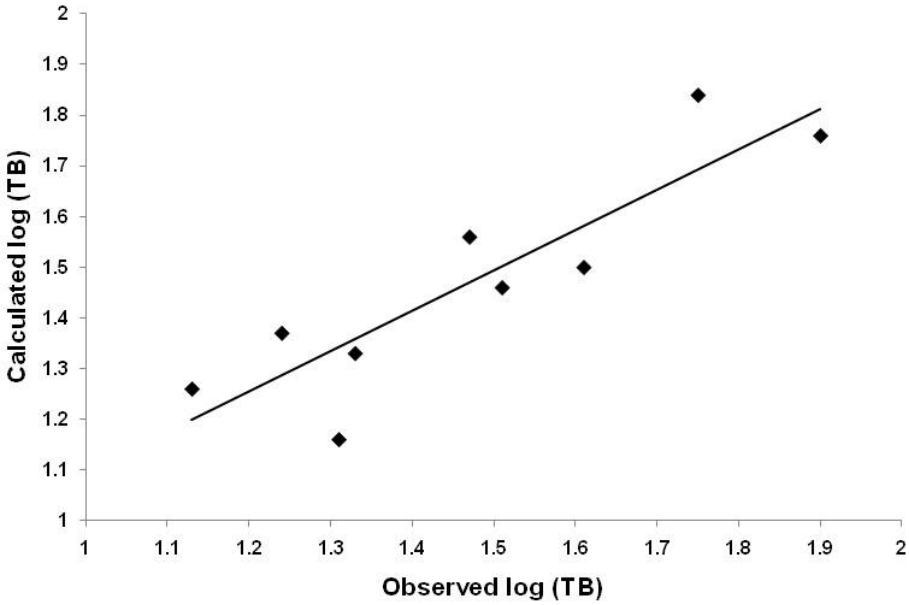

Figure 5. Plot of observed-versus-calculated [^{3}H]-TBOB-binding activities for nine analogs of p-menthane. TB is the difference between the percentage effect of a monoterpenoid on [^{3}H]-TBOB binding and 100.

CHAPTER 4. EFFECTS OF A MONOTERPENOID INSECTICIDE/ACARICIDE, CARVACROL, ON [^{14}C]-NICOTINE BINDING TO THE *MUSCA DOMESTICA* (HOUSE FLY) NICOTINIC ACETYLCHOLINE RECEPTOR

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ABSTRACT

BACKGROUND: Recent studies on a phenolic monoterpenoid, carvacrol which is found in many plant essential oils, such as oils of thyme, oregano, and Alaska yellow cedar, showed a high level of activity against pest arthropods, but the mechanisms of its action as a natural pesticide are not fully understood. Here we address the potential for carvacrol to bind in a membrane preparation containing insect nicotinic acetylcholine receptors (nAChR). RESULTS: [^{14}C]-Nicotine binding assays with *Musca domestica* (house fly) nAChR were used in this study to evaluate carvacrol's binding to nAChR, therefore acting as a modulator of the receptor. Carvacrol showed a concentration-dependent inhibition of [^{14}C]-nicotine binding in a membrane preparation of house fly heads containing nAChR, with an IC_{50} = 1.4 μM. The inhibition demonstrates a non-competitive pattern. CONCLUSION: Carvacrol binds to house fly nAChR at a binding site distinct from nicotine and acetylcholine, and inhibits the function of house fly nAChR in a novel

way.

Key Words: monoterpenoid, carvacrol, nAChR, [^{14}C]-nicotine

1. INTRODUCTION

Carvacrol is present in many plant essential oils, such as thyme oil, pepperwort oil, Alaska yellow cedar oil and oregano oil. It is a phenolic monoterpenoid, which is a class of secondary metabolites found in plant essential oils. Carvacrol has a pleasant oregano smell, a pizza-like taste, and antimicrobial properties,(1-3) so it is widely used as a food additive. Toxicological studies in recent decades illustrated that carvacrol and many other monoterpenoids have very good acute toxicities on various invertebrate pest species including insects, acari, and nematodes.(4-10) Moreover, these naturally occurring compounds are very biodegradable in the environment and have low toxicity to mammals (acute and chronic), fish, and other non-target organisms.(11) All these advantages make monoterpenoids a good choice as an alternative to synthetic chemicals in insect pest management.

