A THESIS

FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

Identification and Characterization of Novel Juvenile Hormone-related Insect Growth Regulators

곤충 유약호르몬의 기능을 교란하는

새로운 곤충 생장 조절 물질의 선별 및 특성 구명

By

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Major in Entomology

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UNDER THE DIRECTION OF ADVISER YEON HO JE
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ABSTRACT

Mosquitoes are medically important insect pests that transmit various diseases when they feed on humans. The Asian tiger mosquito, *Aedes albopictus* Skuse (Diptera: Culicidae), is one of the most invasive vectors of various diseases including dengue fever, chikungunya, and zika virus. Chemical insecticides have been commonly used to control mosquitoes. However, due to their toxicity to environments and development of insect resistance, sustained use of chemical insecticides are limited. Insect growth regulators (IGRs) could become an effective alternative to control mosquitoes and other vector transmitting diseases because they are specific to target insects and relatively low toxic to environment. However, recent studies, mosquitoes and other pests also have developed

resistance to certain IGR insecticides. Hence, there is an urgent need to develop novel IGR insecticides.

In this study, the yeast two-hybrid β-galactosidase assays using the yeast cells transformed with the genes of JH receptor and its partner, Met-FISC of *A. aegypti* were performed to identify novel juvenile hormone (JH)-related IGR compounds. Among the 2,349 chemical compounds, one JH agonist (JHA) candidate and 17 JH antagonist (JHAN) candidates were screened out and loreclezole hydrochloride and penfluridol were selected based on their high levels of JHA and JHAN activity and insecticidal activity against 3rd instar of *A. albopictus* larvae, respectively. Loreclezole hydrochloride and penfluridol are well known as anticonvulsant and neuroleptic dug, respectively. However, these compounds showed not only high levels of JH-related IGR activity, but also high levels of insecticidal activity against 3rd instar of *A. albopictus* larvae compared to those of pyriproxyfen.

To find more effective compounds with JH-related IGRs activity for control of mosquitoes, loreclezole hydrochloride and its derivatives were synthesized. Among these derivatives, K21877 demonstrated high level of JHAN activity. Although both loreclezole hydrochloride and K21877 have similar structures, one compound simulated the binding of *A. aegypti* Met-FISC while the other interfered with the pyriproxyfen-mediated binding of *A. aegypti* Met-FISC. Both of the JHA and JHAN showed much higher larvicidal activities than that of currently used JHA insecticide, pyriproxyfen. There also showed high embryonic lethality and toxicity against *A. albopictus* adults, which were due to the modulation of JH-regulated physiological functions such as expression of JH-responsive genes and follicle development.

To investigate the transcriptional responses of the *A. albopictus* treated with loreclezole hydrochloride and K21877, comprehensive transcriptome sequencing was performed and analyzed. These results suggested that JH-interaction of Met could regulate the expression of genes that are related to metabolic process, nucleotide binding process, and translation pathway and loreclezole hydrochloride and K21877 could modulate the JH-regulated gene expression.

Key words: Insect growth regulators, Juvenile hormone, Juvenile hormone receptor complex, Juvenile hormone agonist, Juvenile hormone antagonist

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INTRODUCTION

The Asian tiger mosquito, *Aedes albopictus*, is medically importance insect pest that transmit various disease including dengue fever (Sang et al., 2015), chikungunya (Reiter et al., 2006), and zika virus (Grard et al., 2014). The global health problem is getting serious because they have spread to at least 28 other countries from Asia (Benedict et al., 2007). Due to its rapid expansion and vectorial capacity, effective control of *A. albopictus* is required for public health (Bonizzoni et al., 2013). Chemical insecticides such as DEET (*N,N*-diethyl-*m*-toluamide), pyrethroids, and temephos have been commonly used to control of mosquitoes (George et al., 2015). However, because of their toxicity to environment and non-targets and development of resistance, there is an urgent need to develop novel effective insecticides (Vontas et al., 2012).

Insect growth regulators (IGRs) could become an effective alternative to control mosquitoes and other vectors. Important advantages of IGRs are relatively low toxic to environment and selectivity (Pener and Dhadialla, 2012). IGRs are chemicals (synthetic or natural) that interfere with insect specific, often arthropod specific, physiological, biochemical and/or molecular processes that are involved in the normal growth and development and reproduction in insects. IGRs are classified into three groups by the mode of action; juvenile hormone agonists (JHAs), ecdysone agonists (EAs), chitin synthesis inhibitors (CSIs). Synthetic pyrethroids are reported high toxic to aquatic organisms, especially fishes, while there is a growing interest in the use of IGRs such as methoprene, pyriproxyfen, novaluron, and diflubenzuron for mosquito control (Chavasse

et al., 1997; Raghavendra et al., 2011). Especially methoprene and pyriproxyfen belonged JHAs that are one of three classes of IGR insecticides are effective larvicides and inhibitor of adult emergence against mosquitoes (Ansari et al., 2005). However, according to recently studies, mosquitoes and other pests also have developed resistance to methoprene and pyriproxyfen (Dennehy et al., 2010; Paul et al., 2005; Silva and Mendes, 2007). Hence, there is an urgent need to develop novel IGR insecticides.

Juvenile hormone (JH) is one of the most important insect hormone regulates development, molting, metamorphosis, reproduction, polyphenism, caste differentiation, and various physiological functions (Hartfelder and Emlen, 2012; Nijhout, 1998; Raikhel et al., 2005; Riddiford, 1994). Because of the key role of JH, JH-related IGR insecticides fatally affect the physiological regulations in insects. Although JHs are very important for insect physiology, their regulatory mechanisms have remained elusive (Riddiford, 2008).

Recent studies identified *methoprene-tolerant* (Met) as the JH receptor (Charles et al., 2011; Jindra et al., 2013). Met is a member of a protein family known as basic-helix-loop-helix (bHLH)-Per-Arnt-Sim (PAS) transcription factors, which have significant roles in JH activity and downstream transcriptional activation. This family of transcription factors must dimerize to regulate transcription (Kewley et al., 2004). Met of *Aedes aegypti* dimerizes with other bHLH-PAS transcription factors, such as *Ftz*-F1-interacting steroid receptor coactivator (FISC) or Cycle (CYC), in a JH-dependent manner (Li et al., 2011; Shin et al., 2012).

Previously, this JH-mediated interaction of Met and its binding partners have been replicated *in vitro* using yeast cells transformed with the *Met* and *FISC/CYC* genes of *A. aegypti* (Lee et al., 2015). Through this *in vitro* yeast two-hybrid *β*-galactosidase assay,

high-throughput screening of JHAs and JHANs could be performed. In this study, novel JHA and JHAN compounds were identified from chemical library through the *in vitro* yeast two-hybrid *β*-galactosidase assay and their larvicidal activities against *A. albopictus* and other agricultural pest were investigated. In addition, structural derivatives of the novel JHA were synthesized to identify more effective JH-related IGR compounds and their biological characteristics, such as larvicidal activities, embryonic lethalities and the effects on ovarian follicle development and expression of JH-response gene, were investigated against *A. albopictus*. Furthermore, the transcriptome sequencing of *A. albopictus* upon IGR treatment was performed to investigate the transcriptional responses to JHA and JHANs.

LITERATURE REVIEW

1. Juvenile hormone

Historical review

It has been almost two centuries since studies relating juvenile hormone (JH) were started. Müller (1,828) described specific organs in the cockroach which were renamed as the corpora allata (CA) in 1899. However, until then, the CA were described as sympathetic ganglia concerned with the innervation of the digestive system. Although Police suggested that the CA were endocrine organs concerned with nervous function in 1910, it had remained to be proved that. In 1934, V. B. Wigglesworth began historical studies on insect JH, making efficient use of surgical techniques (Wigglesworth, 1934). He assumed at first that CA was the source of the molting hormone, an "inhibitory factor" which prevented the first four larval stages from molting directly into adults in *Rhodnius*. In 1936, he showed that the CA was the source of the inhibitory hormone that prevents metamorphosis in young larvae and that CA from young larvae when implanted into fifth instars caused them to undergo a supernumerary molt (Wigglesworth, 1936). Wigglesworth concluded that the concentration of the inhibitory hormone from CA determines the extent of metamorphosis at the next molt. Then, there have been many studies on JH function. The modern era of JH research began with the critical finding by Carroll Williams that he discovered a natural repository for juvenile hormone, "golden oil", and JH was extracted and diluted in peanut oil or mineral oil to conduct hundreds or

even thousands of experiments from male *Hyalophora cecropia* (Williams, 1956). About 10 years after, the structure of JH was identified using gas chromatographic analysis. Röller and colleagues (Röller et al., 1967) identified the first juvenile hormone from lipid extracts of the wild silk moth, *H. 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 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 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 et al., 1984). The trihomosesquiterpenoids JH 0 and its isomer 4–methyl JH I (iso–JH 0) were identified in *M. sexta* eggs (Bergot 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 et al., 1989). These historical studies formed the basis of many JH-related studies today.

