A THESIS

FOR THE DEGREE OF MASTER OF SCIENCE

Screening of Juvenile Hormone Receptor Complex Disruptors from Plant Essential Oil Compounds

식물체 정유로부터 곤충유충호르몬 수용복합체의 교란물질 탐색

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ABSTRACT

Insects cause extensive economic damage every year by harming important crops or transmitting diseases as vectors. Insect growth regulators (IGRs), which are compounds that disrupt the insect endocrine system, are attractive control agents due to their high target specificity, low non-target toxicity and relative safety to the environment. Using a modified yeast two-hybrid system transformed with the *Aedes aegypti* juvenile hormone receptor as a reporter system, juvenile hormone agonists (JHAs) and antagonists (JHANs) were identified from plant essential oil compounds. Two JHA and two JHAN candidates

were selected based on their IGR activity, and their chemical structures were similar to that of juvenile hormone III. Interestingly, JHA candidates have acetate functional groups while JHAN candidates have aldehyde functional groups. All JHA and JHAN candidates displayed potent insecticidal activity against mosquito larvae, with relatively low LC_{50} values. In particular, JHAN candidates showed very high levels of insecticidal activity against 2nd instar larvae of *Aedes albopictus* mosquitoes. At sub-lethal concentrations, application of JHA candidates resulted in slower pupation of 4th instar *A. albopictus* larvae, while application of JHAN candidates led to faster pupation. Both JHA and JHAN candidates affected JH-induced gene expression and ovarian development. Topical application of JHA candidates on adult female mosquitoes caused an increase in *hairy* gene expression while application of JHAN reduced the expression of the same gene. Lastly, both JHA and JHAN candidates induced retardation of ovarian growth. Therefore, the IGR candidates identified in this study could be useful for the development of effective and environmentally safe biological control agents.

Key Words: juvenile hormone, insect growth regulator, plant essential oils, juvenile hormone agonist, juvenile hormone antagonist

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

Every year, insect pests are responsible for causing severe economic damage. Damage to agricultural products inflicted by insects amounts to several billion US dollars annually (Boyer, Zhang et al. 2012). Also, blood feeding insects carry various pathogens and act as vectors of disease. They are responsible for more than one million deaths per annum (Hill, Kafatos et al. 2005). Chemical insecticides including organophosphates, organochlorides and carbamates have been commonly used in order to minimize the losses as much as possible. These chemicals are effective, capable of controlling a wide variety of pests. However, these chemical insecticides have become environmental or health threats, due to their high toxicity. Furthermore, development of insect resistance to these drugs is slowly making them obsolete.

Insect growth regulators (IGRs) are specific to target insects and display relatively low toxicities towards non-target species compared to chemical pesticides, which make them attractive alternatives to conventional chemical pesticides. Commercially available IGRs are divided into three main types depending on their modes of action (Pener and Dhadialla 2012). The first group is known as the juvenile hormone agonists (JHAs) which mimic juvenile hormone (JH) activity and fatally disrupt the endocrine system (Slama 1971). This includes compounds such as methoprene, or pyriproxyfen. The second group consists of the ecdysone agonists and antagonists which interfere with the actions of the molting hormone and hinder normal development. This class includes products such as tebufenozide. Lastly, chitin synthesis inhibitors like buprofezine or lufenuronwhich

prevent chitin formation, make up the third group.

IGRs that affect JH were of particular importance, as JH is an insect specific hormone. The interest in juvenile hormone mimics came with the discovery of juvabione from Balsam Fir., This compound induced abnormal development of the linden bug, *Pyrrhocoris apterus* (Sláma and Williams 1966), which suggested that plants may utilize JH associated IGRs as a part of their defense systems against insect herbivores (Bowers, Fales et al. 1966). However, extensive screening of various plant materials for more JHA candidates, have only revealed a few JHAs (Bowers 2012). Furthermore, the use of JHAs had limitations, as they simulated the status quo effects of JH in insect larvae (Bowers, Fales et al. 1966). Therefore, the need to develop novel IGR alternatives with different modes of action has become a matter of importance.

Previous studies have hypothesized that plant derived compounds interfering with JH receptor-ligand interactions, called JH antagonists (JHANs), might be more effective in plant defense mechanisms than JHAs. JHANs isolated from plants such as *Lindera erythrocarpa*, showed high levels of mosquitocidal activities and affected ovarian development of *Aedes aegypti* mosquitoes (Lee, Oh et al. 2015). This suggested that plants use JHANs as part of their defense mechanisms (Lee, Oh et al. 2015).

Studies in recent years have revealed that the product of the *methoprene-tolerant* (*Met*) gene functions as the JH receptor (Charles, Iwema et al. 2011, Jindra, Palli et al. 2013). Met is a member of a protein family known as basic-helix-loop-helix (bHLH)-Per-Arnt-Sim (PAS) transcription factors. This family of transcription factors must dimerize in order to become active and bind DNA for regulation of transcription (Kewley, Whitelaw et al. 2004). Met of *A*. *aegypti* dimerizes with other bHLH-PAS transcription factors such

as *Ftz*-F1-interacting steroid receptor coactivator (FISC) or Cycle (CYC), and functions in a JH-dependent manner (Li, Mead et al. 2011, Shin, Zou et al. 2012). The JH-mediated interaction of Met and its partners has been replicated through a novel *in vitro* IGR screening system that involves a modified yeast two-hybrid *β*-galactosidase ligand binding assay using cells of the yeast strain Y-187 (Shin, Zou et al. 2012). This screening system allowed efficient identification of substances that display JHA or JHAN activities.

Plant essential oils have been used for the protection of stored grain and legumes from insect pests due to their low toxicity against humans and the environment (Isman 2000). Based on previous discoveries of plant derived JHAs and JHANs, it was hypothesized that plant essential oils may also contain substances with JHA or JHAN activities. In this study, compounds originating from plant essential oils were surveyed for their JHA or JHAN activities with the yeast two-hybrid *β*-galactosidase ligand binding assay. The mosquitocidal activities and physiological effects of selected JHA and JHAN candidates were investigated.

LITERATURE REVIEW

1. Juvenile hormone

Juvenile hormones (JH) are an important class of insect hormones that play a role in many biological processes such as metabolism and molting. They are acyclic sesquiterpenoids that have been identified as "status quo agents" or "metamorphosis inhibitors" (Wigglesworth 1934). Juvenile hormones are secreted from the corpora allata, which are paired endocrine glands. Despite their importance, their functional mechanisms were unidentified for a long period of time (Riddiford 2008).

The first juvenile hormone was identified from lipid extracts of wild silk moth (*Hyalophora cecropia*) by Röller and his team (Röller, Dahm et al. 1967). This compound was initially called *cecropia* JH or C18 JH but is now termed JH I. Another JH, JH II, was identified by Meyer(Meyer, Schneiderman et al. 1968). JH I and II have been found to differ from one another by a single methyl group at C7 in *H. cecropia* extracts. The third homolog was discovered by Judy from suspension media that held isolated corpora allata of tobacco hornworms/*Manduca sexta* (Judy, Schooley et al. 1973). This homolog, which differed from the others by the fact that all three branches of carbons 3, 7 and 11 have methyl groups, is now recognized as JH III and as the most common homologue out of them all (Schooley, Baker et al. 1984). JH 0 and its isomer 4-methyl JH I, which is also known as *iso*-JH 0, have been identified in *M. sexta* eggs (Bergot, Baker et al. 1981). However, most of their activities in the insect body remain unclear. Furthermore, JH III

bis epoxy (JHB3) was discovered from *in vitro* cultures of larval ring gland tissue of *Drosophila melanogaster* (Richard, Applebaum et al. 1989).

2. Juvenile hormone receptor, Methoprene-tolerant

The *Methoprene-tolerant* (*Met*) gene was discovered from an ethyl methane sulfonate mutagenesis screening against the common fruit fly, *D. melanogaster*. Out of Met^1 and Met^2, the two *Met* mutants obtained from the study, Met^1 was almost 100 times more resistant to either JH III or the JH agonist methoprene than wild-type flies (Wilson and Fabian 1986). Since then, *Met* was identified as the gene that encodes a transcription factor of the basic-helix-loop-helix (bHLH)-Per-Arnt-Sim (PAS) domain family (Ashok, Turner et al. 1998).This family has a unique short stretch of basic amino acids that are followed by a bHLH structure as well as two PAS domains (A and B) that are variably spaced (Jindra, Palli et al. 2013). Like other members of the bHLH-PAS family, Met was found to pair with a partner of the same family to form active transcription factors.

