A THESIS

FOR THE DEGREE OF MASTER OF SCIENCE

Identification of Plant Compounds that Disrupt the Insect Juvenile Hormone Receptor Complex

곤충 유약호르몬 수용복합체의 기능을 방해하는

식물 유래 화합물의 특성 연구

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ABSTRACT

Insects impact human health through vector-borne diseases and cause major economic losses by damaging crops and stored agricultural products. Insect-specific growth regulators represent attractive control agents because of their safety to both humans and the environment. We identified plant compounds that serve as juvenile hormone antagonists (PJHANs). Using the yeast two hybrid system transformed with the mosquito JH receptor as a reporter system, we demonstrate that PJHANs affect the JH receptor, methoprene-tolerant (Met), by disrupting its complex with CYCLE or FISC, formation of which is required for mediating JH activity. We

isolated five diterpene secondary metabolites with JH antagonist activity from two plants: *Lindera erythrocarpa* and *Solidago serotina*. They are effective at causing mortality of mosquito larvae at relatively low LD_{50} values. Topical application of two diterpenes caused a reduction in the expression of Met target genes as well as the retardation of follicle development in mosquito ovaries. Hence, the newly discovered PJHANs may lead to the development of a new class of safe and effective pesticides.

Key words: juvenile hormone, receptor, endocrine disruptor

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INTRODUCTION

Insects cause extensive economic damage and human suffering. Diseases transmitted by insects result in a million deaths per year (Hill, Kafatos et al. 2005), and insect infestation leads to annual losses of agricultural products worth billions of US dollars (Boyer, Zhang et al. 2012). To prevent huge losses of both human life and property, chemical insecticides such as organophosphates, methylcarbamates, pyrethroids and neonicotinoids are commonly used. However, the high toxicity of currently available insecticides presents environmental and health risks, and growing resistance and cross-resistance of insects to these existing insecticides gravely complicates the situation. Hence, there is an urgent need to develop novel and effective insecticides.

Insect growth regulators (IGRs) have been devised based on insect-specific functions. Three major classes of IGRs are commercially available (Pener and Dhadialla 2012). These IGRs include juvenile hormone (JH) agonists (methoprene and pyriproxyfen), ecdysone agonists (halofenozide and tebufenozide), and chitin synthase inhibitors (buprofezine). They possess low non-target toxicity and are therefore a much smaller environmental threat. As such, they are even now being used to control pests. JH modulating IGRs are of particular interest because JH is an insect-specific hormone. JH agonists (JHAs) disrupt insect endocrine regulation, causing abnormal development and larval fatality (Slama 1971). However, JHAs have limitations in the scope of their applications because they mimic and enhance

the JH mode of action. Theoretically, JH antagonists (JHANs) could be used as a powerful alternative, but no effective JHANs have yet been developed.

Recent studies identified methoprene-tolerant (Met) as the JH receptor (Charles, Iwema et al. 2011; Jindra, Palli et al. 2013). Met is a member of the family of basic helix-loop-helix (bHLH)-Per-Arnt-Sim (PAS) transcription factors that requires homo- or heterodimerization for DNA binding and transcriptional regulation (Kewley, Whitelaw et al. 2004). In the *Aedes aegypti* mosquito, Met forms a heterodimer with other bHLH-PAS factors, the steroid receptor co-activator (SRC/FISC) or Cycle (CYC) in a JH-dependent manner (Li, Mead et al. 2011; Shin, Zou et al. 2012). JH-mediated Met-CYC binding has been quantitatively simulated using the two-hybrid yeast β-galactosidase assay in cells of the yeast strain, Y187 (Shin, Zou et al. 2012). Thus, it is possible to test both JHAs and JHANs for their interactions with Met and its partners using this assay.

Previously, Balsam fir juvabione has been found to inhibit the metamorphosis of the linden bug, *Pyrrhocoris apterus*, suggesting that plants could use JHAs as resistance factors against insect herbivores (Bowers, Fales et al. 1966; Sláma and Williams 1966). However, screening of plants for JH activity revealed only a few JHAs (Bowers 2012). We hypothesized that JHANs, rather than JHAs, play a significant role in plant defense against insect herbivores. In this study, we observed that plants produce JHANs as secondary metabolites. Through two-hybrid yeast β-galactosidase assay screening of 1,651 plant species, we identified 53 species that exhibited putative JHAN activity based on mosquito larvicidal toxicity tests. We isolated five diterpene secondary metabolites with JHAN activity from two plants:

Lindera erythrocarpa and *Solidago serotina*. They were effective at causing mortality of mosquito larvae at relatively low LD_{50} values. Moreover, topical application of two of these five diterpenes caused the reduction in the expression of Met target genes and the retardation of follicle development in mosquito ovaries.

LITERATURE REVIEW

1. Juvenile hormone

Juvenile hormones (JHs) are a group of acyclic sesquiterpenoids that function as insect hormones. JHs, identified as “metamorphosis inhibitor hormones” (Wigglesworth 1934), are secreted by a pair of endocrine glands called corpora allata (CA).

Röller and colleagues (Röller, Dahm et al. 1967) identified the first juvenile hormone from lipid extracts of the wild silk moth, *Hyalophora cecropia*. This JH, methyl (2E,6E,10–*cis*) –10, 11–epoxy–7–ethyl–3, 11–dimethyl–2, 6–tridecadienoate, was termed *cecropia* JH or C18 JH in older literature, but it is now recognized as JH I. Meyer (Meyer, Schneiderman et al. 1968) identified a minor component that was called JH II, which differed from JH I by a methyl group at C7 in the *H. cecropia* extracts. A third JH homologue, JH III, methyl 10, 11–epoxy–farnesoate, was identified from media in which the CA of the tobacco hornworm, *Manduca sexta*, had been contained (Judy, Schooley et al. 1973). JH III differs from the other homologues in that all three branches of the carbon skeleton, at C3, C7, and C11, are methyl groups. JH III appears to be the most common homologue among the species studied (Schooley, Baker et al. 1984).

The trihomosesquiterpenoids JH 0 and its isomer 4–methyl JH I (*iso* – JH 0) were identified in *M. sexta* eggs (Bergot, Baker et al. 1981), but nothing is currently known of their functions. JH III bisepoxy (JHB3) was identified from *in vitro*

cultures of larval ring glands of *Drosophila melanogaster* (Richard, Applebaum et al. 1989).

JHs play key roles insect development, reproduction, and many other physiological functions. Although JHs are very important, these regulatory mechanisms have remained elusive (Riddiford 2008).

For more information about JHs, see Reference 15 (Gilbert 2011).

2. Methoprene-tolerant as a JH receptor

The *Methoprene-tolerant* (*Met*) gene was recovered from an ethyl methane sulfonate mutagenesis screening against *Drosophila melanogaster*. The screening obtained Met mutants (Met^1 and Met^2), one of which (Met^1) is nearly 100 times more resistant than wild-type to either JH III or methoprene, which is a chemical agonist of JH (Wilson and Fabian 1986). Since then it has been revealed that *Met* encodes a transcription factor of the basic–helix–loop–helix (bHLH)–Per–Arnt–Sim (PAS) domain family (Ashok, Turner et al. 1998). To form active transcription factors, functionally specialized bHLH-PAS proteins pair with a common partner of their family and Met is no exception.

In mammals, the aryl hydrocarbon receptor (AhR), a bHLH-PAS domain family like Met, acts as a transcription factor when interacting with a ligand, such as Dioxin (2,3,7,8–tetrachlorodibenzo–ρ–dioxin, TCDD). In its latent (non-DNA binding) state, the AhR is found in the cytoplasm, stably associated with two molecules of the 90 kDa molecular chaperone heat shock protein 90 (Hsp90), p23 and hepatitis B virus X-associated protein (XAP2/AIP/Ara9). Hsp90 interacts with the AhR via both the

bHLH region and PAS B, which contains the ligand binding region. This association is essential for AhR signaling. Hsp90 appears to be a chaperone which induces the high affinity ligand binding conformation of the AhR and is involved in the retention of the AhR in the cytoplasm, perhaps by masking its nuclear localization sequence (NLS). Following ligand binding, the AhR/Hsp90 complex translocates to the nucleus where Hsp90 is exchanged for its partner protein aryl hydrocarbon receptor nuclear translocator (ARNT) that is also a member of bHLH-PAS domain family. In the nucleus, ligand-bound AhR/ARNT heterodimer recognizes and promotes transcription from xenobiotic response elements (XREs) found upstream of TCDD-responsive genes (Kewley, Whitelaw et al. 2004).

Although responses differ between mammalian and insect bHLH-PAS proteins, this introduction is necessary because compared with the large amount of information about mammalian bHLH-PAS proteins, the molecular action of insect-specific Met is an unexplored territory (Jindra, Palli et al. 2013).

Despite potential differences between factors such as structure and function of individual Met domains, identity and function of its partner proteins, mode of interaction with its target genes, and the role that JH plays within these processes, it is essential to consider the insect model with regards to the mammals.

According to recent studies on *Aedes aegypti* mosquitoes, Met forms a heterodimer with other bHLH-PAS factors, the steroid receptor coactivator (SRC/FISC) or Cycle (CYC) in a JH-dependent manner (Li, Mead et al. 2011; Shin, Zou et al. 2012).

3. Juvabione and Juvenile hormone mimics from plant

Researchers at Harvard University, USA, identified juvabione. They observed that the European bug, *Pyrrhocoris apterus* L., failed to undergo normal metamorphosis, and instead entered a supernumerary larval molt to form sixth instar larvae. Surviving adults yielded eggs with a low hatch rate. These abnormal physiological states were induced by the paper towels used to culture them. This well-known "Paper factor" was identified from balsam fir. Juvabione is a methyl ester of todomatuic acid, proven to be a very target specific insect JH mimic (Sláma and Williams 1966; Sláma and Williams 1966; Slama 1971). The discovery of this highly specific substance received interest from agricultural industries and screen juvenile hormone mimics from plants for developing insect hormone-based insecticides. However, the screening of plant juvenile hormone mimics made known only a few substances suggesting that juvenile hormone mimics are JH activity on lipid soluble, but these in plants are commonly slightly polar Sesquiterpenoid compounds with a very low JH activity (Sláma 1969; Staal 1975).

4. Insect growth regulator

After the research about juvabione and juvenile hormone mimics, Carol Williams proposed the development of insecticides that mimic the action of insect hormones, as "third-generation insecticides" (Williams 1967). The most important advantage of Insect Growth Regulators (IGRs) is their relatively low environmental toxicity profile. In this respect, IGRs clearly differ from conventional insecticides. The

specificity and selectivity of IGRs are also advantageous. IGRs may be highly effective on some taxa of insects but may exert no effect on some other taxa (Pener and Dhadialla 2012). If you wish to access more in-depth information, see reference 11 (Dhadialla 2012).

1. Juvenile hormone agonists

Juvenile hormone is secreted by a pair of endocrine glands, known as the CA, and it controls insect physiological regulation including development, reproduction and metamorphosis. Absence of juvenile hormone at a particular time window in pre-adult instar larvae allows for metamorphosis, the development to adult stages. In the adult, juvenile hormone plays an important role in the reproduction of certain insects (Wyatt and Davey 1996). Juvenile hormone agonists (JHAs) interfere with metamorphosis when they are present at the time window during which endogenous JH is absent (Dhadialla, Retnakaran et al. 2009).