Function

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 CA. JHs play key roles in various physiological functions including molting, metamorphosis, reproduction, polyphenism, caste

differentiation, and various physiological functions in insects (Hartfelder and Emlen, 2012; Nijhout, 1998; Raikhel et al., 2005; Riddiford, 1994). Although JHs are very important for insect physiology, their regulatory mechanisms have remained elusive (Riddiford, 2008).

2. Insect growth regulators

Carol Williams referred to the insecticides that mimic the action of insect hormones as "Third-generation pesticides" because the most important advantage of the insecticides is their relatively low environmental toxicity profile (Williams, 1967). The term insect growth regulators (IGRs) was introduced into the literature in 1972 (Schneiderman, 1972). In the early times, the term means substances which are agonist or antagonist of hormones that regulated insect growth and development such as JH and ecdysones and interfere with normal development of insect. Since then, substances which inhibit chitin synthesis in the epidermis (in the narrow sense) were included in IGRs as chitin synthesis inhibitors (CSI). Therefore, the present term IGRs include JH agonist (JHA) and antagonist (JHAN), ecdysones agonist (EA), and CSI. IGRs have not only relatively low environmental toxicity, but high target specificity (Pener and Dhadialla, 2012). In this respect, IGRs clearly differ from conventional insecticides. As previously mentioned, IGRs are classified into three groups, including JHA, EA and CSI, by the mode of action.

Juvenile hormone agonists

In 1956 when Williams reported the first JH-active lipid extract from cecropia moths, the history of JH-related IGRs had begun (Williams, 1956). Further investigation revealed that these JH-active lipid extract were also present in microorganisms, plants, and various vertebrate organs and the compound with JH activity in lipid extract might be used as the "third-generation pesticides"(Schneiderman and Gilbert, 1959; Schneiderman et al., 1960; Williams, 1967; Williams et al., 1959).

A sesquiterpenoid alcohol, farnesol was isolated from the excrements of yellow mealworm which the first JH-active compounds with the defined chemical structure (Schmialek, 1961). After the discovery of JH-active compounds, identification of novel compounds with JH activity was stimulated. Around the same time, the juvabione, called "paper factor", were identified from the wooden pulp of the Canadian balsam fir (Bowers et al., 1966; Slama and Williams, 1966). The investigation of juvabione led to the hypothesis that certain plants might develop resistance against insect herbivores by evolutionary adaptations leading to synthesis of JHAs (Slama and Williams, 1965, 1966). The JH-based hormonal insect-plant relationships have been investigated experimentally, however, the screening of compounds with JH activity from plants revealed only few compounds, because the plants commonly contain slightly polar sesquiterpenoid compounds (alcohols, acids) with very low JH activities (Bowers, 2012; Sláma, 1969, 1999).

After then, various types of isoprenoid JHAs were synthesized from 1965 to 1975. Especially, In 1972, Zoecon Corporation patented a highly effective JHA, isopropyl 11-methoxy-3,7,11-trimethyldodeca-2,4-dienoate, researched by Henrick (Henrick 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 and is still favor as the least toxic, biodegradable, environmentally safe JHA until this time.

In 1981, Hoffmann-LaRoche laboratories reported the discovery of nonisoprenoid type of new JHA (Masner et al., 1981). The important structural difference was incorporation of the bicyclic, 4-phenoxyphenyl group into the JHA molecule. The first JHA of 4-phenoxyphenyl group registered for practical use was fenoxycarb (Masner et al., 1981). The biological activities, JH-related ovicidal and insecticidal activity, of fenoxycarb were higher than that of isoprenoid JHA, methoprene (Masner et al., 1987). The famous JHA of 4-phenoxyphenyl group is pyriproxyfen. Pyriproxyfen is a 4-phenoxphenoxy type compound with a pyridyl structure in the side chain. The effects of pyriproxyfen have been assayed on a wide range of insect species, including mosquitoes, ants and cockroaches (Banks and Lofgren, 1991; Koehler and Patterson, 1991; Okazawa, 1991). Similar to fenoxycarb, pyriproxyfen also showed a high level of JH-related biological activity.

In 1985, the total amount of JH active compounds was estimated to more than 4000 compounds, which were classified into 9 structural categories: 1. Natural juvenoids from animals and plants; 2. Farnesoates and 2,4-dodecadienoates; 3. Oxa-, thia- and aza-farnesoates; 4. Alicyclic (terpenoid) juvenoids; 5. Heterocyclic juvenoids; 6. Arylterpenoid juvenoids; 7. Simple nonisoprenoid juvenoids; 8. Polycyclic non-isoprenoid juvenoids, and a new category; 9. Juvenogens (Slama, 1985).

The current status of the use of JHA insecticides is focused on the control of urban pests and stored product pests. Especially, the noxious insects of urban communities were favorite targets for JHA insecticides, because the high biological activity and relatively low acute toxicity. The various JHA compounds were found to be effective against mosquitoes, cockroaches, and stored product pests (Bennett and Reid, 1995; Emmerich and Barth, 1968; Letellier et al., 1995; Spielman and Williams, 1966).

Juvenile hormone agonists as insect control agents

There are a number of JHAs, such as methoprene, pyriproxyfen, fenoxycarb, and hydroprene, in common use as insect control agents. They have been widely used for control and eradication of numerous pests in urban environment.

Especially, methoprene has been shown to be highly effective against mosquito (*Aedes* spp., *Culex* spp., and *Mansonia* spp.), storage insect pests (*Rhyzopertha dominica* and *Plutella interpunctella*), dipteran pests of livestock, ants, and flea on domestic pets (Beehler and Mulla, 1993; Beugnet et al., 2011; Butler et al., 2006; Das et al., 1981; Axtell et al., 1979; Gusmao et al., 2011; Krishnamoorthy et al., 1993; Majori et al., 1977; Mian and Mulla, 1982; Wright and Jones, 1976). Pyriproxyfen was also found to be effective against several pests such as mosquito, storage pests, and whitefly and fenoxycarb and hydroprene were used for controlling cockroach and *Tribollium* spp., respectively (Bell and Edwards, 1999; Evans et al., 1995; Ramaseshadri et al., 2012).

3. Methoprene-tolerant as a JH receptor

Methoprene-tolerant (Met) was identified as a potential JH receptor from an ethyl methanesulfonate mutagenesis screen performed by Wilson and Fabian (Wilson and Fabian, 1986). The screening yielded a semidominant *Met* mutation *Drosophila* (Met^{1}) that result from exogenous application of the JHA methoprene. Although "status quo" activity of JH in many insect was well-documented, Met null mutants survived to adulthood and exhibited only minor defects such as reproduction in *Drosophila*.

After then, Met homologs, as a potential JH receptor, have been identified in various insects (Li et al., 2010). One possible explanation for this paradox is that Met was partially backed up by a paralog, *germ-cell expressed* (*gce*), which arose via gene duplication in an ancestor of the drosophilid lineage (Abdou et al., 2011; Baumann et al., 2010a; Baumann et al., 2010b). Met as a JH receptor was confirmed in the red flour beetle *Tribolium castaneum*, where removal of Met expression using RNA interference produced a premature and lethal initiation of pupation because *Tribolium* has only a single homolog in contrast to *Drosophila* (Konopova and Jindra, 2007).

Met is a basic-helix-loop-helix Per-Ahr/Arnt-Sim (bHLH-PAS) protein, including a bHLH and two PAS (A and B) domains (Ashok et al., 1998). Consistent with the role of Met as a JH receptor, it commonly belongs to transcription factor. To be active as a transcription factor, the bHLH-PAS protein dimerizes with other bHLH-PAS, partner protein. Several bHLH-PAS proteins such as Gce, Taiman (Tai), Ftz-F1-interacting steroid receptor coactivator (FISC), CYCLE (CYC), and steroid receptor coactivator

(SRC), are known as JH receptor partner (Godlewski et al., 2006; Li et al., 2011; Shin et al., 2012; Zhang et al., 2011).

In 3D structure of Met, PAS motifs (PAS-A and -B) compose the PAS folds (PAS fold 1 and 2) (Bernardo and Dubrovsky, 2012a; Hefti et al., 2004). Each PAS fold consists of a five-stranded antiparallel β-sheet flanked on one side by three or four α-helices. PAS fold 1 is composed of PAS A motif and three subsequent sequence blocks with intervening loops between PAS A motif and block I, and between block II and III. PAS fold 2 is composed of PAS B motif and PAS-associated C-terminus (PAC) region, which contains a three β-sheet and one α-helix. Among these folds, PAS fold 2 consists of a hydrophobic ligand-binding pocket. When several critical hydrophobic residues in PAS fold 2 were mutated, *Tribolium* Met abolished or reduced JH binding (Charles et al., 2011). Also, use of the Met point mutants incapable of binding JH revealed JH, bound to the PAS fold 2, was necessary for Met to leave the homodimer and to binding partner protein (Charles et al., 2011). These results suggest that Met uses PAS fold 2 as a ligand binding domain of JH and JH, bound to the PAS fold 2, stimulates a conformational change of the Met (Bernardo and Dubrovsky, 2012b).