The mechanism of action of monoterpenoid insecticides on insects is not fully understood, although some possible targets have been suggested and tested including γ-aminobutyric acid (GABA) receptor(12-15), octopamine receptor(16, 17), tyramine receptor and acetylcholinesterase (AChE)(18, 19). The current study addresses the potential for carvacrol to exert its insecticidal effect by binding to the nicotinic acetylcholine receptor (nAChR) in the house fly central nervous system.

The nAChR is a cholinergic receptor that forms ligand-gated ion channels in the plasma membranes of certain neurons in the insect central nervous system. The binding of acetylcholine (ACh), an excitatory neurotransmitter, activates the nAChR, and opens the cation channels on the post-synaptic membrane. The diffusion of Na^+ and K^+ across the receptor causes depolarization that opens voltage-gated sodium channels, which results in firing of the action potential(20, 21). In insects, nAChR is a major target for many insecticides. Nicotine and neonicotinoids act as receptor agonists, which mimic the function of acetylcholine. The binding of neonicotinoids to the nAChR can cause excitatory effects on the insect nervous system(22-24). Another type of insecticide, spinosad, bind to the nAChR and enhance the sensitivity of the nAChR to the endogenous neurotransmitter, acetylcholine (25, 26). In mammalian models, some monoterpenoids, such as borneol and camphor, have been found to inhibit the nAChR-mediated effects non-competitively(27, 28). However, no evidence has been shown that monoterpenoids can modulate the function of nAChR in insects.

The purpose of this study was to determine if carvacrol can bind to nAChR in house fly central nervous system and modulate the function of nAChR.

2. RESULTS

2.1 [^{14}C]-Nicotine saturation binding assay

Nicotine is an agonist for the house fly nAChR. It binds to the same binding site

as the endogenous ligand, acetylcholine(29, 30). Fig. 2 shows the [^{14}C]-nicotine saturation binding assay. The dissociation constant for [^{14}C]-nicotine binding to the membrane homogenate of house fly head containing nAChR, the K_d value, is 308±26 nM, with 50-60% of specific binding out of the total binding. The maximal binding capacity, B_{max} value, for the binding is 15.6 ±0.3 (pmol/mg protein).

2.2 Displacement binding of [^{14}C]-nicotine by nicotine, epibatidine, and imidacloprid

Imidacloprid, nicotine and epibatidine have all been demonstrated to be agonists at insect nAChRs(22-24, 31). We used these three compounds to compare and contrast their effects on [^{14}C]-nicotine binding with carvacrol's effect. As demonstrated in Fig. 3, unlabeled nicotine activated a concentration-dependent inhibition of [^{14}C]-nicotine binding, with an IC_{50} = 66±2 µM (Fig. 3 A). Epibatidine, which is a natural nicotinoid, also inhibited the binding of [^{14}C]-nicotine binding to the receptor, with an IC_{50} = 77±7 µM (Fig. 3 B). Another nAChR agonist, imidacloprid, showed no effects on the [^{14}C]-nicotine binding to the house fly heads membrane preparation in this assay (Fig. 3 C). This finding suggests that house fly native nAChRs may have distinct binding sites for nicotine and imidacloprid, at either the same or different receptors.

2.3 Inhibitory binding of [^{14}C]-nicotine by carvacrol

In this assay, carvacrol induced a concentration-dependent inhibitory effect on

[^{14}C]-nicotine's binding to the membrane homogenate with an IC_{50}= 1.4±0.04 μM (Fig. 4), which suggested that carvacrol binds to the house fly nAChR and inhibits the binding of [^{14}C]-nicotine either competitively or non-competitively. From the [^{14}C]-nicotine saturation binding assay in the presence of 100 μM of carvacrol, we found that the binding of carvacrol enhanced the dissociation constant for [^{14}C]-nicotine binding to the house fly nAChR preparation, the Kd value, from 308 nM to 2,879±167 nM. Carvacrol also decreased the maximal binding capacity, B_{max} value, for the binding from 15.6 (pmol/mg protein) to 10.5 ±0.3 (pmol/mg protein) (Fig. 5 and Table 1). The higher Kd value and lower B_{max} value indicate that carvacrol may non-competitively inhibit the [^{14}C]-nicotine binding by binding to nAChR at an allosteric site.