The first JHAs, farnesol and farnesal, were discovered in laboratory experiments in the 1960s by Schmialek (Schmialek 1961). It came as a surprise that many substances with JH activity had been found before the chemical structure of a natural JH (today known as JH I) was discovered by Röller et al. (1967).

After the discovery of the “paper factor”, the screening of JHA from plants failed. In 1972, Zoecon Corporation patented a highly effective JHA, isopropyl 11-methoxy 3,7,11 trimethyldodeca-2,4-dienoate, researched by Henrick

(Henrick, Staal et al. 1973). This compound, called methoprene, became the first commercialized IGR. Methoprene received full commercial registration in 1975 from the US Environmental Protection Agency (USEPA) to control mosquito larvae. Since the employment of methoprene as a commercial agent, a large number of JHAs have been discovered or synthesized such as fenoxycarb (Dorn, Frischknecht et al. 1981) or pyriproxyfen (HATAKOSHI, AGUI et al. 1986).

2. Ecdysone agonists

20-hydroxyecdysone (20E) is secreted as ecdysone by prothoracic glands and plays a role in almost all molting processes, hence the name insect “molting hormone”. Ecdysone has regulatory roles of its own and may join with 20E to initiate the molting process (Smagghe 2009). Ecdysone, 20E and many other related steroids are collectively termed “ecdysteroids” or “ecdysones”.

There are many natural ecdysteroids in plants called phytoecdysteroids (Dinan, Harmatha et al. 2009), and in animals, they are named zooecdysteroids (Lafont and Koolman 2009). Nevertheless, despite many attempts to develop insecticides based on the structures of natural or synthetic ecdysteroids, none has been developed commercially.

The first reports of a non-steroidal Ecdysteroid Agonist (EA) came from the research laboratories of Rohm and Haas Co. They reported a compound, coded as RH-5849, belonging to the bisacylhydrazine (BAH) class of chemistry, which was found to be effective when ingested by lepidopteran, dipteran and coleopteran larvae. It induced rapid inhibition of feeding during larval stages and

more importantly, caused a premature and unsuccessful molt, interfering with normal cuticle formation and leading to death (Aller and Ramsay 1988; Wing 1988; Wing, Slawecki et al. 1988).

Due to its low potency, RH-5849 was not commercialized. However, the mode of action of this newly discovered member of the BAH class of chemistry became the foundation of extensive research in search of additional compounds with the same mode of action with increased potency and possible insect selective toxicity. Rohm and Haas Co. (now fully owned by the Dow Chemical Company, with the agricultural division owned by Dow AgroSciences, LLC) discovered and developed three commercial BAH EA compounds: tebufenozide, coded as RH-5992 (Heller, Klein et al. 1992); methoxyfenozide, coded as RH-2485 (Le, Thirugnanam et al. 1996); and halofenozide, coded as RH-0345. Halofenozide was commercialized by a joint venture with the American Cyanamide Co. (RohMid 1996). Another BAH EA, chromafenozide, coded as ANS-118, was developed jointly by Nippon Kayaku Co., Ltd. and Sankyo Co., Ltd. It was first registered in Japan (Yanagi, Watanabe et al. 2000; Yanagi, Tsukamoto et al. 2006).

3. Chitin synthase inhibitor

Chitin is a major component of the insect cuticle. It is a straight chain polymer of N-acetyl-D-glucosamine, linked by beta-1-4 bonds. Chitin microfibrils are linked to each other by hydrogen bonds and are mostly, but not always, arranged in chitin lamellae. They are also linked to scleroproteins in the cuticle. The

enzyme, chitin synthase (CS) in the cuticle, transfers N-acetyl-D-glucosamine molecules to construct the polymer chain (Cohen 2010). Chitin Synthase Inhibitors (CSIs) inhibit chitin synthesis which interferes with normal cuticle development. This results in death at, or after the next molt of the target insect. CSIs also affect egg development and thus insect fecundity (Acheuk, Cusson et al. 2012).

From the chemical standpoint, there are two groups of CSIs, benzoylphenyl ureas and compounds not related to benzoylphenyl ureas. Strictly speaking, some of the latter do not inhibit chitin synthesis, but interfere with normal cuticle deposition instead. Nevertheless, they are usually categorized and discussed as CSIs.

One of the most famous CSIs is diflubenzuron. Diflubenzuron is a compound related to benzoylphenyl ureas (1-(4-chlorophenyl-3-(2,6-difluorobenzoyl)-urea). This compound interferes with cuticle deposition. Grosscurt (1978) reviewed the larvicidal and ovicidal effects of diflubenzuron on many species of insects and concluded that the compound interferes with chitin synthase (Grosscurt 1978). Interestingly, these early articles did not use the term “IGR” for benzoylphenyl ureas. Diflubenzuron is still used today as an effective insecticide.

METHODS

1. Yeast two-hybrid binding tests using growth complementation and β-galactosidase assay.

The cDNA fragments encoding the full ORF of each Met and CYC from three insects—*A. aegypti*, *C. pipiens* and *T. castaneum*—and a partial ORF (M1-L689) of *T. castaneum* SRC were synthesized (Bioneer, Daejon, Korea). Bait plasmids were constructed by introducing each insect Met cDNA into the GAL4 DNA binding domain of the vector pGBKT7 (Clontech). GAL4-AD fusion plasmids with each insect CYC and *T. castaneum* SRC were constructed as prey plasmids using the pGADT7 vector (Clontech). In addition, a partial ORF (M1-V510) of *A. aegypti* FISC was cloned into the pGADT7 vector.

These prey plasmids were transformed together with the Met bait plasmid into Y2HGold and Y187 yeast cells for the purpose of the yeast two-hybrid binding test using growth complementation and the quantitative β-galactosidase assay, respectively. For growth complementation, binding was tested in synthetic dropout (SD)-Leu/-Trp/-His/-Ade (QDO) agar medium. The transformed Y187 cells were incubated at 30 °C in SD-Leu/-Trp (DDO) medium until OD_{600} values reached 0.3–0.4, and were then harvested and suspended in twice the volume of the medium. The cells were further incubated for 2h, and then 100μl of the cells in the medium (OD_{600} = 0.2–0.3) were distributed in 96-well plates. Corresponding concentrations of JH or JHA were added into the growth media, and the cells were incubated for a further 3h

and applied to the β-galactosidase assay using the Yeast β-galactosidase Assay kit (Thermo Scientific). The assay reaction mixtures in the 96-well plates were incubated at 24°C for 16 h and then centrifuged. The supernatants were subjected to the OD_{420} measurement.

2. Screening of plant extracts.

The transformed Y187 cells with *A. aegypti* Met-CYC were grown following the protocol described in the previous section. Then, 100 µl of the grown yeast cells (OD_{600} = 0.2–0.3) was treated with 33 ppb pyriproxyfen and 100 ppm of each plant extract (PE) in 96-well plates. A positive control treated with 33 ppb pyriproxyfen and 10 ppm methoprene acid (MA) and a negative control treated with 33 ppb pyriproxyfen and control solvent (DMSO) were placed in each tested plate. The cells were incubated for a further 3 h and subjected to the quantitative β-galactosidase assay. The obtained OD_{420} value for each plant extract-treated sample was converted to an arbitrary unit showing JHAN activity. The OD_{420} value of the negative control was regarded as the 0 unit and one of the 10 ppm MA-treated samples as the 1 unit. A total of 147 extracts from 101 plant species were screened to determine whether they harbored JHAN activity greater than 0.3 units. Repeated tests led to extracts of 83 plant species being selected for use in the subsequent screening step.

$$A = \frac{OD_{420}Control - OD_{420}PE}{OD_{420}Control - OD_{420}MA10}$$

A represents JHAN activity (if A < 0, then A is considered equal to 0). The control was treated with 33 ppb pyriproxyfen. The PE group was treated with 33 ppb

pyriproxyfen and 100 ppm of each plant extract. The MA10 group was treated with 33 ppb pyriproxyfen and 10 ppm MA.

3. Growth inhibition and anti-yeast activity tests.

The transformed Y2HGold (Clontech) yeast cells with *A. aegypti* Met-CYC were incubated at 30 °C in DDO (SD-Leu/-Trp) medium until OD_{600} values reached 0.3–0.4 and then were harvested and suspended in twice the volume of the corresponding medium. QDO medium was used for the growth inhibition test and DDO medium was used for the anti-yeast activity test. The cells were incubated for a further 2 h, and then 200-µl samples were treated with 33 ppb pyriproxyfen and 100 ppm of each screened plant extract in 96-well plates. Each sample was incubated at 30 °C with shaking, and the OD_{600} value of each sample was measured after a 3-h interval.

4. Extraction, isolation and characterization of plant diterpenes.

During October 2013, the fruits of *L. erythrocarpa* were collected from Jeju Island, South Korea, while the roots of *S. serotina* were collected from Hantaek Botanical Garden, South Korea. The dried fruits of *L. erythrocarpa* (58.7 g) and the dried roots of *S. serotina* (32.7 g) were extracted three times with methanol (1 L) at room temperature to obtain 8.2 g and 3.6 g of solid extract respectively. Each methanol extract was subjected to silica gel column chromatography and was further purified by semipreparative HPLC (Gilson) using a Capcell Pak C18 column (Shiseido co, Ltd). LE3B, LE3G and LE3E were isolated from *L. erythrocarpa* fruit, whereas

SS2A and SS5A were purified from *S. serotina* roots. The purified active compounds were analyzed using HRESIMS (Waters Q-TOF Premier), ^{1}H NMR, ^{13}C NMR, DEPT, HMQC, COSY and HMBC (Bruker AM500 MHz FT-NMR spectrometer). 1H–NMR, 13C-NMR, HRESIMS spectra and ULPC chromatograms of five compounds are shown in Figure 1 to 5.