In general, the mechanism of JH-dependent transcription is poorly understood. However, in *Aedes* mosquitoes, the study on two bHLH-PAS protein, Met and FISC mediated JH-induced gene expression was reported that several JH-responsive elements (JHRE), such as *early trypsin* (*ET*) and *Kr-h1*, have been identified in JH-regulated insect genes. Several JHRE contains an asymmetric E-box-like motif characteristic for the bHLH-PAS transcription factor (Li et al., 2011). These results suggest one of the possible mechanisms of JH-dependent transcription in which JH simulates Met to interact with its

bHLH-PAS partner, and then this heterodimer binds to an E-box-like in JH target genes (Bernardo and Dubrovsky, 2012b). This hypothesis is based on the function of bHLH-PAS proteins as DNA-binding transcriptional activators. The other possibility is that Met serves a function as transcriptional coactivators, not direct DNA-binding. In 2012, it was reported that Met interacted with βFtz-F1, a nuclear receptor and functions as a competence factor, in the presence of JH in *Drosophila* (Bernardo and Dubrovsky, 2012a). This interaction is due to the LXXLL motif (Nuclear receptor box, NR box), located in C-terminal of the PAS-B domain. In addition, the interactions of Met with βFtz-F1 are enhanced by JH and deletion in the PAS-B domain disrupts the interaction. These results suggest that Met utilizes the LXXLL motif to participate in JH signaling pathways through nuclear receptor interaction when Met is activated by JH.

These models of mechanism of JH-dependent transcription are based on the ability of translocation to and from the nucleus. For function as JH-dependent transcription factor directly or indirectly, the ability to translocate to and from the nucleus of Met is important. Met contains import and export motifs following the bHLH domain (nuclear localization signal (NLS)-1 and nuclear export signal (NES-1)), within the PAS-B domain (NLS-2 and NES-2), and an unidentified NES in the C-terminus. Among these domains, NES-2 in the PAS-B domain was confirmed that it caused Met to accumulate in the cytoplasm and to enter the nucleus in the presence of JH *in Drosophila* (Greb-Markiewicz et al., 2011). The NLS-related mechanism of JH-dependent nuclear entry is nuclear, however, heat shock protein 83 (HSP83) facilitates nuclear import of Met and expression of JH-induced genes (He et al., 2014). These suggest that Met could import/export to and from the nucleus through the JH-dependent conformational change and interaction with HSP.

4. Screening of compounds with JHA and JHAN activity

It has been reported that the JH-mediated interaction of Met and its binding partners have been replicated *in vitro* using yeast cells transformed with the genes code for Met and FISC/CYC of *A. aegypti* (Lee et al., 2015; Shin et al., 2012). Through this *in vitro* yeast two-hybrid β-galactosidase ligand binding assay, high-throughput screening of JHAs could be performed. Besides, before this study, anti-JH agents were commonly known to inhibit the biosynthesis of JH, such as precocenes (Stall, 1986). Through this study, JH antagonists (JHANs) that interfered with the JH-mediated interaction of Met and its binding partners were identified. As a result, it became possible to screening JHAs and JHANs via *in vitro* screening using yeast cells transformed with the JH receptor and its partner genes.

CHAPTER I. Identification of novel juvenile hormone-related insect growth regulators from chemical library

ABSTRACT

Chemical insecticides have been currently used for control of mosquito are limited in their use because of high toxicity to environment and non-target insects and development of resistance. Insect growth regulators (IGRs) are an effective alternative to control mosquitoes and other pest because they are relatively low toxic to environment and very specific to target insects.

To identify novel juvenile hormone (JH)-related IGRs for control of mosquito, 2,349 chemical compounds were surveyed on their JH agonist (JHA) and antagonist (JHAN) activity using *in vitro* yeast two-hybrid *β*-galactosidase ligand binding assay. Among them, loreclezole hydrochloride was meditated the formation of JH receptor complex and 53 chemical compounds were interfered with the formation of pyriproxyfen-mediated JH receptor complex. Also, loreclezole hydrochloride with JHA activity and penfluridol with JHAN activity showed high level of insecticidal activity against larvae of *Aedes albopictus*. These insecticidal activities of loreclezole hydrochloride and penfluridol were Diptera-specific activity because other pests including Lepidoptera and Hemiptera were not affected by them. These results suggested that a novel JH-related IGR insecticides could be identified by *in vitro* yeast two-hybrid *β*-galactosidase ligand binding assay and loreclezole hydrochloride and penfluridol could be useful for the development of environmentally benign IGR insecticide to control mosquitoes.

INTRODUCTION

Insects cause tremendous economic losses by damaging crops and stored agricultural products (Bowers, 2012). Moreover, public health problems are one of the most important of the threat by vector insects (Hill et al., 2005). Among insect pests, mosquitoes caused public health problems became a biggest threat to many people. Mosquitoes can transmit infectious diseases such as malaria, chikungunya, dengue fever, West Nile fever, yellow fever, and zika between humans or from animal to humans (World Health Organization, 2014). The Asian tiger mosquito, *Aedes albopictus*, originally indigenous to South-east Asia, islands of the Western Pacific and Indian Ocean. Over the last three decades, *A. albopictus* has spread to at least 28 other countries including Africa, the mid-east, Europe, and the North and South America from their original habitats (Benedict et al., 2007; Gratz, 2004). *A. albopictus* act as vector for devastating pathogens and parasites, including malaria, yellow fever, dengue, West Nile, chikungunya, and zika virus (Benelli, 2015; Grard et al., 2014; Reiter et al., 2006; Sang et al., 2015).

To prevent transmission of *A. albopictus*-borne disease, effective and safe control is required (Bonizzoni et al., 2013). Chemical insecticides such as DEET (*N*,*N*-diethyl-*m*-toluamide), pyrethroids, and temephos have been currently used to control mosquito (George et al., 2015). However, these control agents are limited in their use because of high toxicity to environment and non-target insects and development of resistance (Vontas et al., 2012).

Insect growth regulators (IGRs) are an effective alternative to control mosquitoes and

other pest because they are relatively low toxic to environment and very specific to target insects (Pener and Dhadialla, 2012). IGRs are insecticides that disrupt the normal development of target insects based on their mode of action (Dhadialla et al., 2009). In mosquito control, synthetic pyrethroids, one of currently used insecticides, are reported high toxicity to non-target organisms including aquatic organisms, while there is a growing interest in the use of IGRs such as methoprene, pyriproxyfen, novaluron, and diflubenzuron (Chavasse et al., 1997; Raghavendra et al., 2011). However, according to recently studies, mosquitoes and other pests also have developed resistance to methoprene and pyriproxyfen (Dennehy et al., 2010; Paul et al., 2005; Silva and Mendes, 2007). Hence, there is an urgent need to develop novel IGR insecticides.

Juvenile hormone (JH) is one of the most important insect hormone regulates development, molting, metamorphosis, reproduction, polyphenism, caste differentiation, and various physiological functions (Hartfelder and Emlen, 2012; Nijhout, 1998; Raikhel et al., 2005; Riddiford, 1994). Because of the key role of JH, JH-related IGR insecticides fatally affect the physiological regulations in insects. Commercially available JH-related IGRs include methoprene and pyriproxyfen with JH agonist (JHA) activity, which causes abnormal development and larval death (Dhadialla et al., 2009). Recent study, a novel mosquitocidal compound, kanakugiol, that disrupt juvenile hormone receptor complex of *Aedes aegypti*, JH antagonist (JHAN), was identified using *in vitro* yeast two-hybrid *β*-galactosidase ligand binding assay (Lee et al., 2015). Before this study, anti-JH agents were commonly known to inhibit the biosynthesis of JH, such as precocenes (Stall, 1986). After the identification of kanakugiol, novel JHAs and JHANs could be identified through *in vitro* yeast two-hybrid *β*-galactosidase ligand binding assay.

Because chemical insecticides currently used for control of mosquito are limited in their use, the identification of novel mosquitocidal compounds is necessary. In this study, novel JHAs and JHANs were isolated from chemical library using yeast cells transformed with the JH receptor complex of *A. aegypti*, and their biological characteristics were investigated.

MATERIALS AND METHODS

1. Insects

The *A. albopictus* was provided by the Korea National Institute of Health (Cheongwon, Korea). The mosquitos were reared in breeding chambers at 28℃ and 70% relative humidity in aged tap water. The larvae were fed on a diet of TetraMin fish flakes, and the adults were reared using 10% sucrose solution. The chambers were equipped with photocycle-controllers set at 12 h dark/12 h light cycles.