Both mammals and insects have bHLH-PAS proteins. However, compared to the large amount of information about mammalian bHLH-PAS proteins, little is understood about insect specific Met and its molecular actions (Jindra, Palli et al. 2013). Therefore, even though there may be various differences between mammalian and insect specific Met and its partners, it is necessary to consider the insect model with regards to the mammalian system.

The mammalian system uses the aryl hydrocarbon receptor (AhR), which is another bHLH-PAS domain family that acts as a transcription factor upon interacting with a

ligand such as Dioxin/TCDD. When AhR is in its non-DNA binding latent state, it is found in the cytoplasm. At this state, the receptor is associated with two molecules of the 90 kDA chaperone heat shock protein 90 (Hsp90), p23 and hepatitis B virus X-associated protein (XAP2/AIP/Ara9). Both the bHLH region and PAS B (ligand binding region) of the Hsp90 protein are involved in its interaction with AhR. This interaction is requisite for AhR signaling, due to the fact that Hsp90 induces the high affinity ligand binding conformation of the receptor. Hsp90 is also known to be involved in the retention of the AhR in the cytoplasm. The AhR/Hsp90 complex is translocated to the nucleus after ligand binding. At this point, the Hsp90 protein is replaced by aryl hydrocarbon receptor nuclear translocator (ARNT) which is another member of the bHLH-PAS domain family. The ligand-bound heterodimer of AhR and its partner ARNT then induces transcription from xenobiotic response elements (XREs) which are located upstream of the TCDD-responsive genes (Kewley, Whitelaw et al. 2004). An understanding of how bHLH-PAS proteins function in mammals may give more insight to how the same protein families act in the insect body.

The first partners of the insect Met protein in *Drosophila* were none other than Met itself and its paralog, Gce (Jindra, Palli et al. 2013). Dimers of Met-Met and Met-Gce however, were found to dissociate in the presence of JH III or JH agonists such as methoprene. This indicated that Met operates in a ligand-dependent manner (Jindra, Palli et al. 2013). Incapacitation of ligand binding in *Tribolium* Met did in fact cause resistance to methoprene. The nature of bHLH-PAS transcription factors, which requires the formation of a heterodimer to become active, suggested the presence of other partners, as neither Met-Met nor Met-Gce dimers were likely to transduce JH induced signals. In fact,

recent studies of *A. aegypti* Met show that Met forms a heterodimer with other bHLH-PAS factors such as the steroid receptor coactivator (SRC/FISC) or Cycle (CYC) in a JH-dependent manner (Li, Mead et al. 2011, Shin, Zou et al. 2012, Jindra, Palli et al. 2013).

3. Juvenile hormone mimics from plants.

Juvabione is a methyl ester of todomatuic acid that can act as a highly target specific juvenile hormone mimic. This substance, otherwise known as the "Paper Factor," was first identified by researchers at Harvard University, USA. Abnormal development and physiological states of the linden bug, *Pyrrhocoris apterus*, were observed during rearing, including symptoms such as failure to undergo normal metamorphosis and entrance into a supernumerary, sixth larval instar. Furthermore, adults that survived gave rise to eggs with a reduced hatch rate. It was later revealed that the causative agent of these phenomena were the paper towels that were used to rear them (Sláma and Williams 1966, Sláma and Williams 1966, Slama 1971). Upon inspection, these paper towels were found to be derived from balsam fir trees. The discovery of this plant derived, highly specific JH mimic received much attention from various agricultural industries. Other plant derived materials were screened for novel JH mimics in order to develop hormonal insecticides. These attempts however, succeeded in discovering only a handful of plant juvenile hormone mimics.

4. Insect growth regulators

Following the research involving juvabione and other JH mimics, Carol Williams suggested the development of "third-generation insecticides" which operate by mimicking insect hormones (Williams 1967). These insecticides, also known as insect growth regulators (IGRs), have relatively low environmental toxicity, which make them adequate alternatives to other types of insecticides that negatively affect the environment. Furthermore, their high target specificity indicates that they are only effective against specific insect taxa (Pener and Dhadialla 2012).

A. Juvenile hormone agonists

Juvenile hormone plays a key role in processes such as insect development, reproduction and metamorphosis. Also, it has been identified as a "status quo" agent that maintains larval to larval molts by inducing the expression of *Kr-h1*. This gene is known as a repressor of *broad,* which is a gene involved in larval to adult morphogenesis (Jindra, Palli et al. 2013, Jindra 2016). The absence of juvenile hormone at key time points in pre-adult instars thus enables larval to adult metamorphosis. Even in fully developed adult insects, juvenile hormones are essential for reproduction (Jindra, Palli et al. 2013, Jindra 2016). The mechanism through which juvenile hormones affect insect development implies that juvenile hormone agonists (JHAs) can disrupt normal metamorphosis when applied at critical time periods where JH is normally absent.

In 1960, Schmialek discovered farnesol and farnsal, which were the first JHAs (Schmialek 1961). Many substances that had JHA activity were in fact discovered long before the chemical structure of juvenile hormone I was identified by Röller

(Röller, Dahm et al. 1967). After juvabione was discovered, there was much effort to obtain other JHA compounds. In 1972, Henrick of Zoecon Corporation managed to patent a potent JHA called methoprene (isopropyl 11-methoxy 3,7,11 trimethyldodeca-2,4-dienoate), which became the first commercialized IGR insecticide (Henrick, Staal et al. 1973). Methoprene was granted full commercial registration by the US Environmental Protection Agency (USEPA) after thorough testing, and is used to control mosquito larvae. Synthesis or discovery of several other JHA compounds occurred following the commercialization of methoprene, including fenoxycarb (Dorn, Frischknecht et al. 1981) and pyriproxyfen (HATAKOSHI, AGUI et al. 1986).

B. Juvenile hormone antagonists from plants

Recent studies have identified a novel IGR called kanakugiol from plants such as *Lindera erythrocarpa.* These compounds, which are referred to as juvenile hormone antagonists (JHANs), were thought to exist in plant material, but have not been discovered for a long time (Lee, Oh et al. 2015). Insecticidal activity tests of the JHANs showed that these compounds had relatively high toxicity towards mosquito larvae. When topically applied to adult *A. aegypti* females, they induced a reduction of Met target gene expression and slowed down ovarian development (Lee, Oh et al. 2015). The discovery of JHANs, along with plant derived JHAs like juvabione, indicate that plants produce IGRs, and that they use these substances as a part of their defense system against herbivores (Lee, Oh et al. 2015).

C. Ecdysone agonists

The molting hormone, otherwise known as 20-hydroxyecdysone or 20E, is secreted as ecdysone from the insect prothoracic gland. This hormone takes part in almost all molting processes. Ecdysone itself has its own regulatory functions, and may cooperate with 20E to initiate molting (Smagghe 2009). These steroid hormones, along with other related factors, are referred to as "ecdysteroids" or "ecdysones".

Both plants and animals possess a wide variety of natural ecdysteroids. Plant ecdysteroids are called phytoecdysteroids (Dinan, Harmatha et al. 2009), and mammalian ecdysteroids are known as zooecdysteroids (Lafont and Koolman 2009). There have been many attempts at developing novel IGR insecticides based on the structures of natural or synthetic ecdysteroids.

Rohm and Haas *Co*. research labs reported the first non-steroid Ecdysteroid Agonist (EA), with a code name of RH-5849 (Dhadialla, Carlson et al. 1998). This compound belongs to the bisacylhydrazine (BAH) class of molecules, and was proven to be effective against lepidopteran, dipteran and coleopteran larvae when ingested. After application, a quick onset of feeding inhibition occurred during larval stages while simultaneously inducing a premature molt. A closer inspection revealed that the compound fatally disrupted normal cuticle formation (Aller and Ramsay 1988, Wing 1988, Wing, Slawecki et al. 1988).

Even though RH-5849 was unable to be commercialized due to low insecticidal activity, its mode of action became the basis for the search of other compounds with the same mode of action, but with higher potency. Rohm and Haas *Co.* succeeded in developing three commercial BAH EA compounds following their development of

RH-5849. These three products are tebufenozide (Heller, Klein et al. 1992), methoxyfenozide (Le, Thirugnanam et al. 1996) and halofenozide (RohMid 1996), with code names RH-5992, RH-2485 and RH-0345 respectively. Chromafenozide, code name ANS-118, is another commercially registered BAH EA compound that was developed through the collaboration of Nippon Kayaku *Co.*, *Ltd.* and Sankyo *Co., Ltd.* It's first registration occurred in Japan (Yanagi, Watanabe et al. 2000, Yanagi, Tsukamoto et al. 2006).