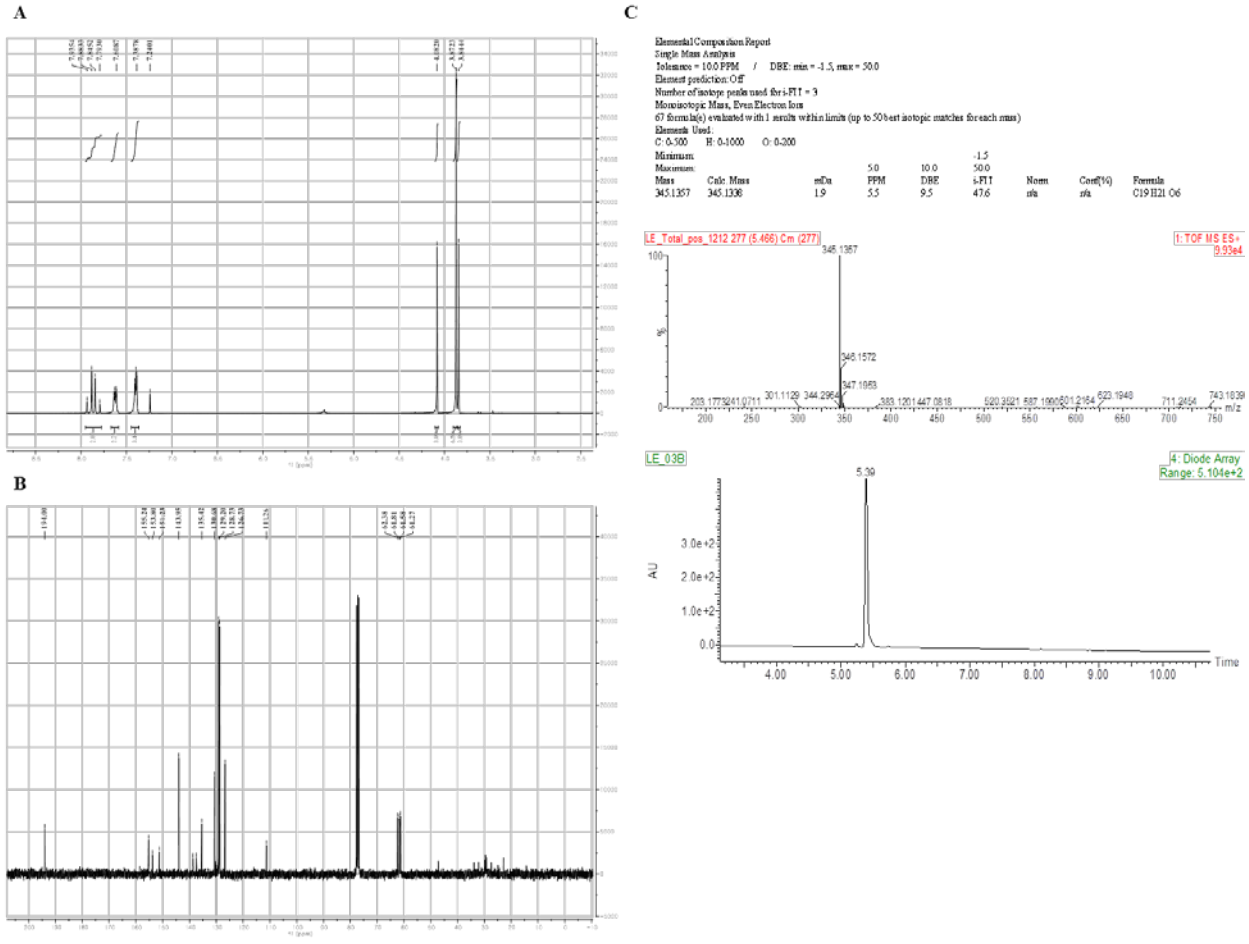

Figure 1 Molecular characterization of LE3B (Kanakugiol) isolated from *L. erythrocarpa*. **A**. ^{1}H NMR spectrum by using 400 MHz Bruker. **B.** ^{13}C NMR spectrum by using 75 MHz Bruker. **C.** Elemental composition report, HRESIMS spectrum and ULPC chromatogram by using waters Q-TOF premier

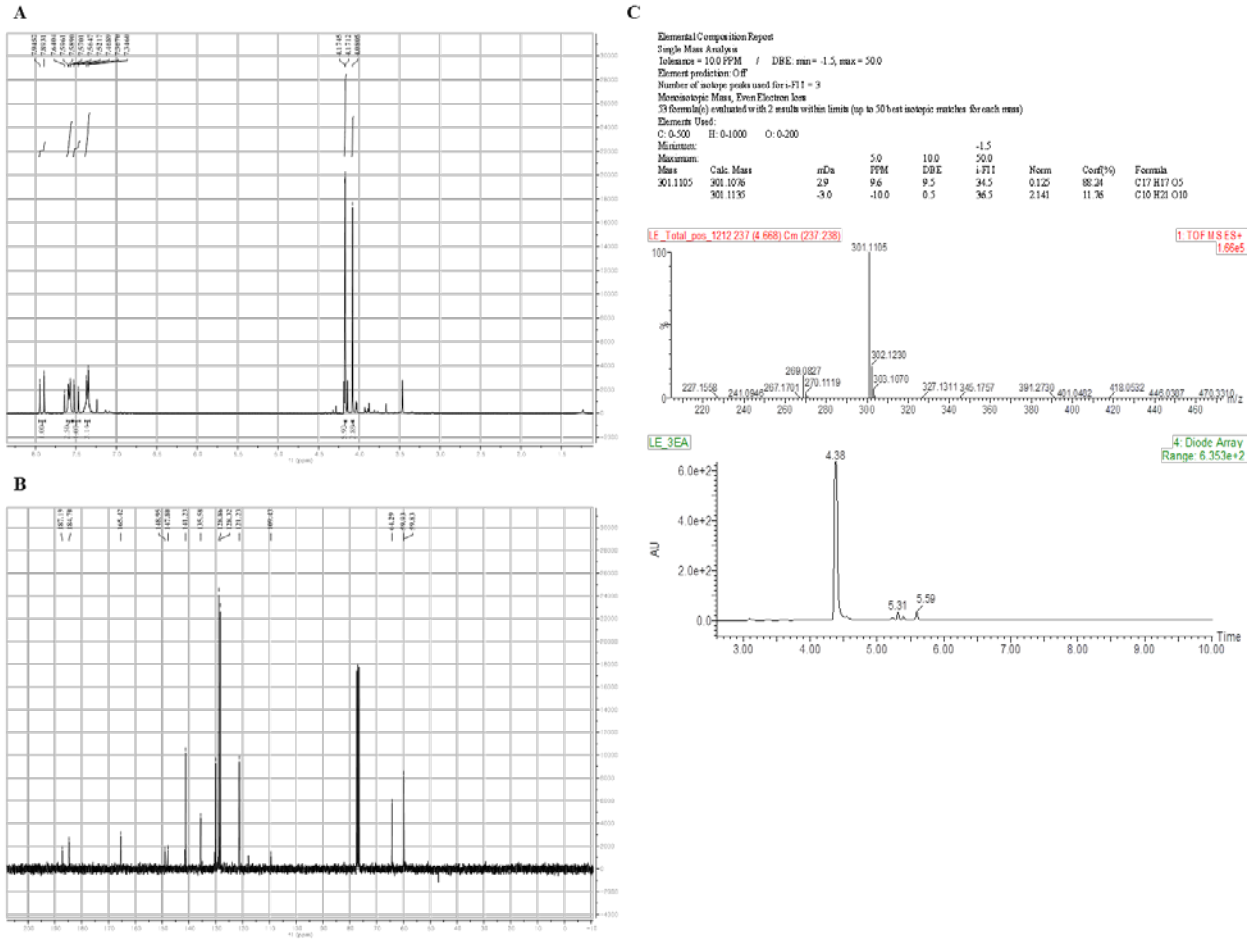

Figure 2 Molecular characterization of LE3E (Methyl linderone) isolated from *L. erythrocarpa*. **A.** ^{1}H NMR spectrum by using 400 MHz Bruker. **B.** ^{13}C NMR spectrum of by using 75 MHz Bruker. **C.** Elemental composition report, HRESIMS spectrum and ULPC chromatogram by using waters Q-TOF premier.

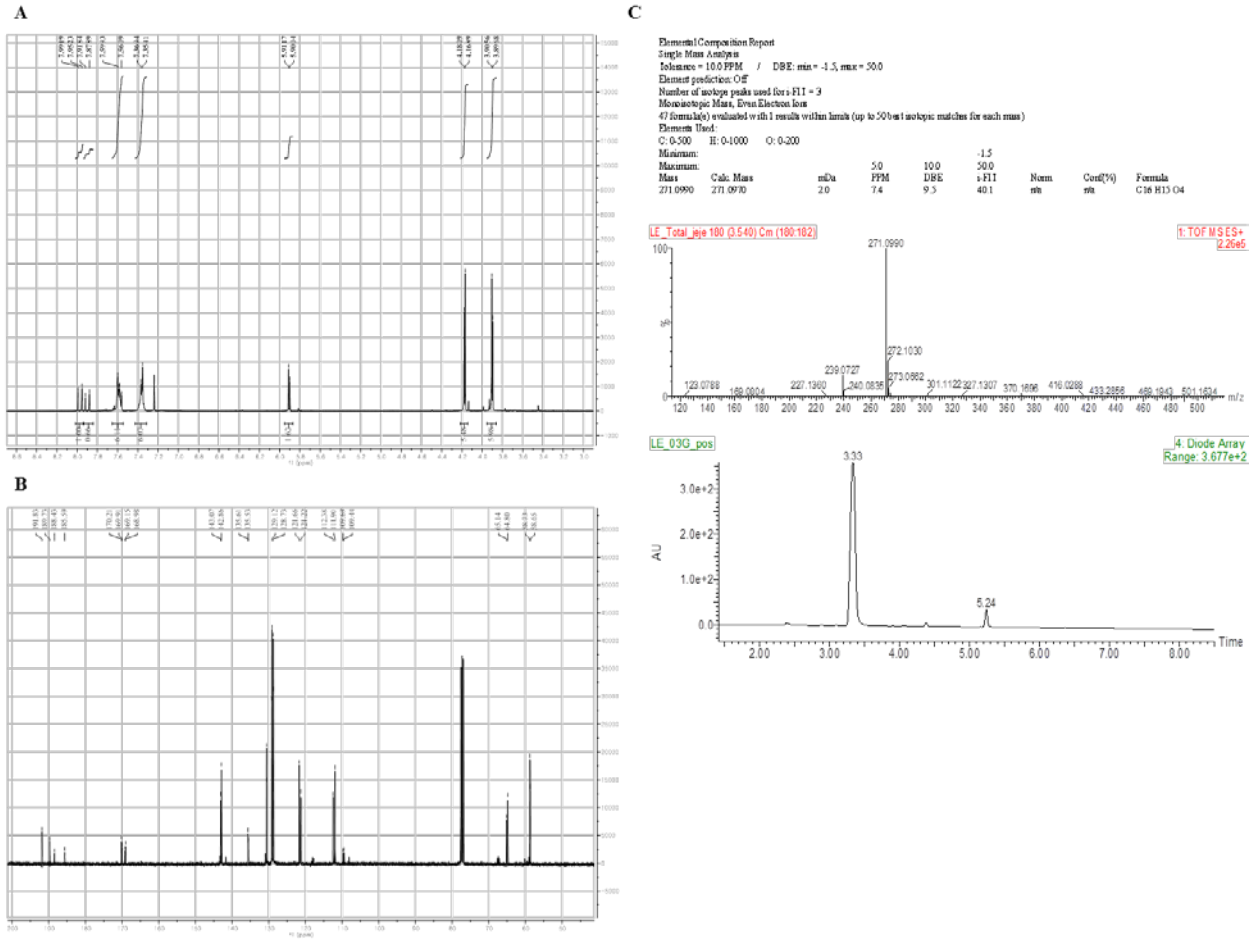

Figure 3 Molecular characterization of LE3G (Methyl lucidone) isolated from *L. erythrocarpa*. **A.** ^{1}H NMR spectrum by using 400 MHz Bruker. **B.** ^{13}C NMR spectrum by using 75 MHz Bruker. **C.** Elemental composition report, HRESIMS spectrum and ULPC chromatogram of by using waters Q-TOF premier.