3. DISCUSSION

The [^{14}C]-nicotine house fly nAChR inhibition binding assay and saturation binding assay with carvacrol demonstrated that carvacrol can bind to the house fly nAChR, and may function as a modulator to the receptor. However, unlike acetylcholine, nicotinoids (e.g. nicotine, and epibatidine), and neonicotinoids (e.g. imidacloprid), carvacrol is not considered to be an agonist of the house fly nAChR for two reasons. First, in structure, carvacrol has neither an easily protonated atom (like the quaternary nitrogen in acetylcholine and nicotine) at a physiological pH, nor an electronegative moiety (like the nitro group in imidacloprid) (Fig. 1), which is

necessary for the binding of nAChR agonists to the tryptophan residue (for acetylcholine and nicotine binding) or lysine/arginine/histidine residues (for imidacloprid binding) at the nAChR binding pocket(32, 33). Secondly, from the [^{14}C]-nicotine binding assay in this study, we demonstrated that carvacrol bound to nAChRs in house fly head at a binding site distinct from nicotine and inhibited the binding of [^{14}C]-nicotine to the house fly nAChRs non-competitively with an IC_{50} = 1.4±0.04 μM. The non-competitive inhibition of [^{14}C]-nicotine binding to the nAChR by carvacrol indicated that carvacrol serves as a non-competitive antagonist of the nAChR in the house fly nervous system, because the binding of carvacrol may also decrease the binding affinity of the endogenous ligand, acetylcholine, to the nAChR, which will reduce the conductance of cations across the post-synaptic membrane and subsequently cause an inhibitory effect on the nervous system. However, from this radioligand binding assay alone, it cannot be confirmed whether carvacrol can interfere with cation movement stimulated by nAChR agonists or not. Further evidence is required to verify the hypothesis that carvacrol acts as a non-competitive inhibitor of insect nAChRs.

Some monoterpenoids, such as borneol and camphor, have been demonstrated to be non-competitive inhibitors of mammalian nAChRs. Park *et al.* reported that, in bovine adrenal cells, both borneol and camphor inhibited the increase of intracellular calcium and sodium level induced by an nAChR agonist,

1,1-dimethyl-4-phenylpiperazinium iodide (DMPP), but did not change the binding of [^{14}C]-nicotine to the nAChR (27, 28).

Besides binding to the insect's nAChRs, there are other possible targets for carvacrol. Previous studies in this laboratory have demonstrated that carvacrol and two other monoterpenoids, thymol and pulgeone, enhanced the binding of [^{3}H]-TBOB to GABA receptors in a preparation from house fly heads, and also increased the chloride uptake activated by GABA in preparations of ventral nerve cord of American cockroach. These findings suggested that carvacrol is a positive allosteric modulator for insect GABA receptors, and can thereby cause an inhibitory effect on the insect nervous system(15). Enan reported that carvacrol was an agonist of the octopamine receptor in American cockroach, with a high sensitivity, based on cAMP production, heartbeat, and [^{3}H]-octopamine binding to the receptor(16, 17). Carvacrol and its isomer, thymol, were also demonstrated in *Drosophila melanogaster* and two nematode species, *Caenorhabditis elegans* and *Ascaris suum*, to interact with a tyramine receptor, which is a G-protein-associated receptor and negatively coupled to adenylate cyclase through interaction with the Gi-protein(19). Carvacrol was shown to inhibit [^{3}H]-tyramine binding to the tyramine receptor expressed from S2 cells, and to mimic the function of tyramine to decrease the cAMP level and enhance the intracellular calcium levels in S2 cells expressing tyramine receptors(19). Targeting octopamine receptors or tyramine

receptors may result in a slow toxicity to insects due to the delayed second-messenger signal transduction activated by G-protein pathways. Targeting the ionotropic receptors, such as GABA receptors and nAChRs, may contribute to fast knockdown of insects, since both GABA receptors and nAChRs are found in the neuromuscular junction in insects.

In addition to targeting these receptors of neurotransmitters, carvacrol was also identified as an activator of fruit fly thermo-transient receptor potential (thermoTRP) channels, which control hot or cold sensation in insects(34, 35). Carvacrol and many other monoterpenoids, such as eugenol, menthol, carveol and thymol, were also observed to be inhibitors of non-thermoTRP channels, which respond to various environmental signals including natural chemicals and mechanical stimuli, in *Drosophila* by using the whole-cell recording patch-clamp method from Schneider 2 and *Drosophila* photoreceptor cells(36). It is not known whether the effects of monoterpenoids on TRP channels are to their toxicities to insects.