D. Chitin synthesis inhibitor

Chitin is a major constituent of the insect cuticle, and is made up of straight chain N-acetyl-D-glucosamine polymers. Chitin synthesis is an enzyme that transfers these N-acetyl-D-glucosamine molecules to form the polymer chains (Cohen 2010). Chitin Synthesis Inhibitors (CSIs) therefore interfere with proper cuticle development, resulting in death at the current stage of development, or after the next molt. Furthermore, CSIs negatively affect egg development and thus overall fecundity (Acheuk, Cusson et al. 2012).

In a broad chemical perspective, there are two major groups of CSIs: those related to benzoylphenyl urea and those unrelated. Some non-benzoylphenyl compounds however disrupt chitin deposition instead of chitin synthesis itself. They are all still referred to as CSIs.

Diflubenzuron is one of the most well known CSIs, and is part of the group that is related to benzoylphenyl ureas. Grosscurt examined the insecticidal activities of diflubenzuron on multiple insect species, and came to the conclusion that

diflubenzuron does interfere with chitin synthesis (Grosscurt 1978).This compound is still used frequently as an effective pest control agent.

5. Plant essential oils as insecticides

Plants became the focus of research on the development of a more environmentally friendly insecticide, due to the richness of their bioactive substances. Assessment of the insecticidal activities of methanol extracts from 30 medicinal plants and 5 plant essential oils against adults of the rice weevil (*Sitophilus oryzae*) and adzuki bean weevil (*Callosobruchus chinensis*) show that extracts from cinnamon oil or horseradish oil caused 100% mortality at 24 hours after treatment (Chantraine, Laurent et al. 1998). High insecticidal activity was observed in plants belonging to the *Apiaceae, Araceae, Lauraceae, Magnoliaceae* and *Myrtaceae* in the same study. Some essential oils or terpenoids have been shown to have high toxicity in *Drosophila* larvae. Furthermore, an evaluation of the effect of essential oils against *A. aegypti* larvae revealed that several components of essential oils such as E-anetol readily kill mosquito larvae, indicating that plant oils are also effective against dipterans (Chantraine, Laurent et al. 1998).

Plant chemicals are often target specific, and can be biodegraded to non-toxic products, which make them attractive candidates for use in pest management programs. They are also environmentally safe, and have reduced risk of pollution. Plant derived compounds were found to be effective even against pesticide resistant insects (Chantraine, Laurent et al. 1998). As plants have been used as natural insecticides for a long period of time, it is reasonable to consider them as sources of new insecticidal compounds.

METHODS

1. Yeast two-hybrid binding test using the *β*-galactosidase assays

The cDNA fragment encoding the full ORF of *A. aegypti* Met was synthesized (Bioneer, Korea). and inserted into the GAL4 DNA binding domain of the pGBKT7 (Clontech, USA) to make the bait plasmid. To construct the prey plasmid, a partial ORF (M1-V510) of *A. aegypti* FISC was introduced into the pGADT7 vector (Clontech, USA).

Both bait and prey plasmids were then transformed together into Y-187 yeast strains for the yeast two-hybrid binding test using quantitative *β*-galactosidase assays. The transformed Y187 cells were incubated at 30 °C in SD -Leu/-Trp (DDO) media until OD_{600} values reached 0.3-0.4. The cells were then harvested and suspended in twice the volume of media. After 2 h of incubation, 100 μl of yeast (OD_{600} = 0.2-0.3) were distributed into wells of 96 well plates. Desired concentrations of JH or JHA were treated into each wells containing yeast, and the plates were incubated for 3 h. Afterwards, the cells were subjected to the *β*-galactosidase assays using the Yeast *β*-galactosidase Assay Kit, purchased from Thermo Scientific (Lee, Oh et al. 2015).

2. Screening of plant essential oils for IGR activities

The transformed Y-187 yeast cells containing *A. aegypti* Met-FISC were cultured as mentioned above. When OD_{600} values reached 0.2-0.3, 100 μl of the cultured cells were

distributed into wells of a 96-well plate. In order to estimate JHA activity, each well with yeast cells was treated with each plant essential oil compound (0.01, 0.1, 1 and 10 ppm). A positive control treated with 0.033 ppm of pyriproxyfen was placed in each plate for JHA tests. To determine JHAN activity, each well was treated with 0.033 ppm pyriproxyfen and each plant essential oil compound (0.01, 0.1, 1 and 10 ppm). A negative control treated with 0.033 ppm pyriproxyfen and control solvent (ethanol) was placed in each plate for JHAN tests. The cells were incubated for 3 h at 30 °C and were subjected to the quantitative β-galactosidase assays. The obtained OD_{420} values for each plant essential oil compound was converted into an arbitrary unit representing JHA or JHAN activity. The formulae used to convert absorbance values to JHA or JHAN activity are as follows:

$$\text{JHAN activity} = \frac{Ab(\text{pyriproxyfen 0.033 ppm}) - Ab(\text{sample 10 ppm})}{Ab(\text{pyriproxyfen 0.033ppm})}$$

$$\text{JHA activity} = \frac{Ab(\text{sample 10 ppm})}{Ab(\text{pyriproxyfen 0.033ppm})}$$

3. Anti-yeast activity test.

Transformed Y187 yeast cells (Clontech, USA) harboring *A. aegypti* Met-FISC were incubated at 30 °C using Double Dropout (DDO) media (SD -Leu/-Trp, liquid) until OD_{600} values reached 0.3-0.4. At this point, they were harvested and resuspended in twice the volume of DDO. The yeast cells were distributed in volumes of 200 µl per well in 96

well plates, and incubated for 2h. The incubated cells were then treated with 0.033 ppm of pyriproxyfen and 10 ppm of each plant essential oil compound. The treated cells were placed in a shaking incubator and incubated at 30 °C. OD_{600} values were measured every 3 h, for 24 h.

4. Mosquito bioassays

The *A.albopictus* mosquitoes provided by the Korea National Institute of Health was reared at 28 °C and 70% relative humidity with a photoperiod cycle of 12h light/12h dark (Zheng, Zhang et al. 2015). Larvae were fed Tetramin fish flakes, and adults were maintained on a 10% sucrose solution as previously reported.

Thirty 3rd instar *A. albopictus* larvae in 5 ml tap water with food mixtures were treated with 10 ppm of each plant essential oil compound. The number of dead larvae for each compound was counted at 24 h after treatment. All experiments were performed as three replicates and the average mortality was calculated.

For determining the LC_{50} of each plant essential oil compound, thirty larvae of *A. albopictus* (2nd and 4th instar, respectively) were treated with different concentrations (50, 25, 10, 5 and 1 ppm). The IRMA QCal program was used to calculate LC_{50} via linear regression.

To determine the effect of candidate compounds on pupation, thirty early 4th instar *A. albopictus* larvae were treated with 0.1 ppm of each compound and the number of pupae for each compound was counted for 5 days at 24 h intervals. All experiments were performed as three replicates and the time at which 50% of the larvae became pupae

(Pupal time 50% or PT_{50}) was determined through linear regression via the IRMA QCal program.

5. qPCR

A. albopictus pupae were collected in separate cages just prior to emergence. Circadian time was taken into account, such that the start of the light phase of a 12L-12D photoperiod was considered as circadian time zero. For each candidate, a total of 0.5 μg was applied topically onto the abdomens of adult females that emerged within a 12 hour time window prior to circadian time 0. Treated individuals were kept alive through a diet of 10% sucrose water. The total RNA of adult mosquitoes were collected at 4 hour intervals from 0h after treatment until 16 hours after treatment, and were converted to cDNA for qPCR analysis of *hairy* gene expression. More specifically, total RNA was prepared with Qiazol (Qiagen).

For the qPCR, the *hairy* gene was selected as the target, and *RPS-7* was used as a reference gene for normalization. QuantiTect Reverse Transcription Kits (QIAGEN, Germany) were used, following the manufacturer's protocol. The qPCR was conducted using the EvaGreen qPCR Mastermix (Applied Biological Materials Inc, Canada) and the CFX96^{TM} Real-Time System (BIO-RAD, USA). The cycling protocol used for the qPCR is as follows: a preheating step for enzyme activation at 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 sec, 55 °C for 15 sec and 72 °C for 30 sec. Relative transcription levels were quantified via the $2^{-\Delta Ct}$ method (Pfaffl 2001). The primers used for qPCR of *hairy* are as follows: *hairy* Fw (5' TGACCGTGAAACATTTGGAA 3') and *hairy* Re (5'

CGGTCTCCAAGGTTTGTCAT 3'). The primers for the reference gene *RPS7* are as follows: *RPS7* Fw (5' TCAGTGTACAAGAAGCTGACCGGA 3') and *RPS7* Re (5' TTCCGCGCGCGCTCACTTATTAGATT 3').