2. Yeast two-hybrid β-galactosidase assays

The yeast two-hybrid binding test using quantitative β-galactosidase assay was carried out using Y-187 yeast cells transformed with JH receptor and its partner, Methoprene tolerant (Met)- Ftz-F1-interacting steroid receptor coactivator (FISC), of *A. aegypti* as previously described (Lee et al., 2015). The transformed Y187 cells were incubated at 30℃ in DDO (SD -Leu/-Trp) media until OD_{600} values reached 0.3-0.4. After harvest, the cells were suspended in the fresh media at a concentration of 2.0×10^6 cells / ml and 100 μl of the cells was distributed in 96-well plates.

For JHA activity, 100 μl of yeast cells (2.0×10^6 cells / ml) distributed in 96-well plates was treated with each 10 ppm of compound. A positive control treated with 0.033 ppm of pyriproxyfen which was identified as the JHA that mediated binding of Met-FISC of *A. aegypti* in the yeast two-hybrid and a negative control treated with solvent (dimethyl sulfoxide, DMSO) was placed in each tested plate (Lee et al., 2015).

To determine JHAN activity, 100 μl of yeast cells (2.0×10^6 cells / ml) distributed in 96-well plates was treated with 0.033 ppm of pyriproxyfen and 10 ppm of compound. A negative control treated with 0.033 ppm of pyriproxyfen and solvent was placed in each tested plate.

The cells treated chemical compounds for JHAN and JHA activity were incubated for further 3 h and subjected to the β-galactosidase assays using the Yeast β-galactosidase Assay Kit (Thermo Scientific, Rockford, IL, USA). The obtained OD_{420} values were converted to an arbitrary unit representing JHA and JHAN activity.

$$\text{JHA activity} = \frac{OD_{420} \text{ of sample}}{OD_{420} \text{ of pyriproxyfen (0.033 ppm)}}$$

$$\text{JHAN activity} = \frac{OD_{420} \text{ of pyriproxyfen (0.033 ppm)} - OD_{420} \text{ of sample}}{OD_{420} \text{ of pyriproxyfen (0.033 ppm)}}$$

3. Growth inhibition tests

The transformed Y187 yeast cells with *A. aegypti* Met-FISC were incubated at 30℃ in DDO (SD -Leu/-Trp) media until OD_{600} values reached 0.3-0.4. After harvest, the cells were suspended in the fresh media at a concentration of 2.0×10^6 cells / ml, and 200 μl of the cells was treated with each 10 ppm of compound in 96-well plates. The treated cells were incubated at 30℃ with shaking, and the OD_{600} of each sample was measured every 3 h for 1 day. The obtained OD_{600} values were converted to an arbitrary unit representing Growth activity.

$$\text{Growth activity} = \frac{OD_{600} \text{ of sample}}{OD_{600} \text{ of solvent}}$$

4. Insecticidal activity tests

Asian tiger mosquito

For screening bioassay, twenty 3rd instar larvae of *A. albopictus* in 5 ml tap water with food mixtures were treated with each 10 ppm of compound. The number of dead larvae was counted at 24 h after treatment for 3 days. To determine the median lethal dose (LC_{50}), twenty larvae of 3rd instar were treated with serial dilutions of each compound, respectively. The number of dead larvae was counted at every 24 h for 3 days. Experiments for determine the LC_{50} were performed in triplicates and the IRMA QCal program (Lozano-Fuentes, et al., 2012) was used to calculate LC_{50} via linear regression.

Diamondback moth

Twenty 3rd instar larvae of *Plutella xylostella* fed on each 200 ppm of chemical treated Chinese cabbage leaf disc (6 cm diameter). The number of dead larvae was counted at 24 h after treatment for 5 days. This experiment was performed in triplicate.

To determine antifeedant properties of compound, twenty 3rd instar larvae of *Plutella xylostella* fed on corresponding concentrations (10, 50, and 200 ppm) of the compound treated Chinese cabbage leaf disc, respectively. The leaf disc was placed on graph paper (1 mm^2) and the ingested area was calculated at 72 h after treatment.

Asian corn borer

Twenty neonates of *Ostrinia furnacalis* fed on each 10 ppm of chemical treated artificial diet. The number of dead larvae was counted at 24 h after treatment for 5 days. This experiment was performed in triplicate.

Small brown planthopper

Twenty nymphs of *Laodelphax striatellus* were treated with dipping in each 200 ppm of chemical for 30 s. The number of dead larvae was counted at 24 h after treatment for 3 days.

Indian mealmoth

Twenty 2nd larvae of *Plodia interpunctella* were treated with dipping in each 100 ppm of chemical for 30 s. The number of dead larvae was counted at 24 h after treatment for 3 days

Fruit fly

Each 2,500 ppm of chemical was topical applied to freshly laid *Drosophila melanogaster* eggs. Over 100 of fresh eggs (laid within 1 h) were treated with 25 μl of acetone containing 2,500 ppm of each chemical. The number of hatching eggs was counted after treatment for 5 days.

RESULTS

1. Screening of compounds that simulated or interfered with the formation of *A. aegypti* receptor complex in yeast

To isolate novel compounds with JHA or JHAN activity, 2,349 chemical compounds (Korea Research Institute of Chemical Technology (KRICT), Daejeon, Korea) were tested whether to simulate the binding of *A. aegypti* Met-FISC or interfere with the pyriproxyfen-mediated binding of *A. aegypti* Met-FISC using *in vitro* yeast two-hybrid *β*-galactosidase assays. For screening of chemical compounds with JHA activity, chemical compounds were applied to yeast cells transformed with *A. aegypti* Met-FISC. Among total samples, K222212, loreclezole hydrochloride (1-[(Z)-2-chloro-2-(2,4-dichlorophenyl)ethenyl]-1,2,4 triazole; hydrochloride, CAS No. 117857-45-1), compound simulated the binding of *A. aegypti* Met-FISC, demonstrating that this compound have high JHA activity (JHAN activity > 0.6) (Fig. 1 and Table 1). To screen chemical compounds with JHAN activity, yeast cells transformed with *A. aegypti* Met-FISC were treated with each compounds and 0.033 ppm of pyriproxyfen. Among them, 53 compounds were found to interfere with the pyriproxyfen-mediated binding of *A. aegypti* Met-FISC. This result demonstrated that these 53 compounds have high JHAN activity (JHAN activity > 0.6) (Fig. 2 and Table 2).

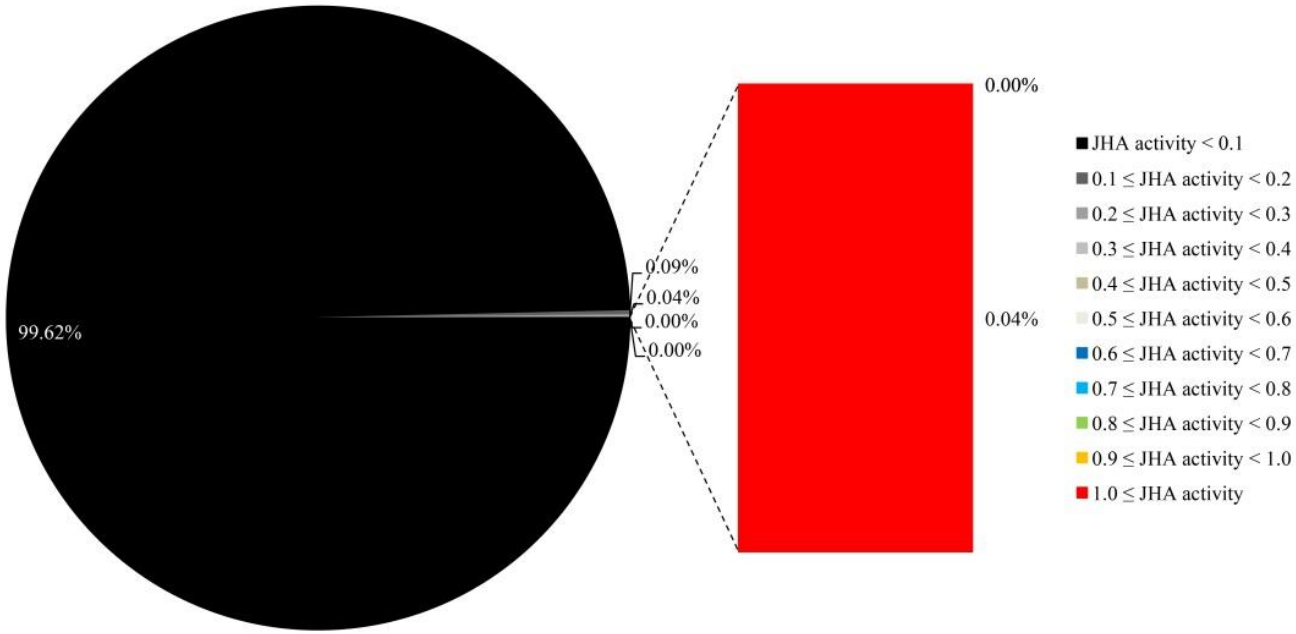


Figure 1. Screening of the chemical compounds with JHA activity. The Met-FISC binding triggered by chemical compound was simulated by β-galactosidase activity in the yeast two-hybrid system. Each chemical compounds was added to the yeast culture at a concentration of 10 ppm.