In summary, the results of the [^{14}C]-nicotine binding assay in house fly indicated that a phenolic monoterpenoid insecticide, carvacrol, can bind to house fly nAChRs at a novel binding site. The binding of carvacrol to this binding site inhibited the binding of [^{14}C]-nicotine non-competitively in a totally different binding pattern from other nAChR agonists, such as acetylcholine, nicotine, epibatidine, and the neonicotinoid insecticide imidacloprid. This finding provides promising

evidence that the insect nAChR could serve as a novel target for monoterpenoid insecticides.

4. MATERIALS AND METHODS

4.1 Materials

Carvacrol (5-isopropyl-2-methylphenol), epibatidine, and imidacloprid were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). Unlabeled nicotine was purchased from Acros Organics (Geel, Belgium). The [^{14}C]-nicotine was purchased from American Radiolabeled Chemicals, Inc. (St. Louis, MO). Structures of carvacrol, epibatidine, nicotine, and imidacloprid are shown in Fig. 1.

4.2 Tissue preparation

House fly heads (0.8 g) were homogenized in 5 ml of 10 mM tris-HCl buffer (pH 7.4) containing 0.25M sucrose (buffer A) with a glass homogenizer. The homogenate was filtered through four layers cheesecloth and centrifuged at 700*xg* for 10 minutes. The pellet was discarded, and the supernatant was re-centrifuged at 125,000*xg* for 60 minutes. The pellet was suspended in 10 mM phosphate buffer (pH 7.4) containing 50 mM NaCl (buffer B) and used directly for the assays or stored at -80℃ and used within a week. The Lowry protein assay was used to determine a final concentration of total protein. (37)

4.3 [^{14}C]-Nicotine binding assay

Membrane preparation containing 200 μg of protein was incubated for 70

minutes at room temperature with 2 μM [^{14}C]-nicotine (specific radioactivity 55 Ci mmol^{-1}), different amounts of candidate ligands, and buffer B. The total assay buffer volume was 200 μl. After incubation, samples were filtered on Millipore glass-fiber filters (pore size 1 μm) and washed with 10 ml ice-cold buffer B three times. After washing, filters were vacuum dried, and put into 10 mL of scintillation cocktail (Ultima Gold, PerkinElmer) overnight. Radioactivity was measured on a Beckman LC5000 CE liquid scintillation counter. Specific binding was calculated as the difference between the total ^{14}C-bound and nonspecific ^{14}C-bound with 1 mM of cold nicotine in buffer B solution. The specific binding was 50-60% of the total binding. For the saturation binding assay, 40 nM, 120 nM, 200 nM, 400 nM, 1.2 μM, 2 μM, and 5 μM concentrations of [^{14}C]-nicotine, with or without 100 μM of carvacrol, were used to test the dissociation constant, K_d value, and maximal binding capacity, B_{max} value.(22, 29, 38)

4.4 Data analysis

Results are shown as mean ± standard error of mean (S.E.M.), and were analyzed by using GraphPad Prism software 5.0 (GraphPad Software, Inc., San Diego, CA, U.S.A.). The K_d value is the dissociation constant indicating the affinity between ligands and the nAChR. The B_{max} value is the maximal binding capacity for the ligand binding to the nAChR. The IC_{50} value is the concentration for half maximal inhibition. The two-tailed Student's *t*-test was used to compare data. A

p-value of less than 0.05 was considered to be statistically significant.