6. Ovary dissection

Pupae were collected in separate cages just prior to emergence. Samples were prepared in the same way as above. Samples were applied topically (0.5 μg per individual) onto the abdomen of each adult female, 48 hours after emergence. Treated mosquitoes were allowed blood meals on lab mice for 6 hours, along with a diet of 10% sucrose water. Adult females were dissected 48 hours after treatment to see changes in ovarian phenotypes. For this experiment, circadian time was not taken into account.

Each dissection took place on a Marienfeld slide glass, with PBS buffer as the dissection media. The mosquitoes were tranquilized on ice before being dissected. Abdomens were separated and submerged in PBS buffer before dissection. Tissue debris was constantly removed by washing with 75% ethanol. The dissections and photos were taken with Leica microscopes. Pictures were calibrated using the Active Measure program.

RESULTS

1. JHA and JHAN activities of plant essential oil compounds

One hundred and seventy three compounds derived from plant essential oils were tested *in vitro* via the yeast two-hybrid binding assay to determine their ability to simulate or interfere with pyriproxyfen-mediated binding of *A. aegypti* MET-FISC (Table 1). As shown in Fig. 1, 4 compounds including E48, E171, E172 and E173 stimulated the binding at a relatively high level, indicating that these compounds have JHA activity. In contrast, another group of 4 compounds including E113, E114, E118 and E121 were found to interfere with pyriproxyfen-mediated binding of *A. aegypti* MET-FISC, which suggest that these compounds have JHAN activity (Fig. 1)

Chemical structures of these 8 compounds were similar to that of juvenile hormone III (Fig. 2). Also, a noticeable difference between JHAN and JHA candidates was found in their functional groups, as JHA candidates have acetates and JHAN candidates have aldehydes. The names of the 4 JHA candidates in order of increasing carbon number are as follows: nonyl acetate (E172), decyl acetate (E171), undecyl acetate (E173) and dodecyl acetate (E48). JHAN candidates in order of increasing number of carbons are nonyl aldehyde (E118), decyl aldehyde (E113), undecyl aldehyde (E121) and dodecyl aldehyde (E114) respectively.

The two pairs of selected samples were then subjected to a concentration dependent *β*-galactosidase binding assay to test the effects of increasing sample concentration on their

JHA and JHAN activities respectively. The absorbance of undecyl and dodecyl acetate (JHAs) increased with increasing concentration in JHA tests (Fig. 3), while no noticeable differences between concentrations were observed in JHAN tests (Fig. 4). On the other hand, undecyl and dodecyl aldehyde showed no JHA activity even at high concentrations, as seen by their minimal absorbance values in Figure 3. However, they displayed a pattern of decreasing absorbance with regards to increasing concentration (Fig. 4).

To eliminate the possibility of false signals from anti-yeast activities of plant essential oil compounds, growth inhibition tests were performed with Y187 yeast strains that were transformed with Met and FISC (Fig. 5). The addition of plant essential oil compounds resulted in normal yeast growth in non-selective DDO media, indicating that these compounds have no anti-yeast activity, and that they directly disrupt the JH receptor complex and exhibit JHAN activity.

Table 1. List of plant essential oil compounds used in this study.

Code Number	Compound Name
E1	Acetophenone
E2	Acetyl eugenol
E3	Acetyl isoeugenol
E4	Allocymene
E5	Angelic acid isoamyl ester
E6	Angelic acid isobutyl ester
E7	Anethole, trans-
E8	Anisaldehyde, m-
E9	Anisaldehyde, ρ-
E10	Anisaldehyde, σ-
E11	Anisole
E12	Aromadendrene, (+)-
E13	Asarone, α-
E14	Asarone, β-
E15	Benzaldehyde Reagent Plus
E16	Bisabolol, α-
E17	Borneol, contain ca 20% Isoborneol
E18	Bornyl acetate
E19	Butyric acid
E20	Camphene, (+)-
E21	Camphene, (-)-
E22	Camphor, (±)-
E23	Camphor, (1S)-(-)-
E24	Carene, 3-
E25	Carvacrol
E26	Carveol, (-)- mixture of isomers
E27	Carvone, (R)-(-)-
E28	Carvone, (S)-(+)-
E29	Carvone, dihydro-(+)- mixture of isomers
E30	Caryophyllene oxide
E31	Caryophyllene, β-

Table 1. Continued

Code Number	Compound Name
E32	Cedrene, (-)-α-
E33	Cineole, 1,4
E34	Cineole, 1,8(=Eucalyptol)
E35	Cinnamaldehyde, trans-
E36	Cinnamyl acetate
E37	Cinnamyl alcohol
E38	Citral mixture of ci and trans
E39	Citronellal, (±)-
E40	Citronellal, (R)(+)
E41	Citronellal, (S)(-)
E42	Citronellol, β-
E43	Copaene, (-)-α-
E44	Cuparene, (+)-
E45	Cymene, m-
E46	Cymene, ρ-
E47	Dipentene, mixture(±Limonene)
E48	Dodecyl acetate
E49	Estragole
E50	Eucarvone
E51	Eugenol
E52	Farnesol
E53	Farnesyl acetate, trans-
E54	Fenchone, (-)-
E55	Fenchone, (+)-
E56	Geranyl acetate
E57	Guaiol, (-)-
E58	Globulol, (-)-
E59	Humulene, α-
E60	3-Phenyl-1-propanol
E61	Isobornyl acetate
E62	Isoeugenol, mixture of cis and trans
E63	Isopulegol, (-)-

Table 1. Continued

Code Number	Compound Name
E64	Isopulegol, (+)-
E65	Isosafrole, mixture of cis and trans
E66	Limonene, (-)-
E67	Limonene, (R)(+)-
E68	Linalool oxide, mixture of isomers
E69	Linalyl acetate
E70	Linolenic acid methyl ester
E71	Menthol, (-)-
E72	Menthone,(-)- contains ca, 5% isomenthone
E73	Methyl acetate
E74	Methyl eugenol
E75	Methyl isoeugenol
E76	Methyl salicylate
E77	Myrcene
E78	Myrtenal, (1R)-(-)-
E79	Myrtenol, (1R)-(-)-
E80	Nopinone, (1R)-(+)
E81	Ocimene
E82	Octyl acetate
E83	Perillaldehyde, (-)-
E84	Perillyl alcohol, (S)-(-)-
E85	Phellandrene, d-
E86	Phellandrene, α-
E87	Phenyl ether
E88	Phenylethanol, 2-
E89	Pinene, (-)-α-
E90	Pinene, (1R)-(+)-α-
E91	Pinene, (-)-β-
E92	Pinocarveol, (-)-trans
E93	Piperitone
E94	Pulegone, (R)(+)-
E95	Pulegone, (S)(-)-

Table 1. Continued

Code Number	Compound Name
E96	Sabinene hydrate
E97	Terpinen-4-ol, (+)-
E98	Terpinen-4-ol, (±)-
E99	Terpinene, α-
E100	Terpinene, γ-
E101	Terpinolene
E102	Thujopsene, (-)-
E103	Thymol
E104	Neral
E105	Nerol
E106	Geranial
E107	Geraniol
E108	trans-2-Heptenal
E109	trans-2-Hexenal
E110	trans-2-Octenal, tech
E111	Acetic acid cis-3-hexenyl ester
E112	cis-2-hexen-1-ol
E113	Decanal,minimum
E114	Dodecyl aldehyde
E115	Heptanal(=n-Heptaldehyde)
E116	Hexanal
E117	n-Hexyl aldehyde(=n-Capronaldehyde)
E118	Nonyl aldehyde
E119	Octyl aldehyde(=Octanal)
E120	Tridecanal, tech
E121	Undecylic aldehyde
E122	(Z)-3-hexen-1-ol
E123	Allyl benzyl ether
E124	Allyl methyl sulfide
E125	Diallyl disulfide
E126	Diallyl sulfide
E127	Isopropyl disulfide