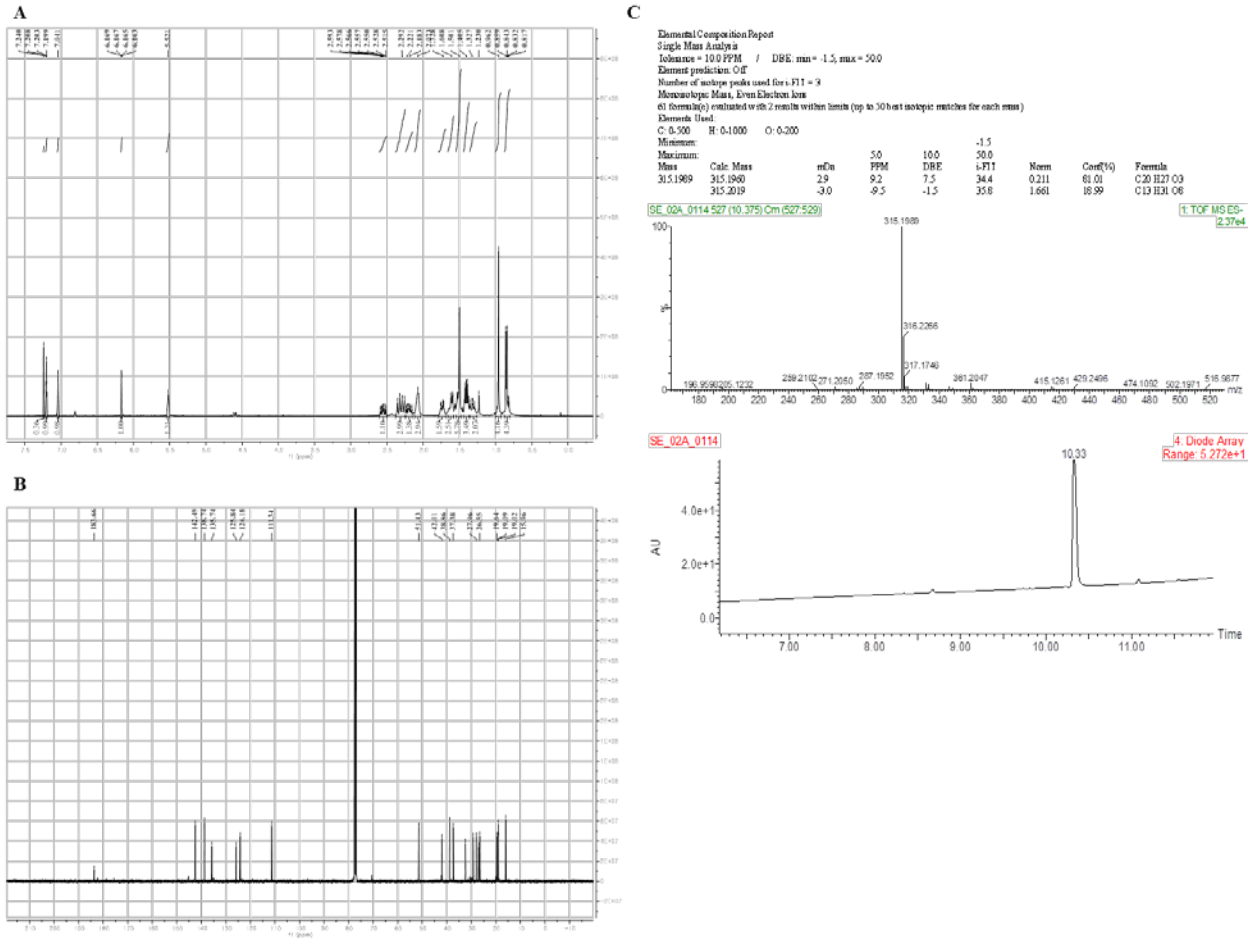

Figure 4 Molecular characterization of SS2A (Solidagoic acid) isolated from *S. serotina.* **A.** ^{1}H NMR spectrum by using 400 MHz Bruker. **B.** ^{13}C NMR spectrum by using 75 MHz Bruker. **C.** Elemental composition report, HRESIMS spectrum and ULPC chromatogram by using waters Q-TOF premier.

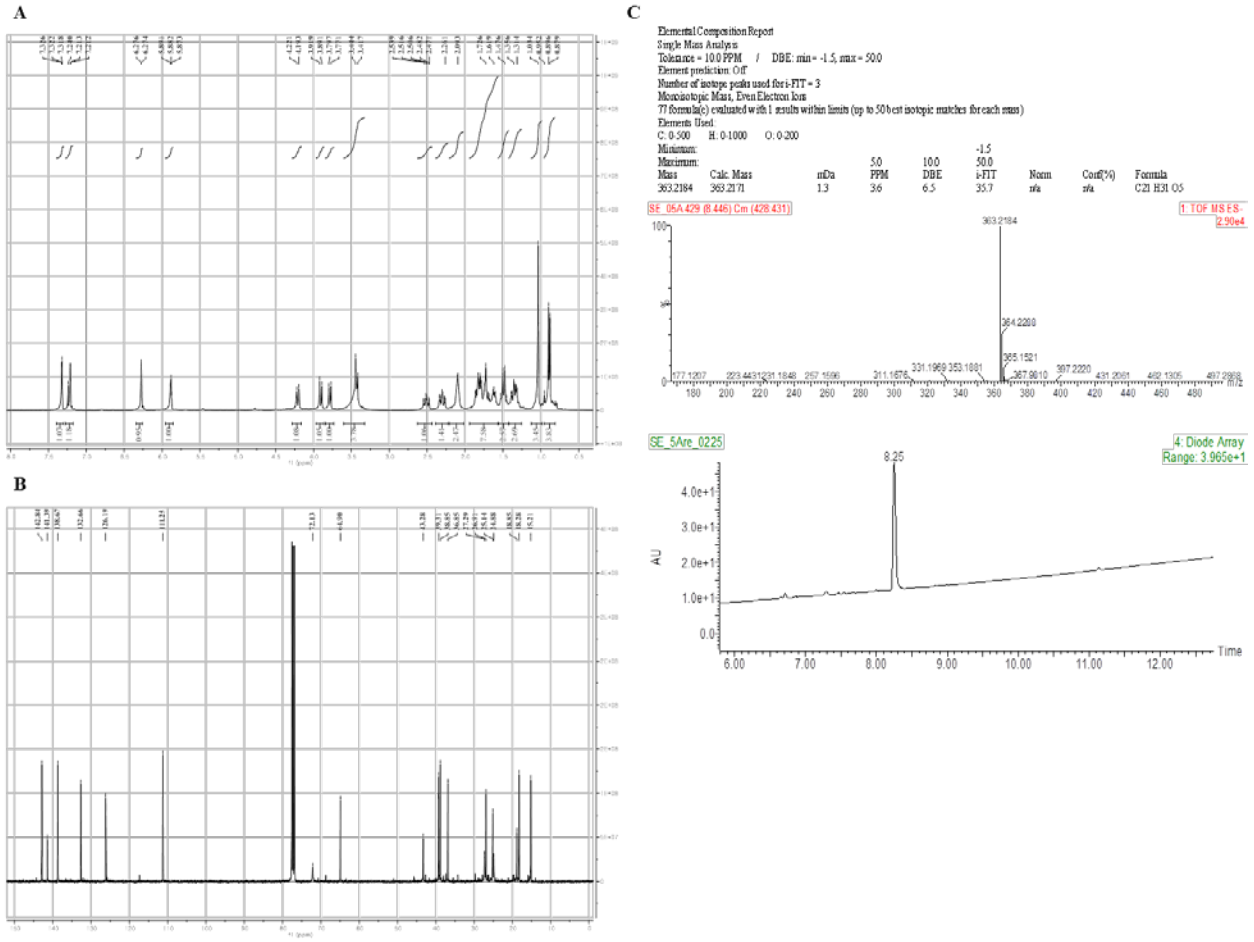

Figure 5 Molecular characterization of SS5A (Kingidiol) isolated from *S. serotina.* **A.** ^{1}H NMR spectrum by using 400 MHz Bruker. **B.** ^{13}C NMR spectrum by using 75 MHz Bruker. **C.** Elemental composition report, HRESIMS spectrum and ULPC chromatogram by using waters Q-TOF premier.

LE3B (Kanakugiol) - yellow oil; UV (MeOH) λmax nm 208, 314; ^{1}H NMR (400 MHz, CDCl3) δ 7.91 (1H, d, J = 15.5 Hz, H-α), 7.82 (1H, d, J = 15.5 Hz, H-β), 7.62 (1H, m, H-2 and H-6), 7.40 (1H, m, H-3, H-4, H-5), 4.08 (3H, s, 2'-OCH3), 3.87 (3H, s, 3'-OCH3 and 5'-OCH3), 3.84 (3H, s, 4'-OCH3); ^{13}C NMR (100 MHz, CDCl3) 194.0 (C-β'), 155.2 (C-2'), 153.8 (C-4'), 151.2 (C-6'), 144.4 (C-β), 138.7 (C-5'), 137.5 (C-3'), 135.4 (C-1), 130.7 (C-4), 129.2 (C-3 and C-5), 128.7 (C-2 and C-6), 126.7(C-α), 111.3 (C-1'), 62.4 (C-2'-OCH3), 61.8 (C-5'-OCH3), 61.6 (C-3'-OCH3), 61.3(C-4'-OCH3); HRESIMS *m/z* [M+H]+ 345.1334, (calculated for $C_{19}H_{21}O_6$, 345.1338).

LE3E (Methyl linderone) - yellow solid; UV (MeOH) λmax nm 248, 364; ^{1}H NMR (400 MHz, CDCl3) δ 7.92 (1H, d, J = 15.9 Hz, H-α), 7.59 (1H, m, H-2 and H-6), 7.50 (1H, d, J = 15.9 Hz, H-β), 7.36 (1H, m, H-3, H-4, H-5), 4.174 (3H, s, 2'-OCH3), 4.171 (3H, s, 3'-OCH3), 4.08 (3H, s, β'-OCH3); ^{13}C NMR (100 MHz, CDCl3) 187.2 (C-5'), 184.7 (C-2'), 165.4 (C- β'), 149.0 (C-3'), 147.8 (C-4'), 141.2 (C-4), 135.6 (C-1), 130.0 (C-β), 128.9 (C-3 and C-5), 128.3 (C-2 and C-6), 121.2 (C-α), 109.4 (C-1'), 64.3 (C- β'-OCH3) 59.9 (C-2'-OCH3), 59.8 (C-3'-OCH3); HRESIMS *m/z* [M+H]+ 301.1104, (calculated for $C_{17}H_{17}O_5$, 301.1076).

LE3G (Methyl lucidone) - yellow solid; UV (MeOH) λmax nm 248, 354; ^{1}H NMR (400 MHz, CDCl3) δ 7.97 (1H, d, J = 15.6 Hz, H-α), 7.60 (1H, d, J = 15.9 Hz, H-β), 7.58 (1H, m, H-2 and H-6), 7.36 (1H, m, H-3, H-4, H-5), 5.92 (1H, s, H-4'), 4.17 (3H, s, β'-OCH3), 3.91 (3H, s, 3'-OCH3); ^{13}C NMR (100 MHz, CDCl3) 191.8 (C-5'), 185.6 (C-2'), 170.2 (C-3'), 169.0 (C- β'), 142.9 (C-β), 135.6 (C-1), 130.5 (C-4), 129.1 (C-3 and C-5), 128.7 (C-2 and C-6), 121.7 (C-α), 111.9 (C-4'), 109.4 (C-1'), 64.8 (C- β'-OCH3), 58.7 (C-3'-OCH3); HRESIMS *m/z* [M+H]+ 271.0990,

(calculated for $C_{16}H_{15}O_4$, 271.0970).