Table 1. Summary of the screening of the chemical compounds with JHA activity

JHA activity	Number of samples
JHA activity < 0.1	2340
0.1 ≤ JHA activity < 0.2	5
0.2 ≤ JHA activity < 0.3	2
0.3 ≤ JHA activity < 0.4	1
0.4 ≤ JHA activity < 0.5	0
0.5 ≤ JHA activity < 0.6	0
0.6 ≤ JHA activity < 0.7	0
0.7 ≤ JHA activity < 0.8	0
0.8 ≤ JHA activity < 0.9	0
0.9 ≤ JHA activity < 1.0	0
1.0 ≤ JHA activity	1
Number of total sample	2349

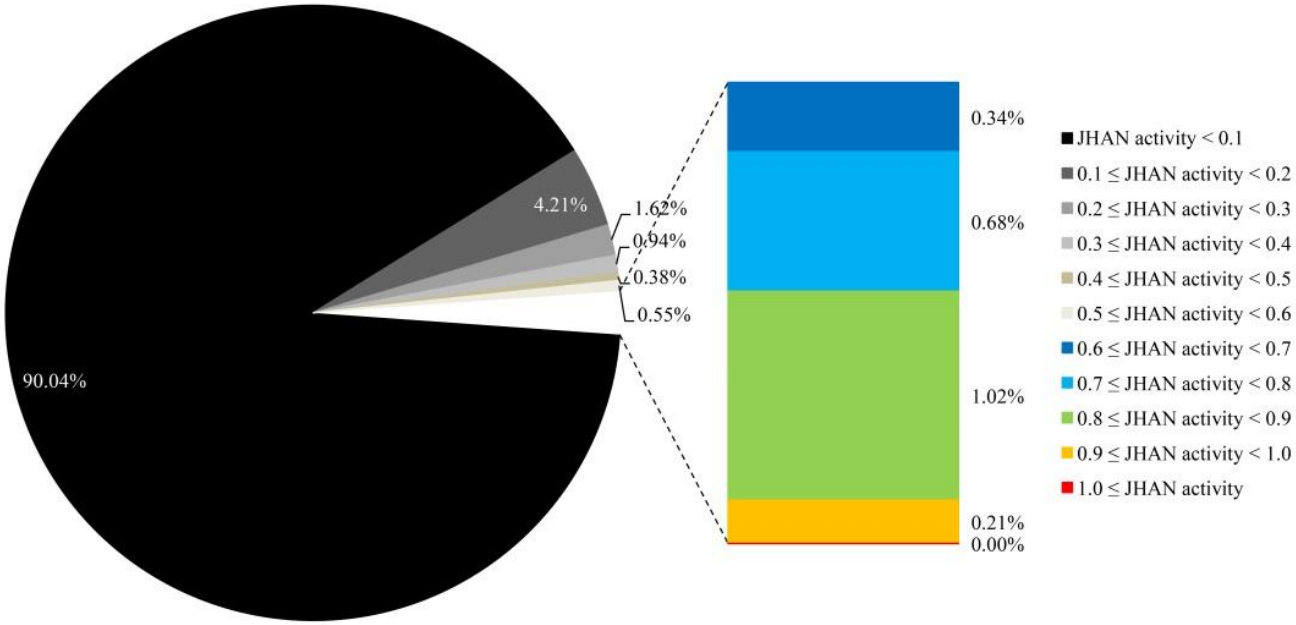


Figure 2. Screening of the chemical compounds with JHAN activity. The Met-FISC binding triggered by 0.033 ppm of pyriproxyfen was simulated by β-galactosidase activity in the yeast two-hybrid system. Each chemical compounds was added to the yeast culture at a concentration of 10 ppm.

Table 2. Summary of the screening of the chemical compounds with JHAN activity

JHAN activity	Number of samples
JHAN activity < 0.1	2115
0.1 ≤ JHAN activity < 0.2	99
0.2 ≤ JHAN activity < 0.3	38
0.3 ≤ JHAN activity < 0.4	22
0.4 ≤ JHAN activity < 0.5	9
0.5 ≤ JHAN activity < 0.6	13
0.6 ≤ JHAN activity < 0.7	8
0.7 ≤ JHAN activity < 0.8	16
0.8 ≤ JHAN activity < 0.9	24
0.9 ≤ JHAN activity < 1.0	5
1.0 ≤ JHAN activity	0
Number of total sample	2349

2. Yeast growth inhibition test

Because screening of compounds with JHAN activity have the possibility of false signals originating from anti-yeast activity of chemical compounds, yeast growth inhibition tests were conducted on 53 compounds with JHAN activity. Among 53 chemical compounds with JHAN activity, 17 chemical compounds showed the normal growth (Growth activity > 0.6) of the Y187 yeast cells transformed with *A. aegypti* Met and FISC (Fig 3 and Table 3).

3. Screening larvicidal activity against *A. albopictus*

To investigate the mosquito larvicidal activity of loreclezole hydrochloride and 17 chemical compounds with JHAN activity, 3rd instar larvae of *A. albopictus* were treated with a 10 ppm concentration of each chemical compound. Among them, loreclezole hydrochloride and K265674, penfluridol (1-[4,4-bis(4-fluorophenyl)butyl]-4-[4-chloro-3-(trifluoromethyl)phenyl] piperidin-4-ol, CAS No. 26864-56-2), with JHA and JHAN activity, respectively, showed high mosquito larvicidal activity (Fig. 4).

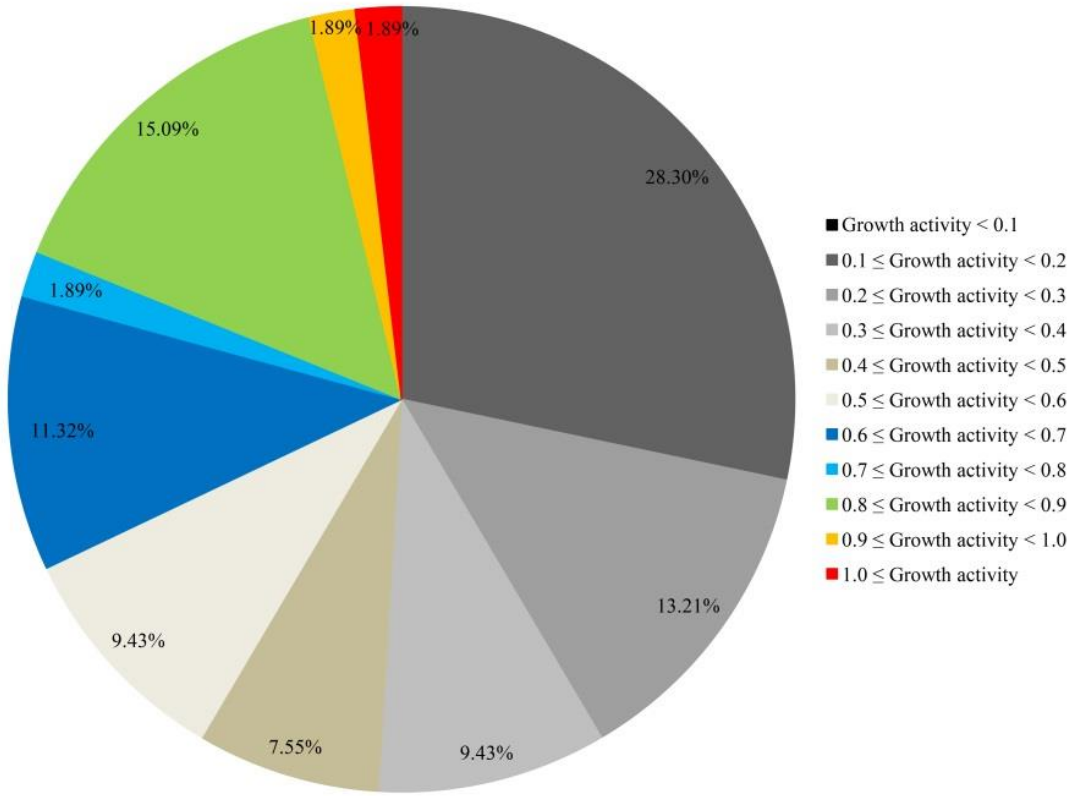


Figure 3. Yeast toxicity tests for chemical compounds with JHAN activity. To eliminate the possibility of false signals originating from anti-yeast activity of chemical compounds, each 10 ppm of compound was applied to yeast cell and were incubated at 30℃ for 24 h.