Table 1. Continued

Code Number	Compound Name
E128	Isopropyl sulfide
E129	Methyl propyl disulfide
E130	Propyl disulfide
E131	Propyl sulfide
E132	Sodium sulfide
E133	Dipropyl trisulphide
E134	Methyl propyl sulphide
E135	Methyl propyl trisulphide
E136	Hexadecanyl acetate C16-Ac
E137	butyl isobutanoate
E138	butyl isovalerate(C4 IV)
E139	butyl 2-methylbutanoate(C4 2MB)
E140	butyl 3-methyl-2-butenoate(C4 IP)
E141	Citromethyl acetate
E142	isoamyl anglate
E143	isoamyl tiglate
E144	isobutyl isovalerate
E145	isobutyl 2-methylbutanate
E146	isobutyl 3-methyl-2-butenoate
E147	methyl cinnamate
E148	methyl N-methylanthranilate
E149	myrtenyl acetate
E150	pentyl isobutanoate
E151	pentyl isovalerate(C5 IV)
E152	pentyl 2-methylbutanoate
E153	pentyl 3-methyl-2-butenoate
E154	Pinocarvyl acetate
E155	1-phenyl-1-ethanol
E156	2-methylbutyl angelate
E157	2-methylbutyl isobutyrate
E158	2-methylbutyl isovalerate
E159	2-methylbutyl 2-methylbutanoate

Table 1. Continued

Code Number	Compound Name
E160	2-methylbutyl 3-methyl-2-butenoate
E161	2-phenylethyl acetate
E162	3-methylbutyl isobutyrate
E163	3-methylbutyl isovalerate
E164	3-methylbutyl 2-methylbutanoate
E165	3-methylbutyl 3-methyl-2-butenoate
E166	3-methyl-2-butenyl isobutanoate
E167	3-methyl-2-butenyl isovalerate
E168	3-methyl-2-butenyl 2-methylbutanoate
E169	3-methyl-2-butenyl 3-methyl-2-butnoate
E170	3-phenyl-1-propan-1-ol
E171	Decyl acetate C10-Oac
E172	Nonyl acetate C9-Oac
E173	Undecyl acetate C11-Oac

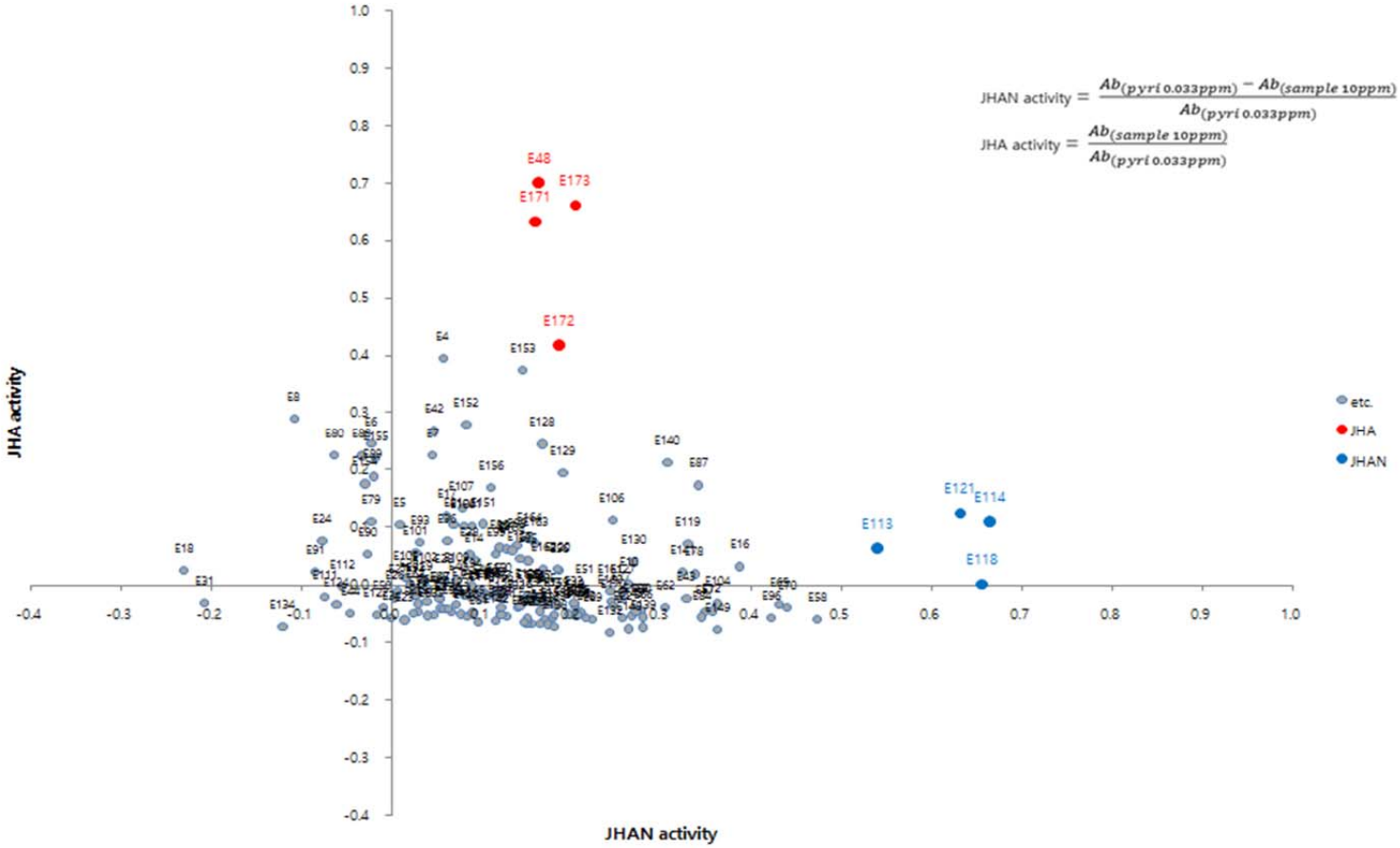

Fig 1. JHA and JHAN activities of plant essential oil compounds. Red dots indicate JHA candidates and blue dots indicate JHAN candidates.

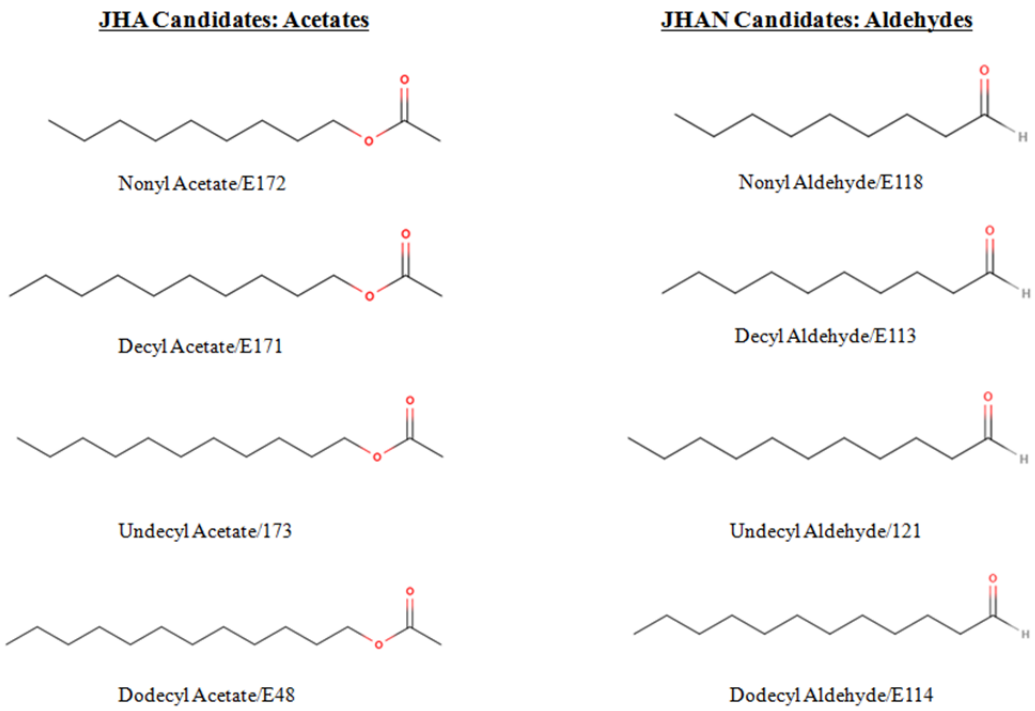

Fig 2. Chemical structures of plant essential oil compounds showing JHA or JHAN activity. Chemical formulae with corresponding names and code numbers are shown in order of increasing carbons.