SS2A (Solidagoic acid A) - white solid; UV (MeOH) λmax nm 217; ^{1}H NMR (400 MHz, CDCl3) δ 7.20 (1H, t, J = 1.6 Hz, H-15), 7.04 (1H, s, H-16), 6.17 (1H, dd, J = 1.6, 0.8 Hz, H-14), 5.52 (H, br s, H-3), 2.56 (1H, m, H-12a), 2.33 (1H, m, H-10), 2.27 (1H, m, H-6a), 2.19 (1H, m, H-12b), 2.07 (2H, m, H-2), 1.74 (2H, m, H-1), 1.59 (1H, m, H-7a, H-8), 1.40 (1H, m, H-6a, H-6b, H-11), 1.50 (3H, s, H-18), 1.29 (1H, m, H-7b), 0.96 (3H, s, H-19) 0.84 (3H, d, J = 6.4 Hz, H-17); ^{13}C NMR (100 MHz, CDCl3) 183.7 (C-20), 142.5 (C-15), 138.7 (C-16), 135.7 (C-4), 125.8 (C-13), 124.2 (C-3), 111.3 (C-14), 51.4 (C-5), 42.0 (C-10), 38.9 (C-9), 37.4 (C-8), 32.4 (C-11), 29.3 (C-6), 28.0 (C-7), 27.1 (C-19), 26.5 (C-2), 19.6 (C-1), 19.1 (C-18), 19.0 (C-12), 16.0 (C-17); HRESIMS *m/z* [M-H]- 315.1989, (calculated for $C_{20}H_{27}O_3$, 315.1960).

SS5A (Kingidiol) – white solid; UV (MeOH) λmax nm 202; ^{1}H NMR (400 MHz, CDCl3) δ 7.32 (1H, t, J=1.6 Hz, H-15), 7.21 (1H, d, J = 0.4 Hz, H-16), 6.28 (1H, d, J = 0.8 Hz, H-14), 5.89 (1H, t, J = 4.8 Hz, H-3), 4.21 (1H, d, J = 11.2 Hz, H-19a), 3.91 (1H, d, J = 11.2 Hz, H-19b), 3.79 (1H, d, J = 10.6 Hz, H-20a), 3.43 (1H, d, J = 10.6 Hz, H-20b), 2.52 (1H, m, H-12a), 2.31 (1H, m, H-12b), 2.10 (2H, m, H-2), 1.85 (1H, m, H-11a), 1.83 (1H, m, H-1a), 1.81 (1H, m, H-6a), 1.73 (1H, m, H-11b), 1.69 (1H, m, H-1b), 1.62 (1H, m, H-8), 1.49 (1H, m, H-6b, H-7a), 1.35 (1H, m, H-10), 1.32 (1H, m, H-7b), 1.03 (3H, s, H-18), 0.89 (3H, d, J = 6.8 Hz, H-17); ^{13}C NMR (100 MHz, CDCl3) 142.8 (C-15), 141.4 (C-4), 138.7 (C-16), 132.7 (C-3), 126.2 (C-13), 111.3 (C-14), 72.1 (C-20), 64.9 (C-19), 43.3 (C-5), 39.3 (C-10, C-11), 38.9 (C-9), 36.9 (C-8), 27.3 (C-6), 26.9 (C-7), 25.1 (C-2), 24.9 (C-18), 18.8 (C-1), 18.3 (C-12), 15.2 (C-17); HRESIMS *m/z* [M-H+FA]- 363.2184, (calculated for $C_{20}H_{29}O_3$•CH_2O_2,

363.2171).

5. Larval toxicity tests and LD_{50} determination.

Ten 3rd instar mosquito larvae in 3 mL tap water with food mixtures were treated with a 500-ppm concentration of each plant extract. The numbers of dead larvae were counted 24 h after treatment. No dead larva was found in the negative controls treated with the solvent DMSO. All experiments were performed with three replicates, and the average mortality rate was calculated. For the determination of LD_{50} of PJHAN compounds, ten larvae were treated with different concentrations of each compound with three replicates, and the numbers of dead larvae were counted 24 h after treatment. The average larval mortality data from gradual concentration were subjected to PROBIT analysis to calculate LC_{50}.

6. *In vivo* tests of PJHANs.

Half-microgram samples of the diterpene compounds in 0.2 μl acetone were topically applied onto abdomens of newly emerged mosquitoes. Acetone was used as a control. Two days after treatment, total RNA was prepared for Real-Time PCR analysis of kr-h1 and Hairy gene expression. To assess the development of ovarian follicles, the mosquitoes were dissected 3 days after treatment. To test the prolonged effect of LE3B, twenty 2nd instar mosquito larvae in 10 mL tap water with food mixtures were treated with a 10ppm concentration of LE3B. The numbers of dead larvae were counted twenty-four hours after treatment and surviving larvae were

maintained at 26 °C until adult emergence. The density of larvae was adjusted by adding more tap water with food mixture to control samples. The larvae were left undisturbed until adult emergence and then the ovaries of emerged female mosquitoes were dissected 4 days after emergence.

7. RNA preparation and Real-Time RT-PCR.

Total RNA was prepared using TRIzol (GIBCO/BRL). Real-time RT-PCR experiments were performed following a previously described method (Li, Mead et al. 2011).

8. Maintenance of mosquitoes and vertebrate approval.

The mosquito *A. aegypti* UGAL/Rockefeller strain was raised as described previously (Roy, Hansen et al. 2007), and all animal experiments were performed with the approval of the Institutional Animal Use and Care Committee of the Korea Research Institute of Bioscience and Biotechnology.

RESULTS

1. Yeast two-hybrid binding tests using growth complementation and β-galactosidase assays.

The cDNAs encoding full open reading frames (ORFs) of *Met* and *CYC* genes from two mosquitoes, *A. aegypti* and *Culex pipiens*, and a storage crop pest beetle, *Tribolium castaneum*, were synthesized and introduced into yeast two-hybrid bait and prey plasmids respectively. In this two-hybrid yeast assay, binding of Met and CYC from mosquitoes occurred in the presence of JHA and pyriproxyfen, whereas that from the beetle did not (Fig. 6). Instead, we observed pyriproxyfen-mediated binding between the beetle Met and SRC (Fig. 6). Although the pyriproxyfen-mediated binding of *A. aegypti* Met-CYC, *C. pipiens* Met-CYC, and the beetle Met-SRC was apparent in Y2HGold yeast cells (Fig. 6), only the binding between *A. aegypti* Met-CYC was successfully simulated by two-hybrid yeast β-galactosidase assays in Y187 yeast cells (Fig. 7). Therefore, we used *A. aegypti* Met-CYC for all subsequent experiments.

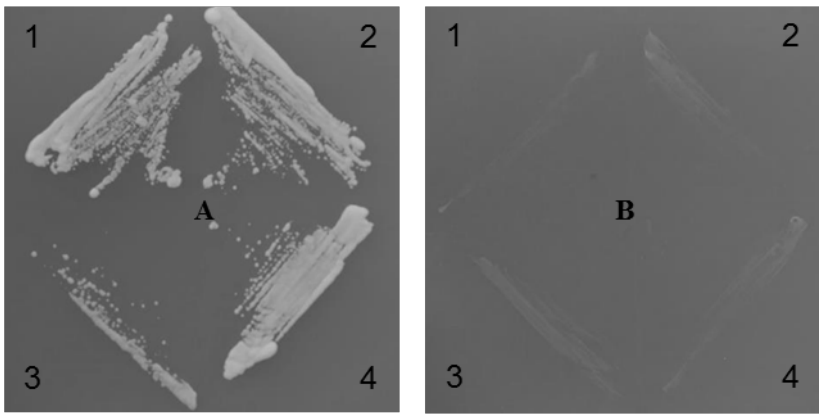

Figure 6 Pyriproxyfen-mediated binding of *A. aegypti* Met-CYC, *C. pipiens* Met-CYC and *T. castaneum* Met-SRC was observed by means of the growth complementation test of Y2HGold yeast cells transformed with each corresponding pair of plasmids. **A**: + pyriproxyfen, QDO agar media, **B**: - pyriproxyfen, QDO agar media, **1**: *A. aegypti* Met-CYC, **2**: *C. pipiens* Met-CYC, **3**: *T. castaneum* Met-CYC, **4**: *T. castaneum* Met-SRC.

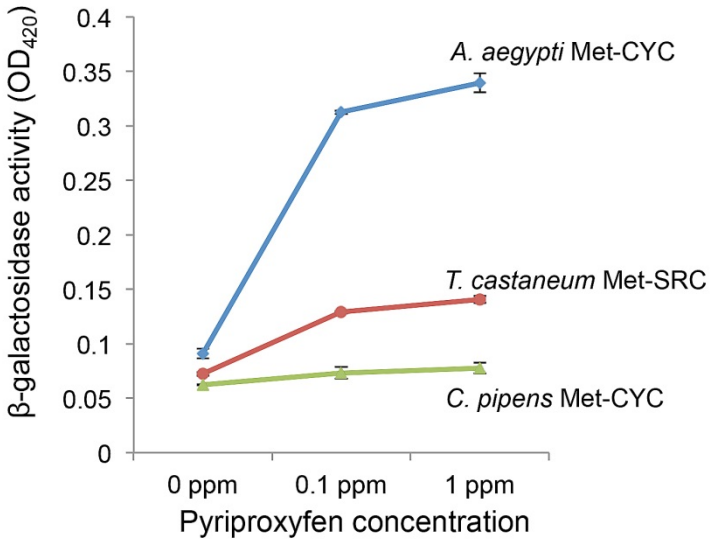

Figure 7 Quantitative β-galactosidase assay with Y187 yeast cells transformed with each corresponding pair of plasmids.

2. Screening of plant extracts.

First, we investigated whether the pyriproxyfen-mediated Met-CYC binding could be disrupted by plant extracts in the two-hybrid yeast assay. We tested methanol extracts prepared from 1,651 plant species (Korean Plant Extracts Bank, Daejeon, Korea), directly adding each extract to the yeast culture. Extracts from 83 plants were found to interfere with pyriproxyfen-mediated binding of *A. aegypti* Met-CYC. Figure 8A shows a few examples. In a parallel study, we tested the effect of methoprene acid (MA) on the pyriproxyfen-mediated Met-CYC binding in the two-hybrid yeast assay. Because MA interfered with the binding in a dose-dependent manner, it was used as a positive control during screening (Fig. 8A and 9B).

To eliminate the possibility of false signals originating from anti-yeast activities of plant extracts, we tested growth inhibition of the Y2HGold yeast strain transformed with Met and CYC (Fig. 8B for examples). The addition of several plant extracts interfered with Met-CYC binding, resulting in normal yeast growth in non-selective double dropout minimal media (DDO, -leu/-trp), whereas retarded growth was recorded in selective quadruple dropout minimal media (QDO, -leu/-trp/-his/-ade) (Fig. 8B). The plant extracts that caused yeast growth retardation in both DDO and QDO media, such as extracts from *Magnolia obovata* trunks and *Dioscorea septemloba* roots, were eliminated from further screening (Fig. 8A and 8B). Among 83 extracts from plant species that were initially screened, 72 passed this test but 11 failed due to their inhibitory action on yeast growth. Hence, we concluded that these 72 plant extracts that directly disrupt the JH receptor complex exhibit JHAN activity.