Table 3. Summary of yeast toxicity tests for chemical compounds with JHAN activity

Growth activity	Number of samples
Growth activity < 0.1	0
0.1 ≤ Growth activity < 0.2	15
0.2 ≤ Growth activity < 0.3	7
0.3 ≤ Growth activity < 0.4	5
0.4 ≤ Growth activity < 0.5	4
0.5 ≤ Growth activity < 0.6	5
0.6 ≤ Growth activity < 0.7	6
0.7 ≤ Growth activity < 0.8	1
0.8 ≤ Growth activity < 0.9	8
0.9 ≤ Growth activity < 1.0	1
1.0 ≤ Growth activity	1
Number of total sample	53

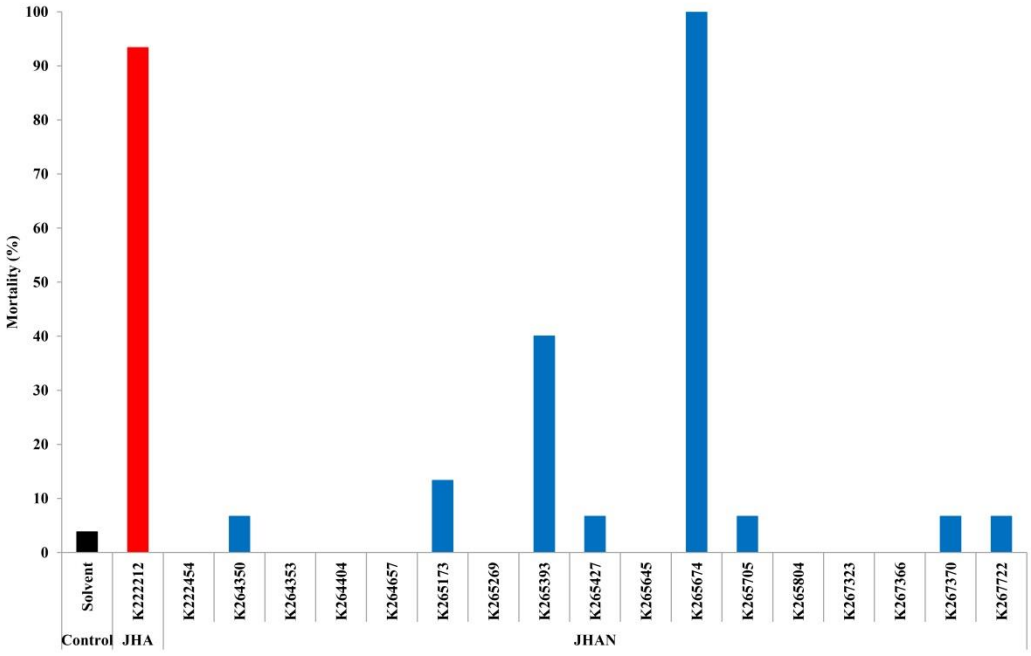


Figure 4. Mosquitocidal activities of chemical compounds with JHA or JHAN activity. Third instar larvae of *A. albopictus* were treated with 10 ppm of corresponding compounds and the mortality was calculated at 72 h after treatment. Red and blue bar represent mortalities of JHA and JHAN candidates, respectively.

4. Selection of JHA and JHAN compounds from chemical compounds

The chemical compounds, loreclezole hydrochloride and penfluridol, were selected based on their availability for the strength of JHA and JHAN activity and mortality against 3rd instar larvae of *A. albopictus*. Loreclezole hydrochloride has been known as a $GABA_A$ receptor positive allosteric modulator using a sedative and an anticonvulsant (Fig. 5A). Penfluridol has been known as a typical antipsychotic and a dopamine receptor blocking agent using typical antipsychotics (Fig. 5B).

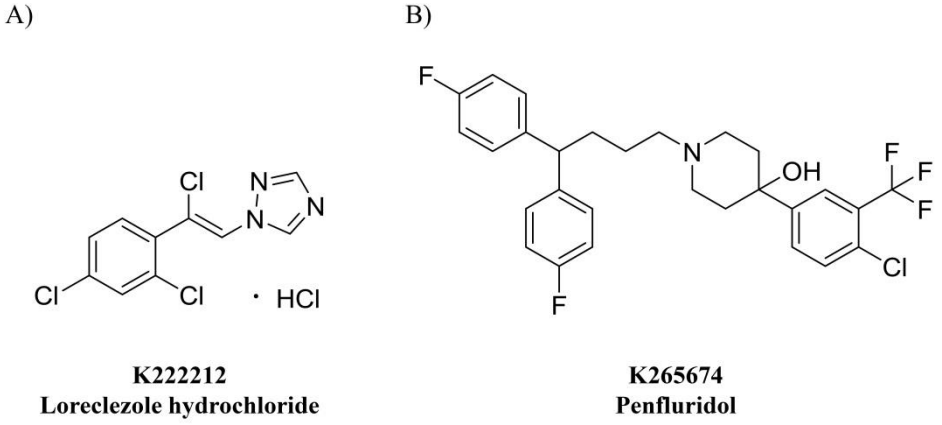


Figure 5. Structure of chemical compounds with JHA or JHAN activity. (A) Loreclezole hydrochloride with JHA activity. (B) Penfluridol with JHAN activity.

5. Dose-dependent JHA and JHAN activity of loreclezole hydrochloride and penfluridol

To determine ability of loreclezole hydrochloride and penfluridol to simulate the binding of *A. aegypti* Met-FISC or to interfere with the pyriproxyfen-mediated binding of *A. aegypti* Met-FISC in a dose-dependent manner using *in vitro* yeast two-hybrid β-galactosidase assays. Loreclezole hydrochloride successfully simulated the binding between *A. aegypti* Met-FISC, and penfluridol showed no JHA activity in dose dependent manner (Fig. 6). In contrast, penfluridol interfered with pyriproxyfen-mediated Met-FISC binding in the β-galactosidase assay, but loreclezole hydrochloride didn't interfere with pyriproxyfen-mediated binding even at high concentrations (Fig. 7).

6. Median lethal concentration (LC_{50}) against 3rd larvae of *A. albopictus*

To further investigate the mosquito larvicidal activity of loreclezole hydrochloride and penfluridol, the median lethal concentrations (LC_{50}) against 3rd instar larvae of *A. albopictus* were determined (Table 4). Against 3rd instar larvae, both loreclezole hydrochloride and penfluridol showed high level of mosquito larvicidal activities, with 3.14 and 2.03 times lower LC_{50} values compared to those of pyriproxyfen, respectively.

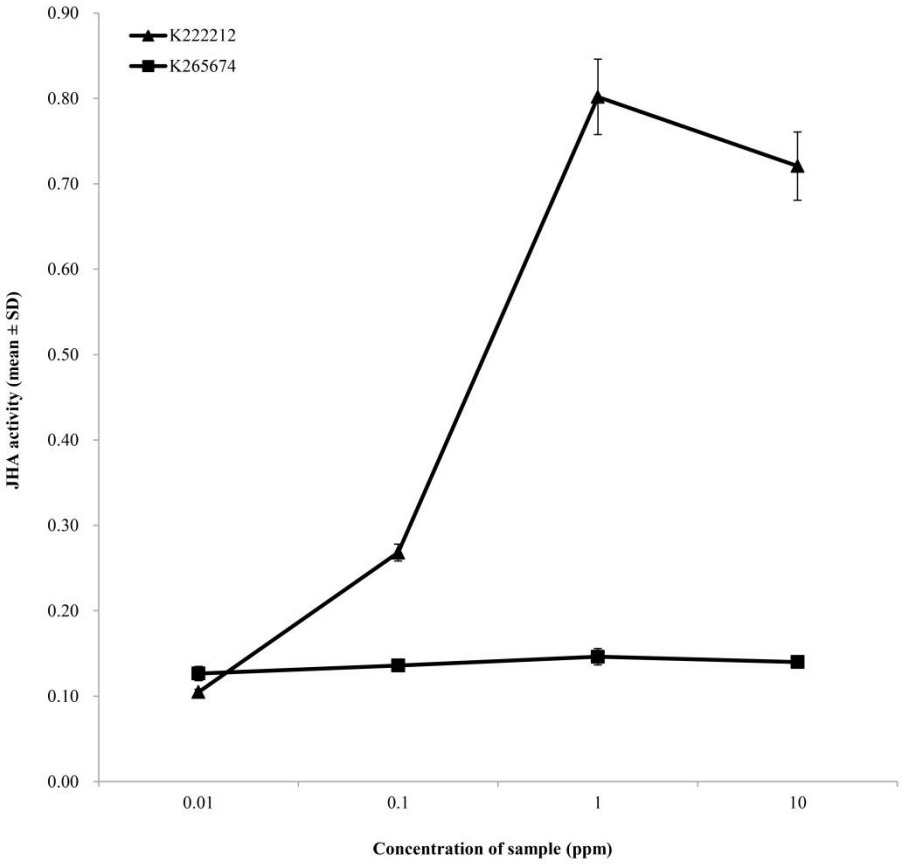


Figure 6. Concentration-dependent JHA activities of K222212 and K265674.