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TABLES

Table 1 [^{14}C]-nicotine saturation binding data

	B_{max}(pmol/mg protein)	K_d (nM)
[^{14}C]-nicotine saturation binding	15.6 ±0.3	308±26
[^{14}C]-nicotine saturation binding with 100 µM of carvacrol	10.5 ±0.3	2,879±167

FIGURES

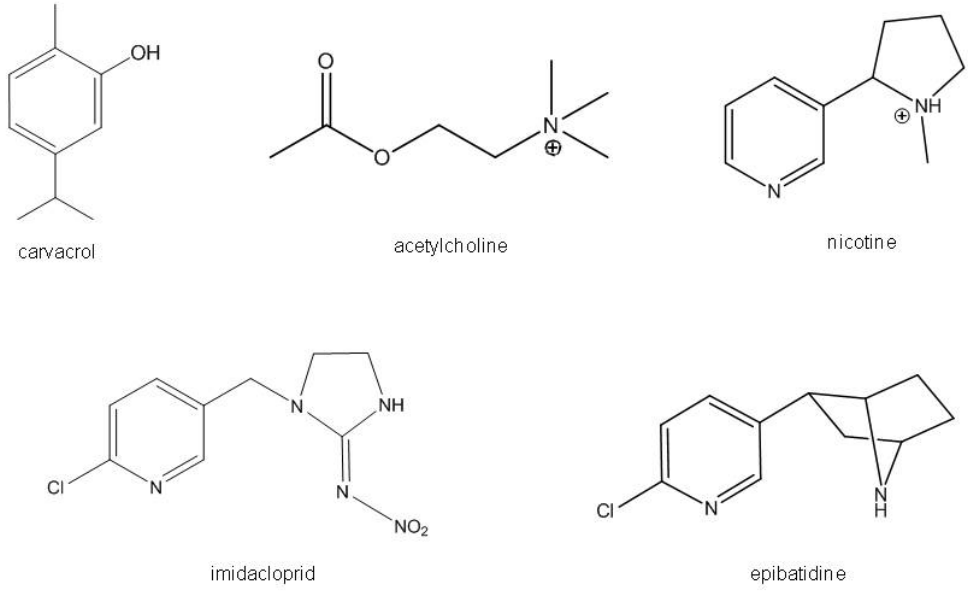

Fig. 1 Structures of carvacrol, acetylcholine, nicotine, imidacloprid, and epibatidine

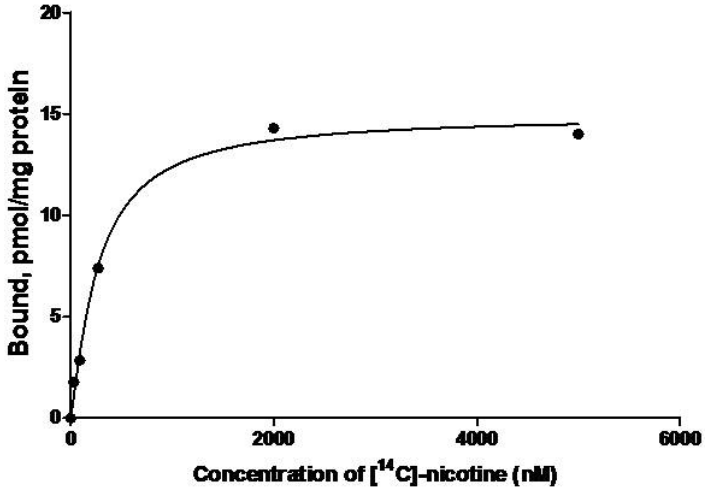

Fig. 2 Saturation binding curve for [^{14}C]-nicotine binding to membrane preparation of house fly heads