In a third screening step, we investigated mosquito larvicidal toxicity of selected

plant extracts. Ten 3rd instar mosquito larvae in 3 ml tap water with food mixtures were treated with 500 ppm concentration of each plant extract. Of these 72 extracts, 53 (74%) caused more than 50% larval mortality, suggesting that the larvicidal toxicity of these plant extracts is associated with PJHAN activity (Fig. 8C and Table 1). In contrast, when we tested toxicity of extracts from 110 randomly selected plant species, we found that extracts from only 22 plant species (20%) caused a similar larval mortality (Fig. 8C).

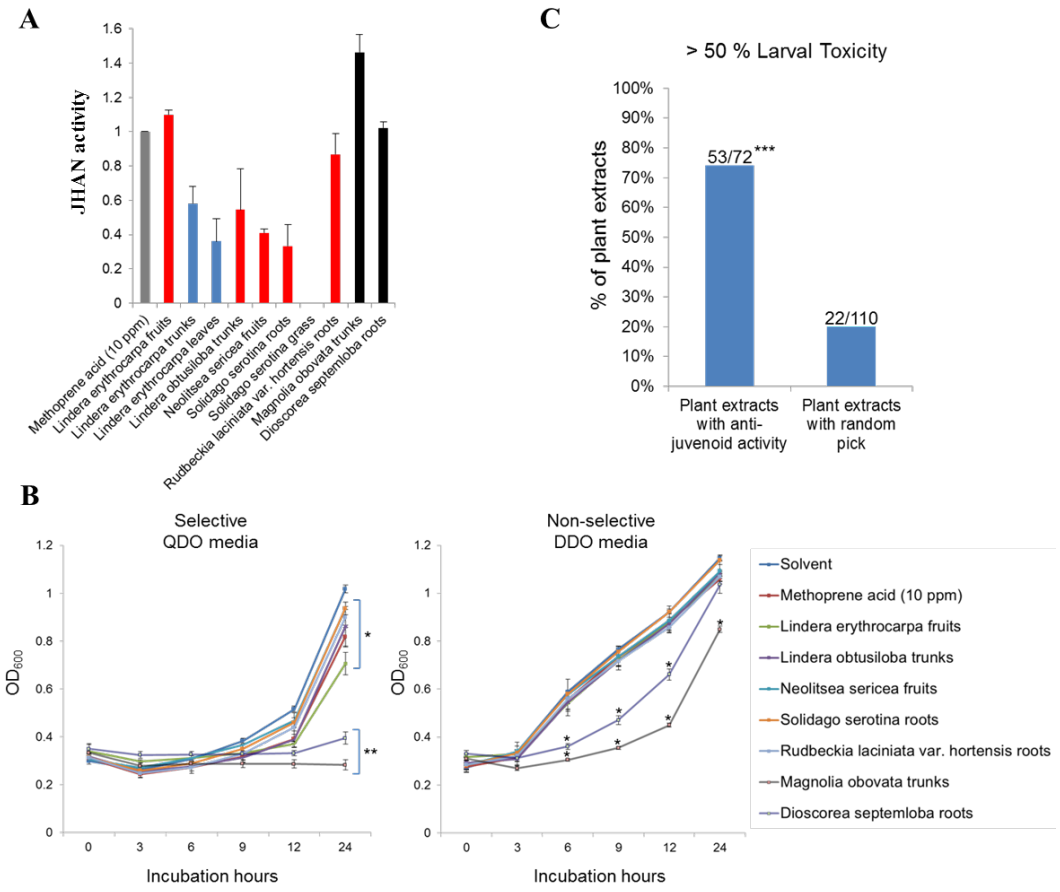

Figure 8 Screening of the plant extracts harboring JHAN and mosquito larvicidal activity. **A.** JHAN activity of some plant extracts. The Met-CYC binding triggered by 33ng/ml pyriproxyfen was simulated by β-galactosidase activity in the yeast two hybrid systems. Inhibition of β-galactosidase activity by treatment with 10 ppm methoprene acid was used as a positive control. Each plant extract was added to the yeast culture at a concentration of 100 ppm. When multiple extracts from the same plant species were screened to have JHAN activity, only the extract with the strongest activity was selected for further screening (red column). For example, the trunks and leaves of *L. erythrocarpa* were excluded from screening (blue column) because the extract from the plant fruits had the strongest JHAN activity. **B.** Distinction between JHAN and anti-yeast activity. Two plant extracts from *Magnolia obovate* trunks and *Dioscorea septemloba* roots (**A**, black column) were excluded from the screening because they significantly inhibited yeast growth when compared with the control solvent. * $P < 0.01$ (student t-test), ** $P < 1.0$ E-5 (student t-test). **C.** Correlation between JHAN activity and larvicidal toxicity in the screened plant extracts. *** $P < 0.0001$ (two-sample z-test).

Table 1 The list of 53 plant species extract which exhibited JHAN activity and mosquito larvicidal toxicity**.**

plant family	plant species	plant part
Aceraceae	Acer takesimense	trunk-bark
Araliaceae	Hedera rhombea	fruit
Araliaceae	Kalopanax pictus	trunk-bark
Araliaceae	Acanthopanax chiisanensis	trunk
Araliaceae	Oplopanax elatus	trunk
Araliaceae	Aralia continentalis	whole
Aristolochiaceae	Aristolochia manshuriensis	trunk
Betulaceae	Betula schmidtii	leaf
Betulaceae	Alnus japonica	flower
Betulaceae	Alnus firma	flower
Celastraceae	Euonymus sieboldiana	fruit
Compositae	Aster koraiensis	flower
Compositae	Solidago serotina	root
Compositae	Rudbeckia laciniata var. hortensis	root
Compositae	Carpesium abrotanoides	flower
Compositae	Siegesbeckia glabrescens	whole
Compositae	Helianthus annuus	fruit-seed
Cupressaceae	Thuja orientalis	trunk-bark
Cupressaceae	Juniperus virginiana	leaf-trunk
Cupressaceae	Juniperus communis	trunk-bark
Ericaceae	Rhododendron micranthum	leaf-trunk
Euphorbiaceae	Euphorbia ebracteolata	root
Euphorbiaceae	Mallotus japonicus	exciple
Lauraceae	Actinodaphne lancifolia	whole
Lauraceae	Neolitsea sericea	fruit
Lauraceae	*Lindera obtusiloba*	trunk-bark
Lauraceae	*Lindera erythrocarpa*	fruit

plant family	plant species	plant part
Leguminosae	*Trifolium pratense*	whole
Leguminosae	*Sophora flavescens*	root
Leguminosae	*Albizzia julibrissin*	trunk-bark
Liliaceae	*Majanthemum dilatatum*	flower
Magnoliaceae	*Magnolia sieboldii*	trunk
Moraceae	*Ficus carica*	trunk-bark
Pinaceae	*Pinus thunbergii*	trunk-heartwood
Pinaceae	*Pinus banksiana*	trunk
Pinaceae	*Pinus densiflora*	root
Pinaceae	*Pinus koraiensis*	root
Pinaceae	*Pinus densiflora for. multicaulis*	trunk
Pinaceae	*Pinus bungeana*	trunk
Primulaceae	*Primula modesta var. fauriae*	whole
Pteridaceae	Matteuccia orientalis	whole
Rosaceae	*Spiraea microgyna*	seed
Rutaceae	*Citrus junos*	root
Salicaceae	*Salix hallaisanensis*	leaf
Saxifragaceae	*Hydrangea macrophylla for. otaksa*	grass
Sterculiaceae	*Firmiana simplex*	leaf-trunk
Taxodiaceae	*Sciadopitys verticillata*	leaf
Umbelliferae	*Angelica czernevia*	root
Umbelliferae	*Peucedanum japonicum*	root
Urticaceae	*Pilea hamaoi*	whole
Valerianaceae	*Patrinia scabiosaefolia*	seed
Verbenaceae	*Caryopteris divaricata*	seed
Zingiberaceae	*Zingiber officinale*	root

3. Extraction, isolation and characterization of plant diterpenes

We then purified putative plant juvenile hormone antagonist (PJHAN) compounds from two of the screened plants. The extracts of the two plants were selected based on their availability for collection and the strength of their JHAN activity—specifically *Lindera erythrocarpa* fruits and *Solidago serotina* roots, which have relatively strong and mild activities respectively (Fig. 8A and 8B). Purification resulted in isolation of three diterpene compounds from *L. erythrocarpa* (LE3B [Kanakugiol], LE3E [Methyl linderone] and LE3G [Methyl lucidone]) and two diterpenes from *S. serotina* (SS2A [Solidagoic acid A] and SS5A [Kingidiol]) (Fig. 9A) (McCrindle and Nakamura 1974; Bohlmann, Zdero et al. 1984; Ng, Lee et al. 1990; Sosa, Tonn et al. 1994; Lee, Lee et al. 2002). Details of the purification process and molecular characterization are shown in Figures 1 to 5. The molecular structures of these diterpene molecules differed from both the sesquiterpenoid structure of JHs and various terpenoid structures of JHAs (Fig. 2A).

These diterpene molecules interfered with pyriproxyfen-mediated Met-CYC binding in the β-galactosidase assay using the Y187 yeast strain in a dose-dependent manner (Fig. 9B). Binding between Met and SRC/FISC/Taiman was observed in *A. aegypti* (Li, Mead et al. 2011), *T. castaneum* (Jindra, Palli et al. 2013) and *Bombyx mori* (Kayukawa, Minakuchi et al. 2012). When we tested whether the plant diterpenes interfered with the pyriproxyfen-mediated binding between *A. aegypti* Met and FISC, we found that the diterpenes interfered with JHA-dependent Met-FISC binding in a very similar manner with Met-CYC binding (Fig. 9B). These results show that the diterpenes disrupt both the hormone-mediated heterodimer of

Met-CYC and that of Met-FISC. Of the five diterpenes, four also exhibited a significant larvicidal toxicity against 3rd instar mosquito larvae, with an LD_{50} of less than 100 ppm at 24 h after treatment (Table 2). Yet, they were minimally toxic towards Human Embryonic Kidney 293 cells, except for LE3E compound (Fig. 10).