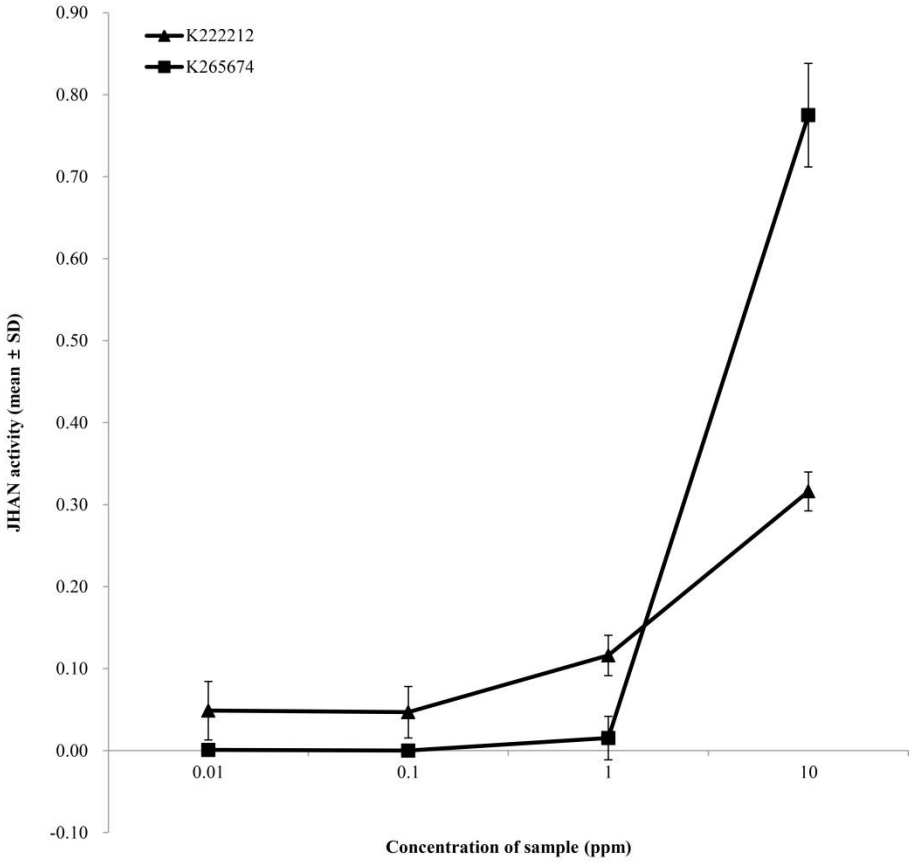


Figure 7. Concentration-dependent JHAN activities of K222212 and K265674.

Table 4. Median lethal concentration (LC_{50}) of chemical compounds with JHA or JHAN activity against 3rd instar larvae of *A. albopictus*.

	3rd instar	
Samples	LC_{50} (ppm)	Fiducial limits
Pyriproxyfen	6.25	5.42 ~ 7.20
K222212	1.99	1.83 ~ 2.16
K265674	3.07	2.57 ~ 3.67

7. Insecticidal activity of loreclezole hydrochloride and penfluridol against other agricultural pests

In order to define spectrum of insecticidal activity of loreclezole hydrochloride and penfluridol, insecticidal activity of these chemical compounds against *P. xylostella, O. furnacalis, L. striatellus, P. interpunctella* and *D. melanogaster* was evaluated. The screening bioassays were performed on proper stage by various methods, respectively.

To evaluate the insecticidal activity of loreclezole hydrochloride and penfluridol against *P. xylostella,* twenty 3rd instar larvae of *P. xylostella* fed on each 200 ppm of chemical treated Chinese cabbage leaf disc. Penfluridol showed significant insecticidal activity with mortality above 80% (Dunnett T3, $p < 0.001$) (Fig. 8).

The larvicidal activity of both chemical compounds against *O. furnacalis* was evaluated. Twenty neonates of *O. furnacalis* fed on each 100 ppm of chemical treated artificial diet. Like screening bioassay against *P. xylostella,* 100% mortality was observed in penfluridol-treated group (Tukey-HSD, $F = 3376.000$; $df = 2, 6$; $p < 0.001$) (Fig. 9).

Interestingly, mortality of penfluridol against 3rd instar larvae of *P. xylostella* and neonate of *O. furnacalis* based on its antifeedant properties (Fig. 10). Antifeedant property of penfluridol was dose-dependent manner (Fig 11). At 50 and 200 ppm, there were only about one percent of leaf damage (1.19 and 0.67%, respectively). But at 10 ppm, 9.52% area of leaf disc was damaged. However, loreclezole hydrochloride showed no significant insecticidal activity against 3rd instar larvae of *P. xylostella* and neonate of *O. furnacalis.*

Additionally, the screening bioassays were performed against nymphs of *L. striatellus* (Fig. 12), 2nd instar larvae of *P. interpunctella* (Fig. 13) and eggs of *D. melanogaster* (Fig.

13). Among these bioassays, loreclezole hydrochloride caused embryonic lethality of *D. melanogaster* at a concentration of 2,500 ppm (Fig. 14). But no significant insecticidal activities were observed against *L. striatellus* and *P. interpunctella.*

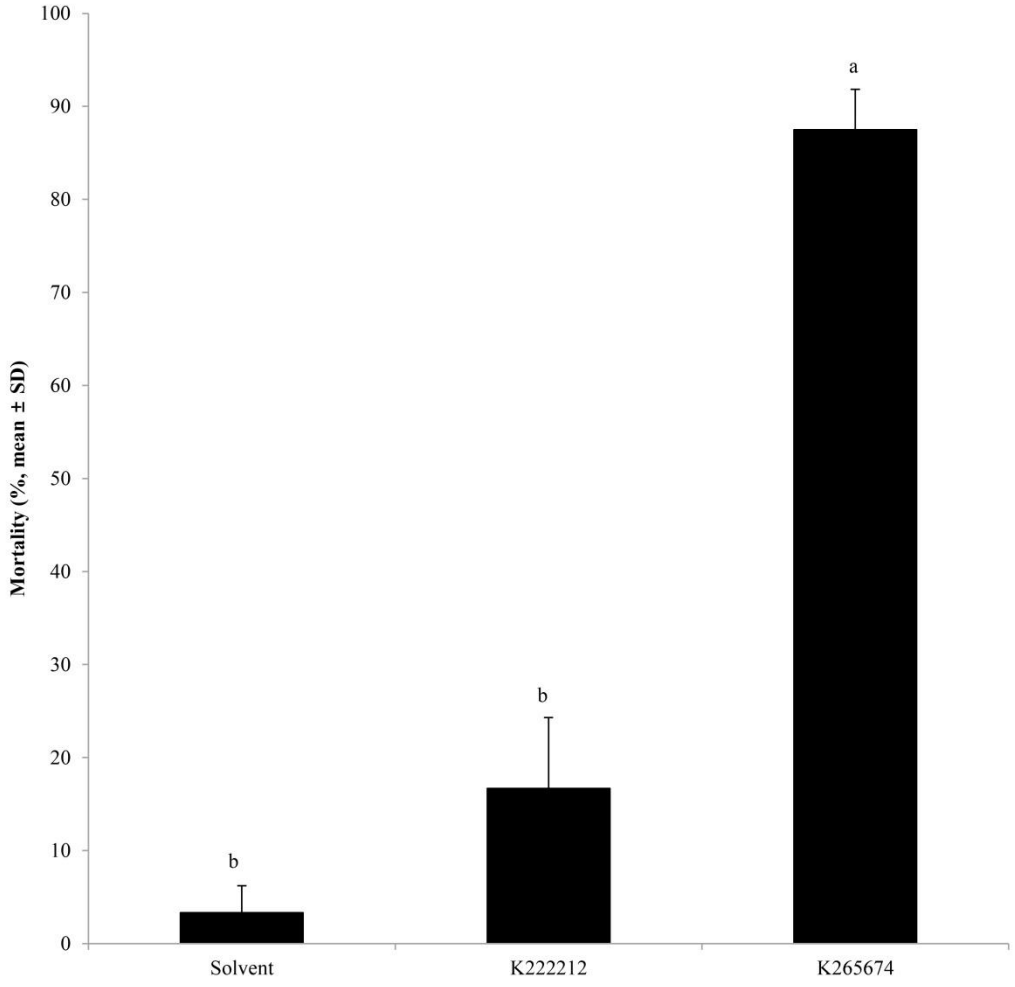


Figure 8. Insecticidal activities of chemical compounds with JHA or JHAN activity against 3rd instar larvae of *P. xylostella.* ($p < 0.001$)