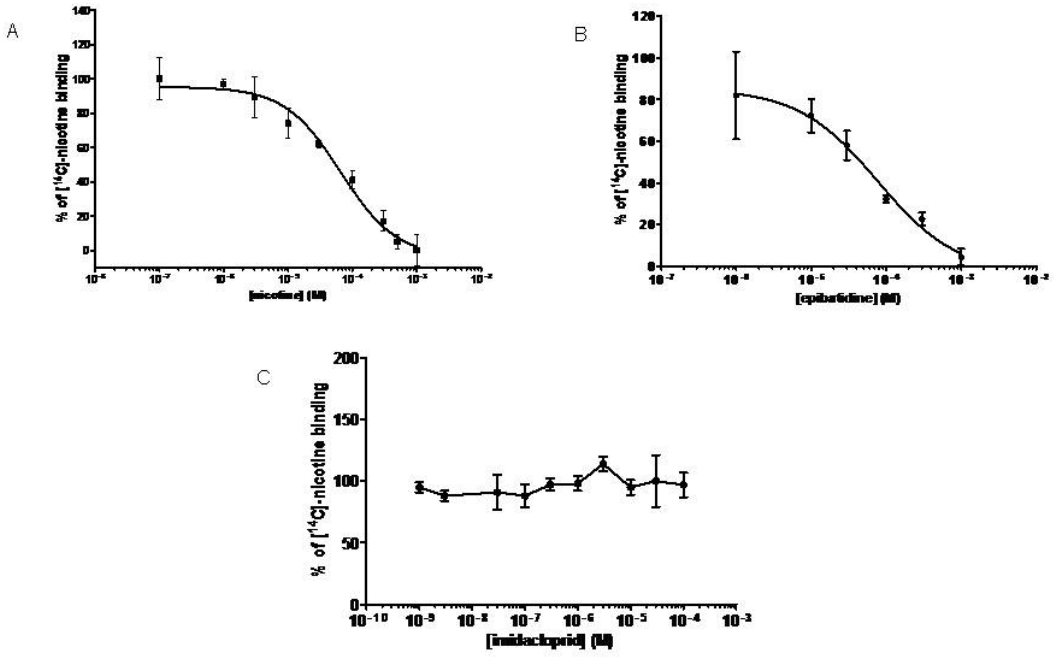

Fig. 3 Displacement binding of [^{14}C]-nicotine by typical nAChR agonists, nicotine (A), epibatidine (B) and imidacloprid (C), to membrane homogenates of house fly heads. Results are expressed as percentages of [^{14}C]-nicotine binding that occur with various concentrations of an agonist, compared to [^{14}C]-nicotine binding in the absence of an agonist (set to 100%). Each point represents the mean ± S.E.M., n=3 (3 unique membrane preparations).

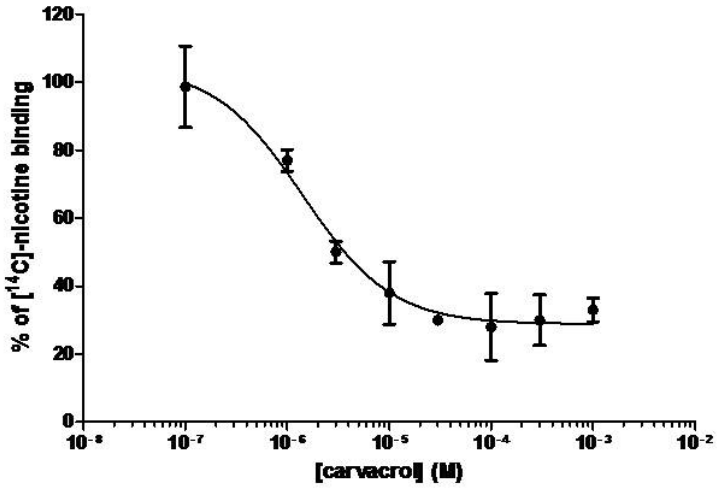

Fig. 4 Inhibitory effects of carvacrol on [^{14}C]-nicotine binding in membrane preparations of house fly heads. The [^{14}C]-nicotine binding in the absence of carvacrol is expressed as 100%. Each point represents the mean ± S.E.M., n=3 (3 unique membrane preparations).

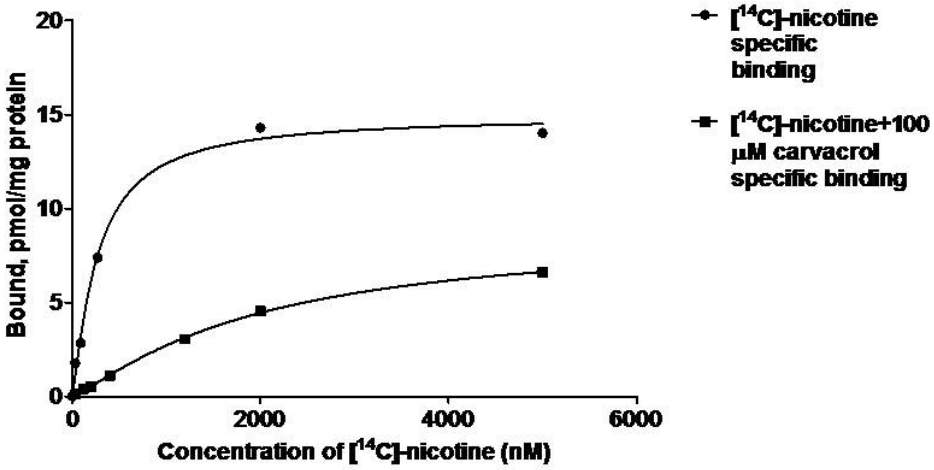

Fig. 5 Saturation binding curves for [^{14}C]-nicotine binding to membrane preparations of house fly heads with/without 100 µM of carvacrol.