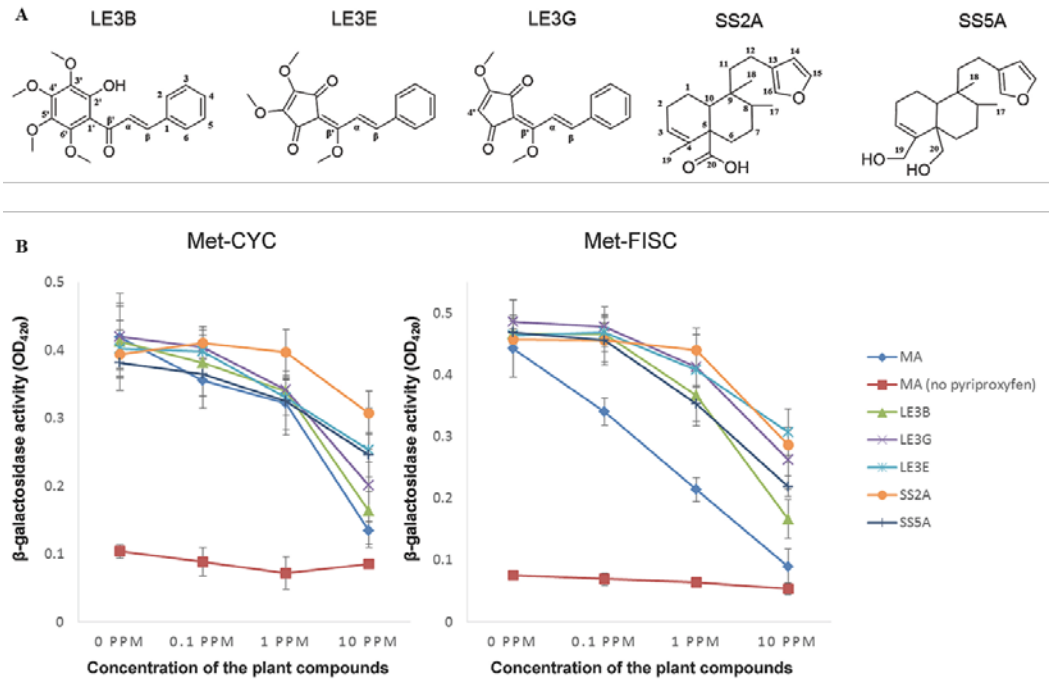

Figure 9 PJHAN compounds and their JHAN activities. **A**. chemical structures of five PJHAN diterpene molecules isolated from *Lindera erythrocarpa* fruits and *Solidago serotina* roots identified by 1H-NMR, 13C-NMR, and HRESIMS. **B**. Concentration-dependent inhibition of pyriproxyfen-mediated Met-CYC or Met-FISC binding by the isolated diterpenes. To initiate the binding, 0.033 ppm of pyriproxyfen was applied into each reaction.

Table 2 Characteristics of isolated plant diterpenes

Plant extracts	Compounds	JHAN activity (10 ppm) ‡	LD_{50} at 24 h†	Effect on development (0.5 μg per insect) ‡	Impaired gene expression (0.5 μg per insect) ‡
Lindera erythrocarpa fruits	LE3B	0.89	23 ppm	defect of ovarian follicles [1), 2)]	Significant reduction
	LE3E	0.62	47 ppm	ND*	NS**
	LE3G	0.76	31 ppm	ND*	NS**
Solidago serotina roots	SS2A	0.39	>100 ppm	ND*	NS**
	SS5A	0.62	85 ppm	defect of ovarian follicles [3)]	Significant reduction
Methoprene acid		1	>100 ppm	ND*	NS**

1) Control solvent-treated, follicle number per ovary, 102.32 ± 16.14; Control follicle size, 102.31 ± 15.76

2) LE3B treated, follicle number per ovary, 62.25 ± 10.94 (p=3.7E-12, *t*-test); Control follicle size, 66.41 ± 19.10 (p= 7.0E-49)

3) SS5A treated, follicle number per ovary, 86.33 ± 6.98 (p=0.029); Control follicle size, 91.27 ±16.64 (p=0.054)

***, not-detected; **, not significant; †, average of independent two replication data, ‡, independent three replication data set.**

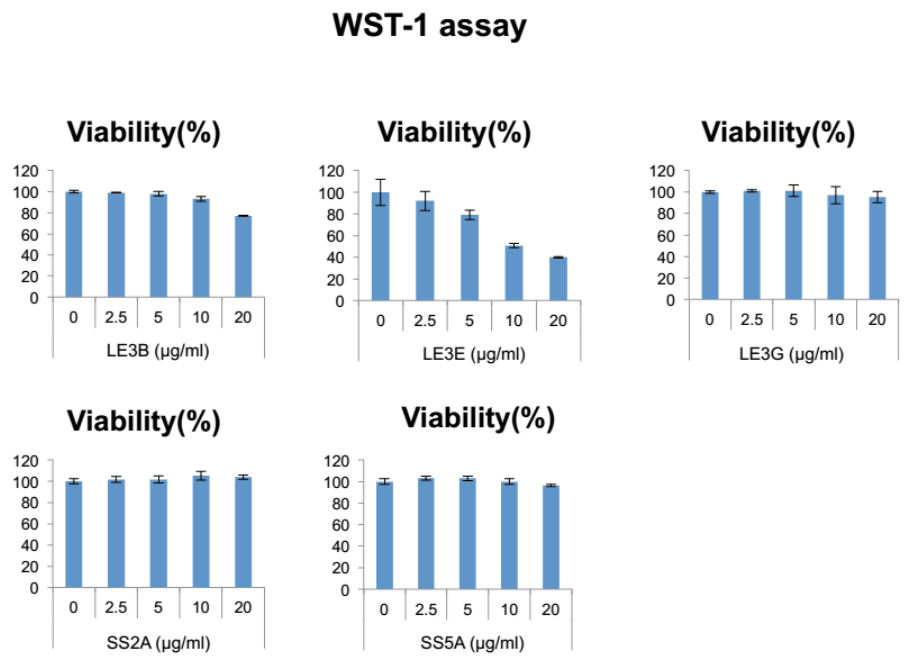

Figure 10 Cytotoxicity tests of purified compounds with HEK293 cells by WST-1 assay

4. *In vivo* tests of PJHANs

Next, we conducted *in vivo* tests using the purified diterpene molecules. In the female *A. aegypti* mosquito, JH removal or Met RNA interference silencing negatively affected the gene expression of two transcriptional regulators, Hairy and Kr-h1 (Bowers 2012). In addition, these treatments resulted in arrest of ovarian development with a significant reduction in the size of ovarian follicles (Zou, Saha et al. 2013). We applied 0.5 μg of either LE3B or SS5A, each purified in 0.2 μl acetone, topically onto the abdomens of newly emerged mosquitoes (12-24 h posteclosion). Acetone was used as a control. Two days after treatment, total RNA was prepared for real-time PCR analysis of Kr-h1 and Hairy gene expression. Application of two molecules, LE3B and SS5A, phenocopied the effects of JH and Met depletions. The expression of both Hairy and Kr-h1 were significantly reduced (Fig. 11A). To assess whether these compounds affect development of ovarian follicles, the mosquitoes were dissected 3 days after treatment, after application of either LE3B or SS5A. These treatments resulted in the retardation of ovarian development, with significantly smaller and fewer ovarian follicles than solvent-treated mosquitoes from the control group (Fig. 11B and Table 2). These results indicate that these two diterpenes disrupt JH-dependent regulation. Mosquitoes treated with the other three diterpene molecules exhibited no significant changes in either Hairy or Kr-h1 expression or in the size of ovarian follicles.

We also tested the effect of LE3B on mosquito development through longer exposure to 2nd instar mosquito larvae. We treated them with a sub-lethal concentration of LE3B (10 ppm), which caused 25% (15/20) to 50% (10/20)

mortality overnight (Table 3). In the control group, 90-95% of DMSO-treated larvae survived the night (Table 3). All of the surviving larvae in both the LE3B- or DMSO-treated samples were allowed to grow until adult emergence. Four days after emergence, female mosquitoes were dissected to observe ovary development (Fig. 12). We observed that the treatment caused neither morphological disturbance nor lethality through premature or precocious metamorphosis. Instead, we observed early adult emergence (Table. 3) and a corresponding defect on ovary development in LE3B-treated mosquitoes (Fig. 12). This indicates that the forced early metamorphosis triggered by the plant compound may cause defects in ovary development.

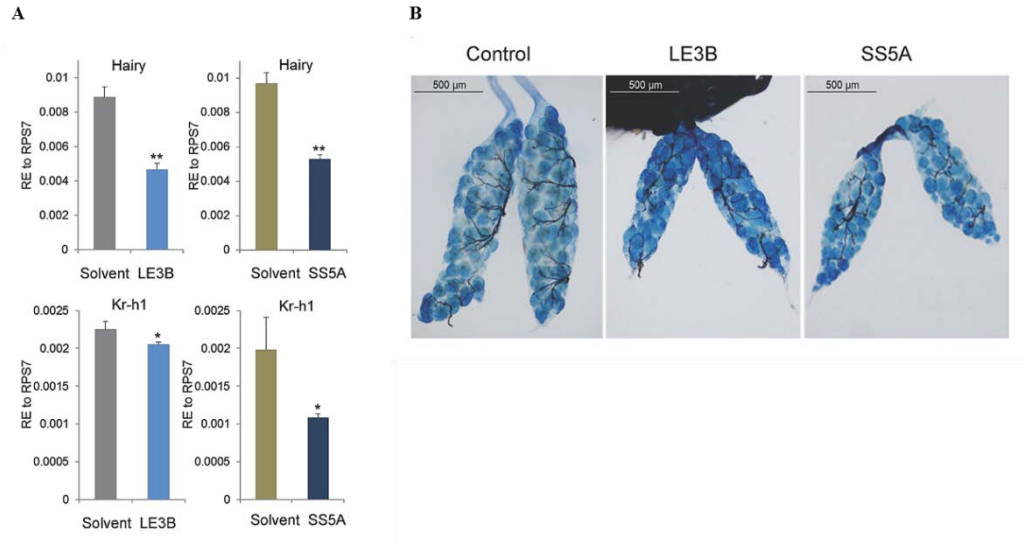

Figure 11 JHAN activities of LE3B and SS5A in adult female mosquitoes. **A**. The impaired expression of JH-activated genes, Hairy and Kr-h1, 48 h after treatment of female mosquitoes with 0.5 µg of these compounds. **B**. LE3B and SS5A impaired ovarian development in treated female mosquitoes. (Left) Normal development of ovarian follicles from a female mosquito treated with solvent. (Center and Right) Ovaries from a 3-day-old female mosquito after treatment with 0.5 µg LE3B or SS5A per mosquito.

Table 3 Prolonged effect of LE3B on mosquito development.

	Overnight survival	Days to adult emergence	Peak day to adult emergence	Length of ovaries (㎛)	Ovarian follicles : Mean Diameter (㎛)	Numbers of follicles per a ovary
LE3B-1	10/20	8-14 days	10	7 individuals 968.1 (±224.9)*	89.5 (±11.4)*	60.8 (±24.2)*
LE3B-2	15/20	10-18 days	11			
LE3B-3	13/20	9-18 days	16			
Control-1	19/20	14-22 days	19	7 individuals 1529.3 (±215.2)	115.1 (±16.1)	102.8 (±17.4)
Control-2	18/20	14-23 days	19			
Control-2	19/20	13-22 days	18			

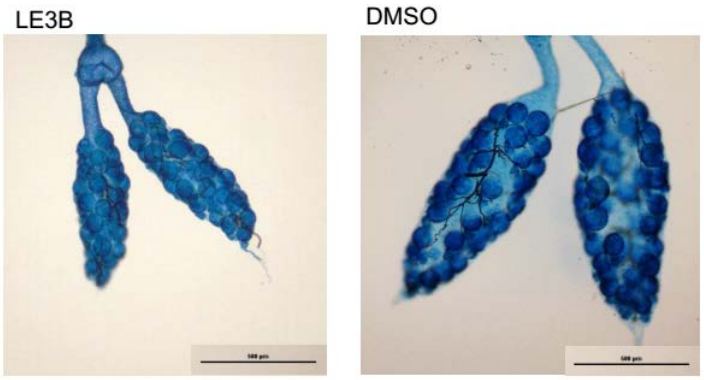

Figure 12 The exposure of 2nd instar mosquito larvae to LE3B impaired the ovary development in adult female mosquitoes emerging from the diterpene-treated larvae.