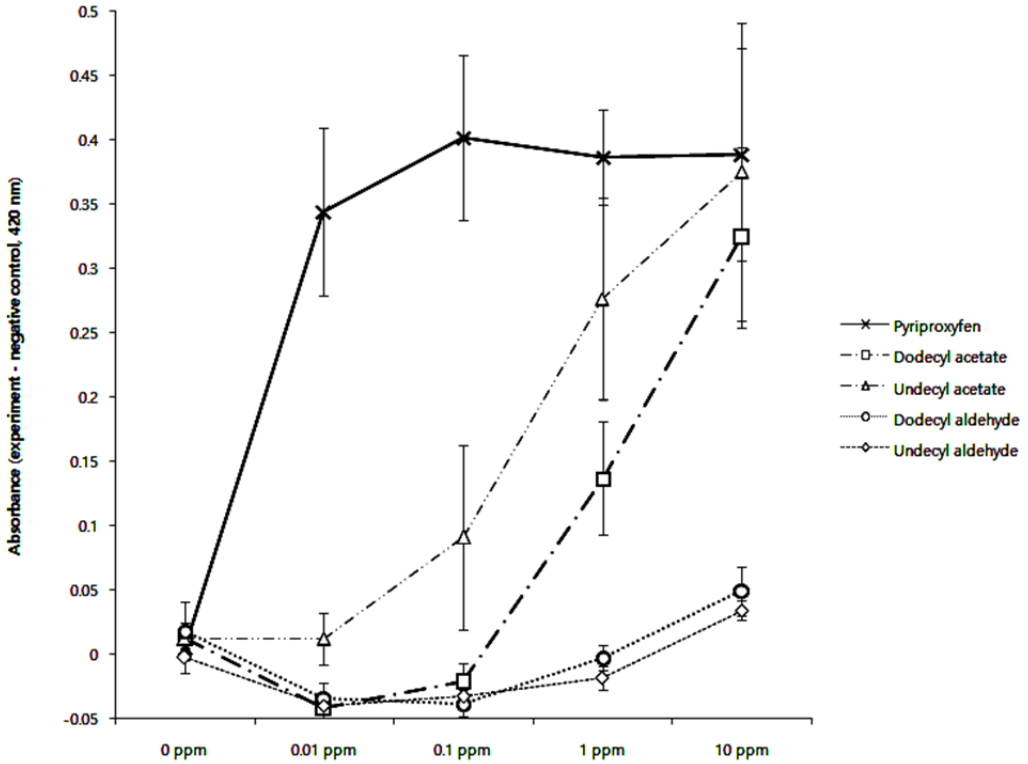

Fig 3. Concentration-dependent JHA activities of plant essential oil compounds.

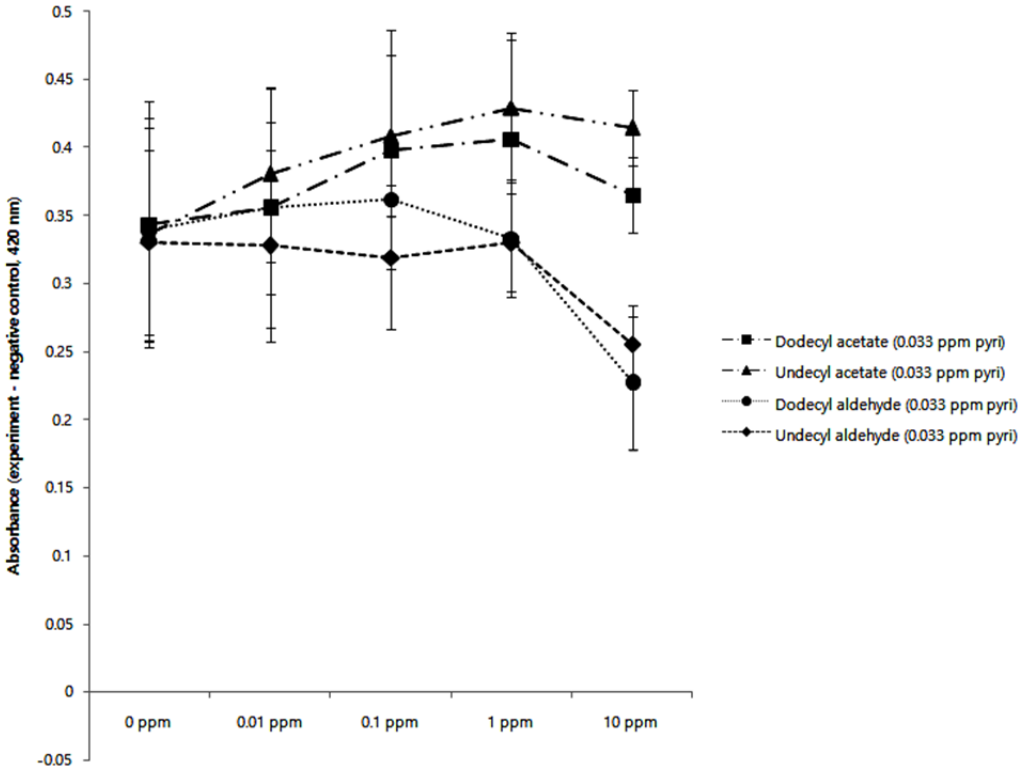

Fig 4. Concentration-dependent JHAN activities of plant essential oil compounds.

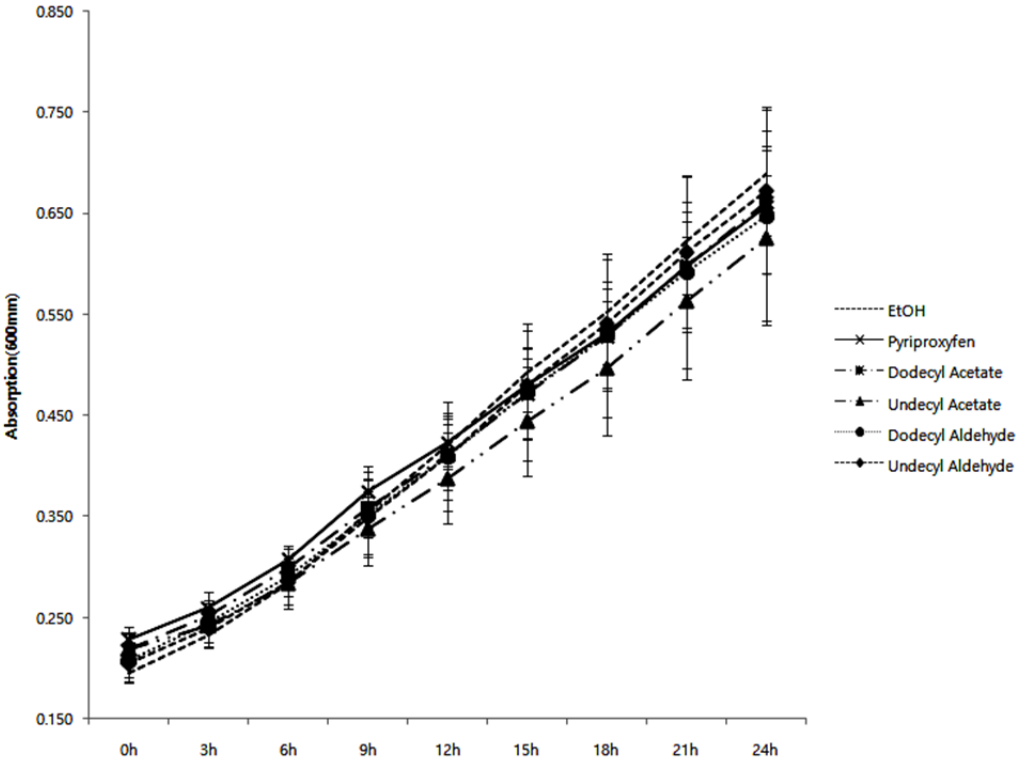

Fig 5. Anti-yeast activity tests against plant essential oil compounds.

2. Insecticidal activities of IGR compounds from plant essential oils

To evaluate the insecticidal activities of plant essential oil compounds with JHA or JHAN activity, 3rd instar *A. albopictus* larvae were treated with each compound, and mortalities were determined (Fig. 6). All compounds tested showed high levels of insecticidal activities, with mortalities above 50%. Among them, two pairs of JHA and JHAN candidates with equal numbers of carbons, (undecyl acetate/undecyl aldehyde and dodecyl acetate/dodecyl aldehyde) showed larval mortalities above 60%.

To further examine the insecticidal activities of plant essential oil compounds showing larval mortalities above 60%, median lethal concentration (LC_{50}) of each compound was determined (Table 2). Against 2nd instar larvae, undecyl and dodecyl aldehyde, which are both JHAN candidates, showed high levels of insecticidal activities compared to pyriproxyfen. Their LC_{50} values were approximately 6.6-10.4 times lower than the LC_{50} of pyriproxyfen. The difference in insecticidal activities between the JHA and JHAN candidates against 4th instar larvae however, was not quite as significant.