CHAPTER 5. GENERAL CONCLUSIONS

To discover and develop safe and potent insecticides is a global perpetual need. Botanical secondary metabolites, monoterpenoids, show potential to be a good alternative to conventional insecticides due to their relatively high toxicity to insect pests, low toxicity to non-target organisms, and biodegradability in the environment. This dissertation has investigated the possible mechanisms of action of monoterpenoid insecticides in the insect nervous system, including an inhibitory neurotransmitter receptor, the GABA receptor, and an excitatory neurotransmitter, the nicotinic acetylcholine receptor (nAChR). By using radioligand binding assays, $^{36}Cl^-$ uptake assays, and quantitative structure-activity relationship assays, monoterpenoid insecticides were suggested to bind to these two receptors, and could interfere with the function of them in the nervous system, which might be the reason why these naturally occurring compounds have toxicity to insects.

In the study on the effects of monoterpenoids on GABA receptors in house flies and American cockroaches (Chapter 2), five monoterpenoids were evaluated by using [^{3}H]-TBOB binding assays and $^{36}Cl^-$ uptake assays. Carvacrol, pulegone, and thymol all showed enhanced effects on both [^{3}H]-TBOB binding in house fly head membrane homogenates, and $^{36}Cl^-$ uptake induced by GABA in ventral nerve cords of American cockroaches. These findings indicated that these three monoterpenoids are all positive modulators of the insect GABA receptor, and they

could cause inhibitory effects on the insect nervous system. The other two monoterpenoids, α-terpineol and linalool, did not show significant effects on either TBOB binding in house flies or $^{36}Cl^-$ uptake in American cockroaches, which indicates that the GABA receptor is not the major target for these two compounds. These results supported the hypothesis that insect GABA receptor is a potential target for some monoterpenoid insecticides.

In the QSAR study of monoterpenoids' binding to house fly GABA receptor (Chapter 3), [^{3}H]-TBOB binding effects in house fly head membrane homogenates of 22 monoterpenoids from different chemical categories were screened, and various chemical and structural parameters were calculated. Two QSAR models were developed from two subsets of monoterpenoids, and demonstrated that log P, total energy, and slight charges on certain carbon atoms were the key factors for monoterpenoids binding to house fly GABA receptor. In the first QSAR model for 9 monoterpenoids with significant effects on TBOB binding in house fly head membrane preparations, log P and the Mulliken charge on carbon 4 were found to have good correlation with the binding. In the second QSAR model for 9 analogs of p-menthane, total energy and the Mulliken charge on carbon 6 were identified to relate to the binding of the receptor. These parameters can be used to help predict the binding activities of monoterpenoids as well as develop new insecticides from untested monoterpenoids and their derivatives with better binding activities.

In the third study, a potent monoterpenoid insecticide, carvacrol, was tested to determine if it has modulating effects on another neurotransmitter in insects, nAChR, by using [^{14}C]-nicotine binding assay in house fly head membrane preparations. From the inhibitory binding assays and saturation binding assays, the results showed that carvacrol inhibited the binding of [^{14}C]-nicotine in house fly head membrane non-competitively. This finding suggested that carvacrol could bind to house fly nAChR at an allosteric binding site other than the nicotine binding site, and inhibit the endogenous neurotransmitter, nACh, binding to the receptor. Based on this finding, the binding of carvacrol to the nAChR could cause an inhibitory effect on the insect nervous system.

Data presented in this dissertation can help reveal the reasons for monoterpenoids' toxicity to insects, and will be also valuable for discovery and development of novel insecticides with higher binding affinity to targets. However, the research on mechanisms of action of monoterpenoids still faces a lot of challenges, and still needs to answer many further questions, such as what are the binding sites of these monoterpenoids on their targets? Are the monoterpenoids' toxicities correlated to the binding activities on these receptors? Are there any other modes of action contributing to the toxicity? By answering all these questions completely, we may get a more thorough and comprehensive understanding of those safe, green, and natural insecticides.

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