5. Comparison of antagonist activities of plant diterpenes against JH III and pyriproxyfen.

Only LE3B and SS5A among five diterpenes exhibited both *in vivo* antagonistic activity against JH/JHA on Met target gene expression and ovary development. Though the other three diterpenes and methoprene acid harbored *in vitro* antagonistic activity against JH/JHA, they did not demonstrate *in vivo* antagonistic activity. When we tested whether the plant diterpenes antagonize JH III-mediated binding between Met and CYC, we found that LE3B and SS5A interfered more specifically with natural JH rather than the other three diterpenes (Fig. 13). We also observed a similar result when we tested interference by the plant compounds in JH III mediated Met-FISC binding (Fig. 13). This may contribute to the observed *in vivo* activity of these two diterpenes.

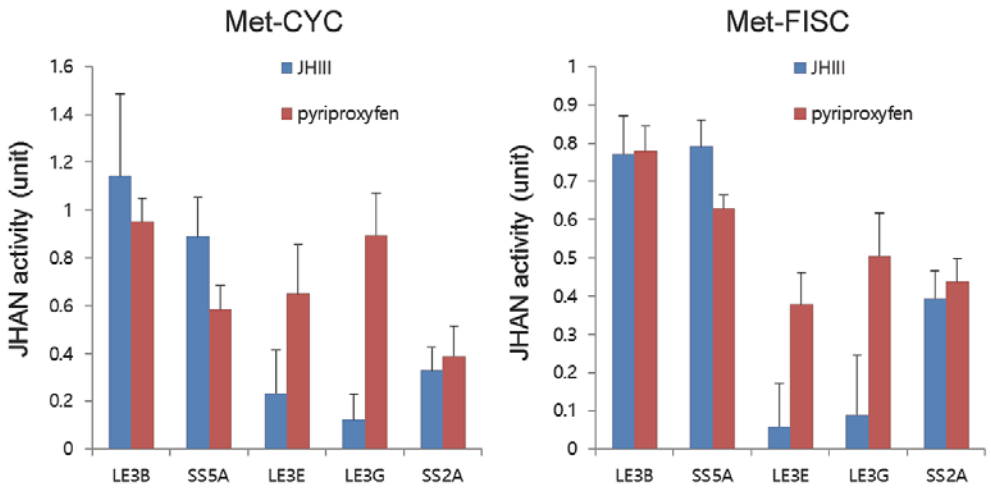

Figure 13 Comparison of antagonist activities of plant diterpenes against pyriproxyfen and JH III in hormone-mediated binding of the JH receptor and its partners. The binding initiated by 1 ppm of JH III or 33ppb of pyriproxyfen was applied with 10 ppm of each plant diterpene.

DISCUSSION

We identified plant compounds that serve as juvenile hormone antagonists (PJHANs). Using the yeast two hybrid system transformed with the mosquito JH receptor as a reporter system, we demonstrate that PJHANs affect the JH receptor, methoprene-tolerant (Met), by disrupting its complex with CYCLE or FISC, formation of which is required for mediating JH action.

In yeast two-hybrid binding tests using growth complementation and β-galactosidase assays, only the binding between *A. aegypti* Met-CYC was successfully simulated by Y187 yeast cells. The weak β-galactosidase activity observed in the binding of *C. pipiens* Met-CYC and the beetle Met-SRC does not necessarily indicate that the affinity of the binding itself is weak, because the affinity could be affected by many additional arbitrary factors during the simulation, such as folding of recombinant proteins.

MA did disrupt both JH III and pyriproxyfen-mediated binding between Met and its partners, but we could not observe any *in vivo* antagonistic activity. The molecular details regarding why only LE3B and SS5A had *in vivo* antagonistic activities against natural JH and the association of JHAN activity of plant extracts and diterpenes with larvicidal toxicity, need to be addressed in future studies.

Overall, we found that 53 plant species from 28 families exhibited JHAN activity and larvicidal toxicity against 3rd instar mosquito larvae (Table 1). Of these samples, four plant families were significantly over-represented (hypergeometrical distribution,

$p < 0.01$): 1) *Araliaceae* and 2) *Lauraceae* from *Archichlamydeae*; and 3) *Cupressaceae* and 4) *Pinaceae* from *Coniferopsida* (Table 4). This suggests that the presence of PJHAN compounds in plants is closely associated with their evolution. Plants deploy secondary metabolites as major constituents of defense against herbivores. It has been suggested that secondary metabolites may improve the producer's survival or fitness by acting on specific receptors in competing organisms (Williams, Stone et al. 1989). All five purified diterpenes belong to secondary metabolites that have no known roles in plant functions. Thus, PJHAN diterpenes likely play a role in plant defense against insect herbivores.

Through using *in vitro* yeast two-hybrid screening assays and following *in vivo* results of PJHANs on marker gene expression and ovary development in the mosquito, we demonstrated that they disrupt JH regulation by interfering with the interaction between Met and CYC or FISC. The JH receptors belong to a family of bHLH-PAS transcription factors that is composed of nine phylogenetic clusters (Bowers 2012). Met factors from insects form a unique phylogenetic cluster within the bHLH-PAS protein family. Thus, PJHANs block the endocrine regulation of insect specific receptors, indicating that they are likely to be environmentally safe. Our results indicate that newly discovered PJHAN compounds could be used as the starting material for development of novel insecticides.

Table 4 Phylogenetic distribution of plants with the JHAN activity. Plant extracts which had either JHAN activity or both JHAN activity and larvicidal toxicity were grouped into the corresponding plant families. Significantly over-represented families (hypergeometric distribution, $P < 0.01$) are highlighted in yellow.

phylum	class	subclass	family	72 species with anti-juvenoid activity	Hyper-geometrical distibution	53 species with anti-juvenoid and larvicidal Toxicity	Hyper-Geometrical distibution	1,651 total plant species
Angiospermae	Dicotyledoneae	Archichlamydeae	Aceraceae	2		1		21
Angiospermae	Dicotyledoneae	Archichlamydeae	Amaranthaceae	2		0		9
Angiospermae	Dicotyledoneae	Archichlamydeae	Araliaceae	5	0.0001	5	2.90E-05	13
Angiospermae	Dicotyledoneae	Archichlamydeae	Aristolochiaceae	1		1		4
Angiospermae	Dicotyledoneae	Archichlamydeae	Berberidaceae	1		0		7
Angiospermae	Dicotyledoneae	Archichlamydeae	Betulaceae	3		3		19
Angiospermae	Dicotyledoneae	Archichlamydeae	Celastraceae	2		1		14
Angiospermae	Dicotyledoneae	Archichlamydeae	Euphorbiaceae	2		2		22
Angiospermae	Dicotyledoneae	Archichlamydeae	Lauraceae	4	0.0016	4	0.0005	13
Angiospermae	Dicotyledoneae	Archichlamydeae	Leguminosae	4		3		71
Angiospermae	Dicotyledoneae	Archichlamydeae	Magnoliaceae	2		1		9
Angiospermae	Dicotyledoneae	Archichlamydeae	Moraceae	1		1		18
Angiospermae	Dicotyledoneae	Archichlamydeae	Rosaceae	1		1		107
Angiospermae	Dicotyledoneae	Archichlamydeae	Rutaceae	1		1		15
Angiospermae	Dicotyledoneae	Archichlamydeae	Salicaceae	1		1		18
Angiospermae	Dicotyledoneae	Archichlamydeae	Saxifragaceae	2		1		32
Angiospermae	Dicotyledoneae	Archichlamydeae	Simaroubaceae	1		0		2
Angiospermae	Dicotyledoneae	Archichlamydeae	Sterculiaceae	1		1		3
Angiospermae	Dicotyledoneae	Archichlamydeae	Theaceae	2		0		9
Angiospermae	Dicotyledoneae	Archichlamydeae	Umbelliferae	3		2		38
Angiospermae	Dicotyledoneae	Archichlamydeae	Urticaceae	1		1		15
Angiospermae	Dicotyledoneae	Sympetalae	Caprifoliaceae	1		0		30
Angiospermae	Dicotyledoneae	Sympetalae	Compositae	7		6		158
Angiospermae	Dicotyledoneae	Sympetalae	Ericaceae	1		1		6
Angiospermae	Dicotyledoneae	Sympetalae	Lentibulariaceae	1		0		1
Angiospermae	Dicotyledoneae	Sympetalae	Primulaceae	1		1		9
Angiospermae	Dicotyledoneae	Sympetalae	Valerianaceae	1		1		7
Angiospermae	Dicotyledoneae	Sympetalae	Verbenaceae	1		1		10
Angiospermae	Monocotyledoneae		Cyperaceae	1		0		32
Angiospermae	Monocotyledoneae		Gramineae	1		0		60
Angiospermae	Monocotyledoneae		Liliaceae	3		1		63
Angiospermae	Monocotyledoneae		Zingiberaceae	1		1		2
Gymnospermae	Coniferopsida		Cupressaceae	4	0.0008	3	0.004	11
Gymnospermae	Coniferopsida		Pinaceae	8	1.51E-07	6	7.40E-06	17
Gymnospermae	Coniferopsida		Taxodiaceae	1		1		4
Pteridophyta			Pteridaceae	2		1		32

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ABSTRACT IN KOREAN

곤충 유약호르몬 수용복합체의 기능을 방해하는 식물 유래 화합물의 특성 연구

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초 록

곤충은 질병을 매개하고 작물과 저장곡물에 가해를 함으로써 인간에게 피해를 준다. 이러한 피해를 효과적으로 관리 할 수 있는 방법 중 하나인 곤충 특이적 성장 조절물질 (Insect-specific growth regulators, IGRs)은 사람과 환경에 비교적 안전하여 매력적인 곤충 방제 물질로 알려져 있다. 우리는 식물 유래의 곤충유약호르몬 수용복합체 (juvenile hormone receptor complex)에 대한 antagonist (Plant Juvenile Hormone Antagonist, PJHAN)를 발견했다.

Yeast two hybrid 시스템을 이용하여 곤충유약호르몬 수용체로 알려진 methoprene-tolerant (Met)과, Met과 복합체를 이루는 것으로 알려진 CYCLE 이나 FISC를 각각 재조합 했고, PJHANs를 처리하여 곤충유약호르몬이 매개하는 곤충유약호르몬 수용복합체 형성을 방해하는 것을 확인했다. 또한 우리는 비목나무와 미국미역취로부터 PJHAN 활성을 나타내는 다섯 개의 diterpene 이차대사산물을 분리 정제했다. 이 물질들은 상대적으로 낮은 LD_{50} 수준의 모기 유충에 대한 살충성을 보였다. 그 중 2개의 물질은 Met이 곤충유약호르몬의 존재 시 유도하는 유전자의 발현을 감소시켰고, 암컷 모기 성충의 난소 난포(ovarian follicle)의 발달을 저해했다. 따라서 PJHAN의 발견은 새로운 유형의 효과적이고 안전한 살충제의 발전을 이끌 수 있을 것이다.

Key words: 곤충유약호르몬, 곤충유약호르몬 수용복합체, 내분비조절 교란

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