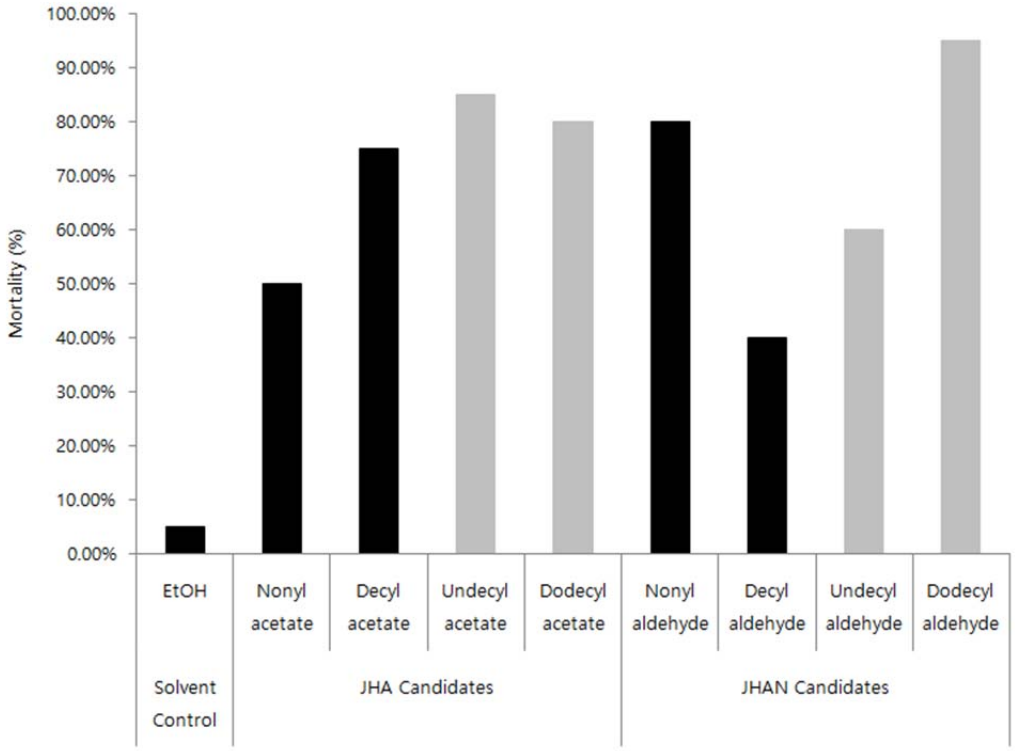

Fig 6. Insecticidal activities of plant essential oil compounds. Mortalities of *A. albopictus* larvae treated with each compound were recorded after 24h.

Table 2. Median lethal concentration (LC_{50}) of plant essential oil compounds against *A. albopictus* larvae

Sample Name	LC_{50} (ppm)	
	2nd Instar	4th Instar
Pyriproxyfen	1.25 (0.78~2.01)	9.7 (6.6~14.2)
Dodecyl acetate	7.01 (2.46~20.09)	24.0 (17.3~33.4)
Undecyl acetate	4.70 (1.99~11.02)	15.2 (11.0~20.9)
Dodecyl aldehyde	0.19 (0.16~0.23)	14.4 (10.1~20.5)
Undecyl aldehyde	0.12 (0.06~0.21)	35.3 (21.8~57.4)

3. *In vivo* tests of IGR compounds from plant essential oils

Upon observation, it was revealed that most of the larvae either pupated or died at around 120 hours after treatment. Cumulative pupation rate was therefore measured until 120 hours, and median pupation time (PT_{50}) was estimated (Table 3). Results showed that larvae treated with undecyl and dodecyl acetates, both JHA candidates, pupated approximately 10.5~11.5% slower in comparison to larvae treated with solvent controls. Larvae treated with pyriproxyfen, the JHA positive control, also pupated about 24.7% slower than solvent controls, indicating a similar pattern. On the other hand, larvae treated with JHAN candidates undecyl and dodecyl aldehydes, pupated 18.5~20.0% faster than larvae treated with solvent only. Treatment with kanakugiol, the JHAN control, also resulted in pupation rates that were accelerated by approximately 11.2%.

To determine the effects of candidates on the expression of JH induced genes, female *A. albopictus* adults were topically applied with plant essential oil compounds. The results of qPCR analyses of *hairy* gene expression are shown in Figure 7.Treatment with undecyl acetate, a JHA candidate, led to an increase in *hairy* gene expression that was about 2.6 times greater than that of solvent controls. On the other hand, treatment with undecyl aldehyde, a JHAN candidate, has resulted in about a 40% decrease in *hairy* gene expression.

Dissections at 48 hours after blood meals have revealed that both JHA and JHAN candidates resulted in severe retardation of ovarian growth in comparison to solvent controls (Fig. 8). Ovaries of females treated with acetone solvent had already matured by 48 hours, and a change in egg shape and overall size of the ovary was observed. Eggs

have changed shape from circular to elliptical, and the entire ovary became much larger relative to the midgut and malpighian tubules. Adults treated with pyriproxyfen, dodecyl acetate and aldehyde however, all maintained immature, circular eggs and significantly smaller ovary sizes.

Table 3. Median pupation time (PT_{50}) of *A. albopictus* larvae treated with plant essential oil compounds

Merged (3 Trials)	Pupal Time 50%
EtOH	109.5h (80.6h~148.4h)
Pyriproxyfen	136.6h (83.9h~221.4h)
Kanakugiol	96.1h (75.9h~121.5h)
Dodecyl Acetate	121.0h (80.6h~181.3h)
Undecyl Acetate	122.1h (83.9h~177.7h)
Dodecyl Aldehyde	85.4h (66.7h~108.9h)
Undecyl Aldehyde	89.2h (71.5h~112.2h)

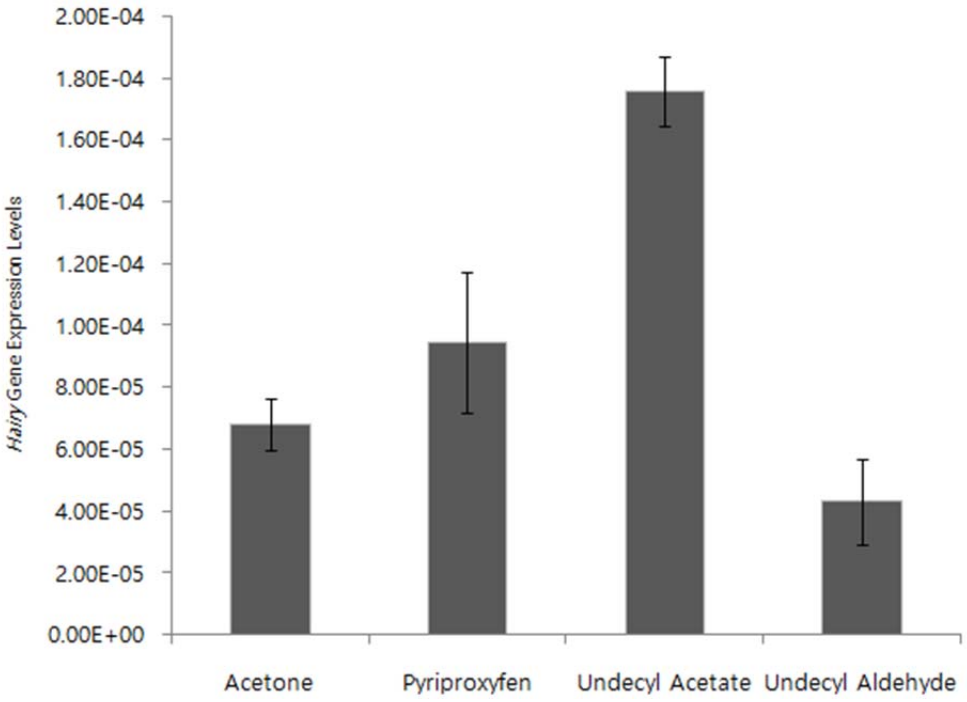

Fig 7. Expression of *Hairy* gene in female adults of *A. albopictus* mosquitoes treated with plant essential oil compounds.

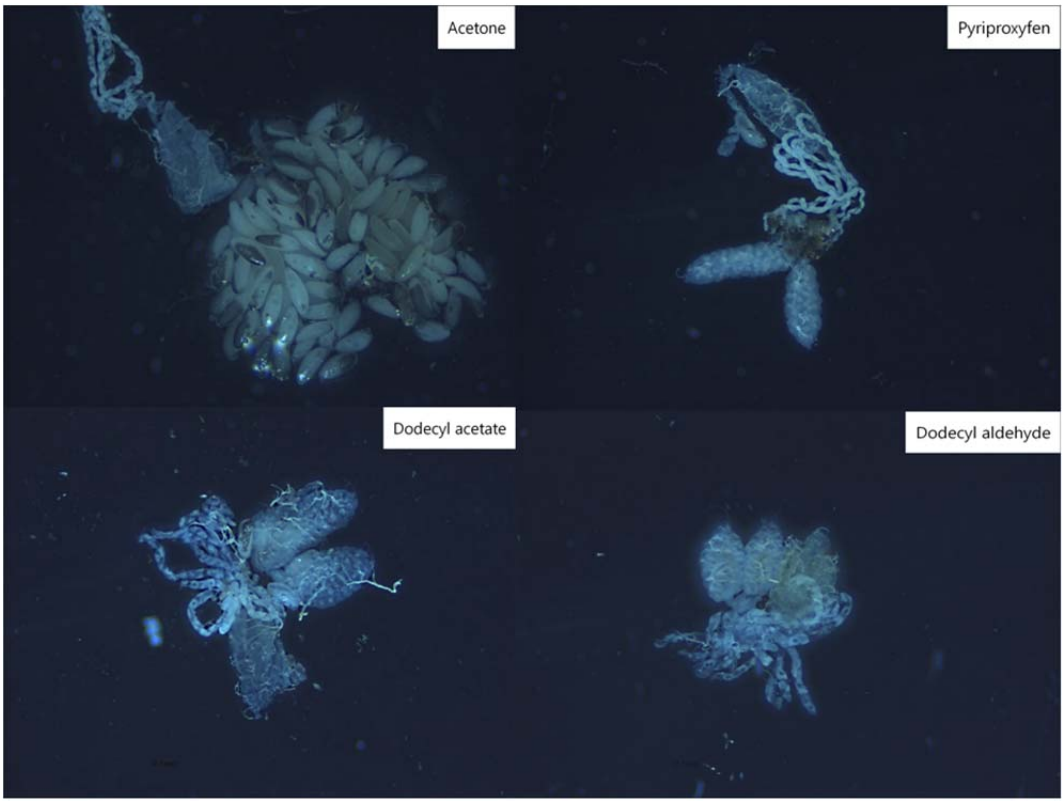

Fig 8. Impaired ovarian development of female adult *A. albopictus* mosquitoes treated with plant essential oil compounds.

DISCUSSION

In this study, through a novel *in vitro* IGR screening system, chemical compounds derived from plant essential oils displaying JHA or JHAN activities respectively, were identified and characterized.

By screening all 173 samples, 4 candidates with high levels of JHA activity and 4 candidates with high levels of JHAN activity were selected. A structural analysis of these 8 compounds has revealed that all 8 candidates share a similar chemical structure as that of juvenile hormone III. Furthermore, JHA candidates were shown to have acetates as functional groups while JHAN candidates had aldehydes as functional groups (Figure 2). The different functional groups of JHA and JHAN candidates may explain their differences in IGR activity, but there have been no prior studies to support this. Further research is therefore required to investigate any possible relationships between functional groups and IGR activity.

Insecticidal activities of all candidates against 3rd instar *A. albopictus* larvae were investigated, and it was revealed that they all displayed high toxicity (above 50% mortality) compared to solvent controls. Their high insecticidal activities may be attributed once again, to their similarity in shape to JH III and their ability to bind and interfere with the juvenile hormone receptor complex.

We further verified the differences in IGR activities of the 2 pairs of candidates. through concentration dependent JHA and JHAN activity tests (Fig. 4 and 5). These results demonstrated that the JHA candidates simulate pyriproxyfen-mediated Met-FISC

binding, while the JHAN candidates interfere with the process in a dose dependent manner. This indicates that plants prepare secondary metabolites with IGR activities in order to defend themselves against insect herbivores. Despite the cost of production, synthesis of defensive secondary metabolites including IGR compounds likely increase the host plant's chances of survival and overall fitness (Williams, Stone et al. 1989). The ability of plant derived JHA and JHAN candidates to disrupt JH receptor complexes therefore provides further support that IGR compounds have coevolved with plants as a part of their defense systems.

Second instar mosquito larvae were much more susceptible to JHAN candidates than JHA compounds (Table 2). However, there were no significant differences between the insecticidal activities of JHA and JHAN candidates against 4th instar larvae. This may be due to the fact that lower instar larvae are more heavily influenced by the presence and absence of JH in their system. Also, it has been reported that the titer of JH in the insect body fluctuate, depending on circadian rhythm (Shin, Zou et al. 2012). There may be longer periods of high JH titers in the hemolymph in younger instars compared to last instar larvae, in which JH titers are slowly decreasing in preparation for pupation. This may be why 2nd instar larvae are more readily affected by JHANs which interfere with the JH pathway.

Sub-lethal concentrations of pyriproxyfen and JHA candidates resulted in delayed pupation while treatment with kanakugiol and JHAN candidates accelerated pupation time. This result is in accordance with previous studies on other insects like *P. apterus*, suggesting that JHA substances extend the current developmental stage (Bowers, Fales et al. 1966, Sláma and Williams 1966) while JHAN substances prematurely terminate it.

In vivo results of JH-induced gene expression and female ovary development, in combination with the *in vitro* binding assays, show that the candidates selected in this study interfere with the actions of the JH receptor complex. They therefore can disrupt normal endocrine function in insects. Furthermore, since this receptor complex is insect specific, selected candidates should also have low non-target toxicity (Lee, Oh et al. 2015).

In conclusion, the JHA and JHAN candidates that have been determined through this experiment have the potential to play a significant role in the development of novel, environmentally friendly IGR pesticides.

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ABSTRACT IN KOREAN

식물체 정유로부터 곤충 유충호르몬 수용 복합체의 교란 물질 탐색

서울대학교

농생명공학부 곤충학전공

하규백

초록

곤충은 매해 막대한 농작물 피해뿐만 아니라 질병 매개를 통한 인명 피해를 유발하고 있다. 이러한 해충들을 관리하기 위해 화학적 살충제와 같은 여러 가지 방법이 사용되어 왔으나 이들은 인축에 유해하고 환경에 악영향을 미치는 등의 단점이 있다. 곤충성장조절물질은 (Insect Growth Regulators, IGRs) 기주 특이성이 높고 인축과 환경에 비교적 안전하기 때문에 친환경살충제로 널리 이용되고 있다. 본 실험에서는 *Aedes aegypti* 모기의 유약호르몬 수용복합체 (juvenile hormone receptor complex)를 yeast two-hybrid 시스템에 reporter system으로 도입한 신규 IGR활성 탐색 방법을 이용하여 여러

가지 식물체 정유 유래물질로부터 유약호르몬 수용복합체를 교란시키는 물질들을 선발하고 그 특성을 구명하였다. 총 173개의 식물체 정유 유래물질에 대한 *in vitro* screening을 통하여 높은 수준의 JHA 또는 JHAN 활성을 보이는 후보물질 2종을 각각 선발하였으며, 선발된 후보물질들은 모두 ,juvenile hormone III와 유사한 terpenoid 구조를 가지고 있는 것을 확인하였다. 또한 JHA 후보 물질의 경우 acetate 작용기를 가지고 있는 반면 JHAN 후보 물질들은 aldehyde 작용기를 가지는 것으로 나타났다. 선발된 후보 물질들은 모두 *Aedes albopictus* 모기 유충에 대해 뛰어난 살충활성을 보였다. JHAN 후보 물질의 경우 2령 유충에 대해 JHA 후보물질보다 더 높은 살충활성을 가지는 것으로 나타났다. JHA 후보 물질들의 경우 4령 유충의 용화를 늦추는 반면 JHAN 후보 물질들은 용화 속도를 향상시켰습니다. 또한 JHA 후보 물질들은 JH III에 의해 발현이 유도되는 *hairy* 유전자의 발현을 증가시키는 반면 JHAN 후보 물질들은 이 유전자의 발현을 억제하였다. 한편 JHA 및 JHAN 후보 물질 모두들 암컷 모기 성충의 난소 발달을 저해하였다. 이러한 결과는 본 연구에서 선발된 IGR 후보 물질들이 보다 더 효율적이고 친환경적인 살충제의 개발에 유용하게 이용될 수 있음을 시사 하였다.

Key Words: 곤충 유약호르몬, 수용복합체 교란 물질, *Aedes aegypti*, *Aedes albopictus*, 식물체 정유

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