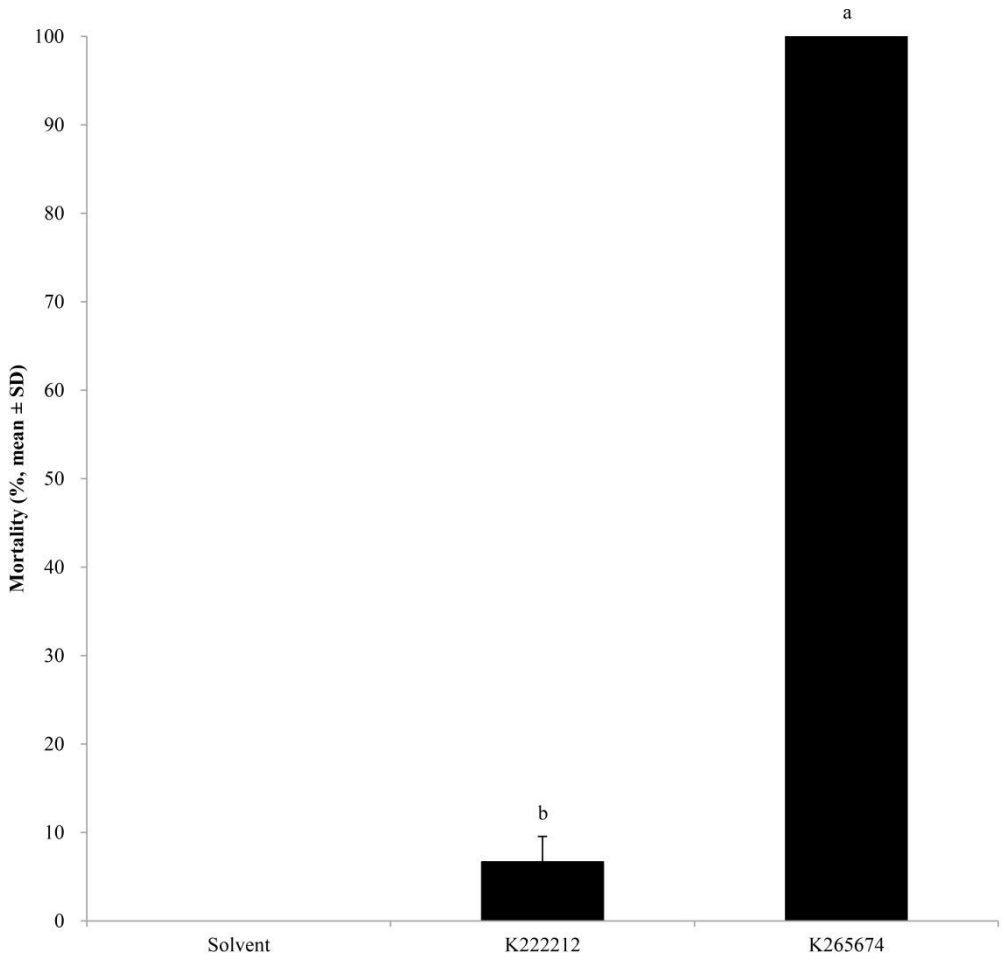


Figure 9. Insecticidal activities of chemical compounds with JHA or JHAN activity against neonates of *O. furnacalis.* ($p < 0.001$)

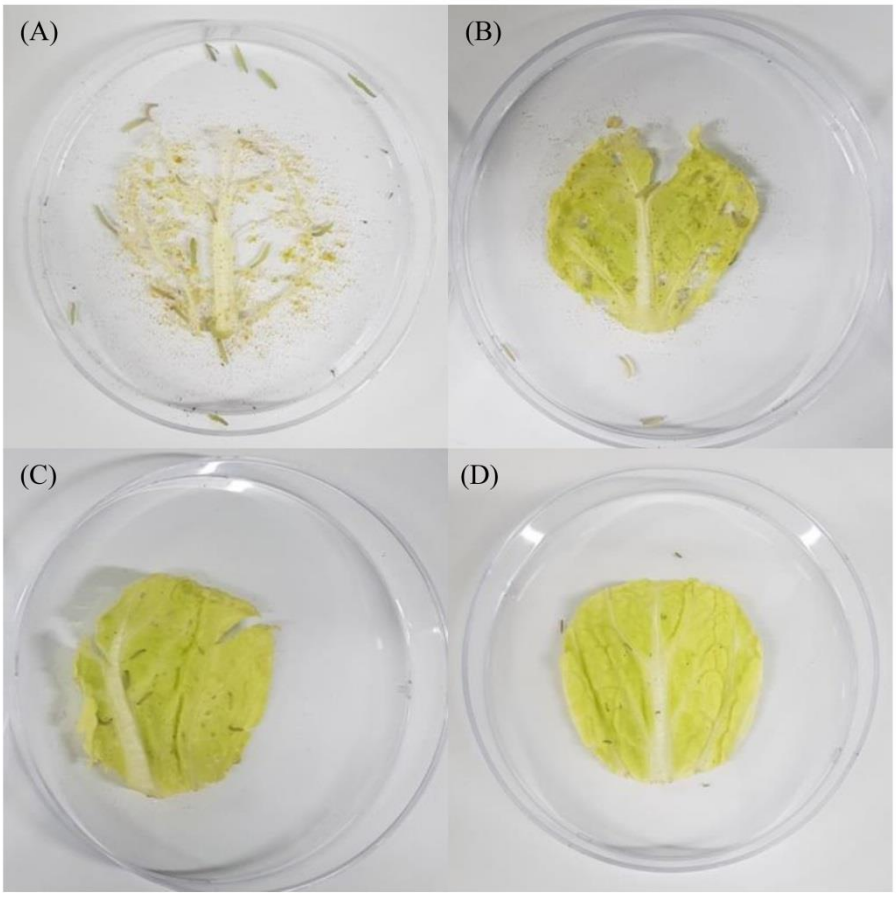


Figure 10. Concentration-dependent antifeedant properties of K265674 against 3rd instar of *P. xylostella* larvae. (A) Solvent-treated control. (B) 10 ppm, (C) 50 ppm, (D) 200 ppm of K265674 treatment.

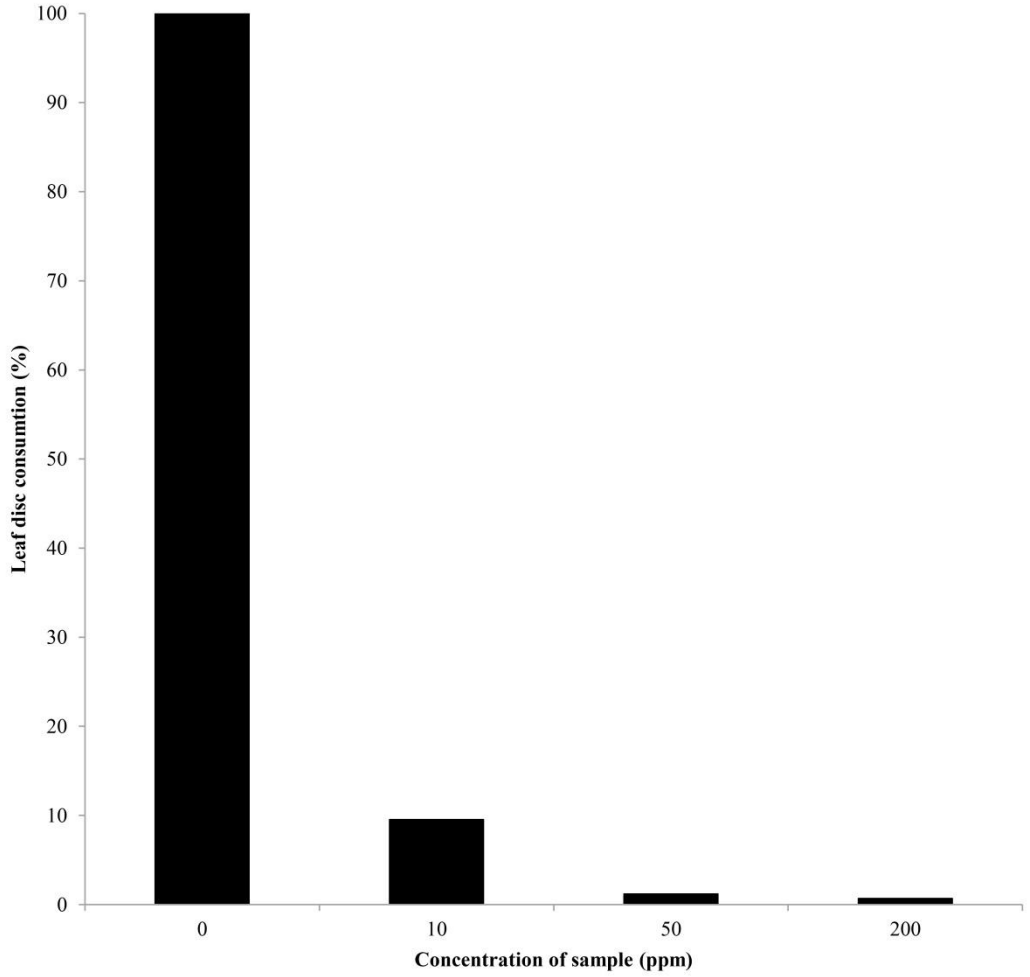


Figure 11. Concentration-dependent antifeedant properties of K265674 against 3rd instar larvae of *P. xylostella.*

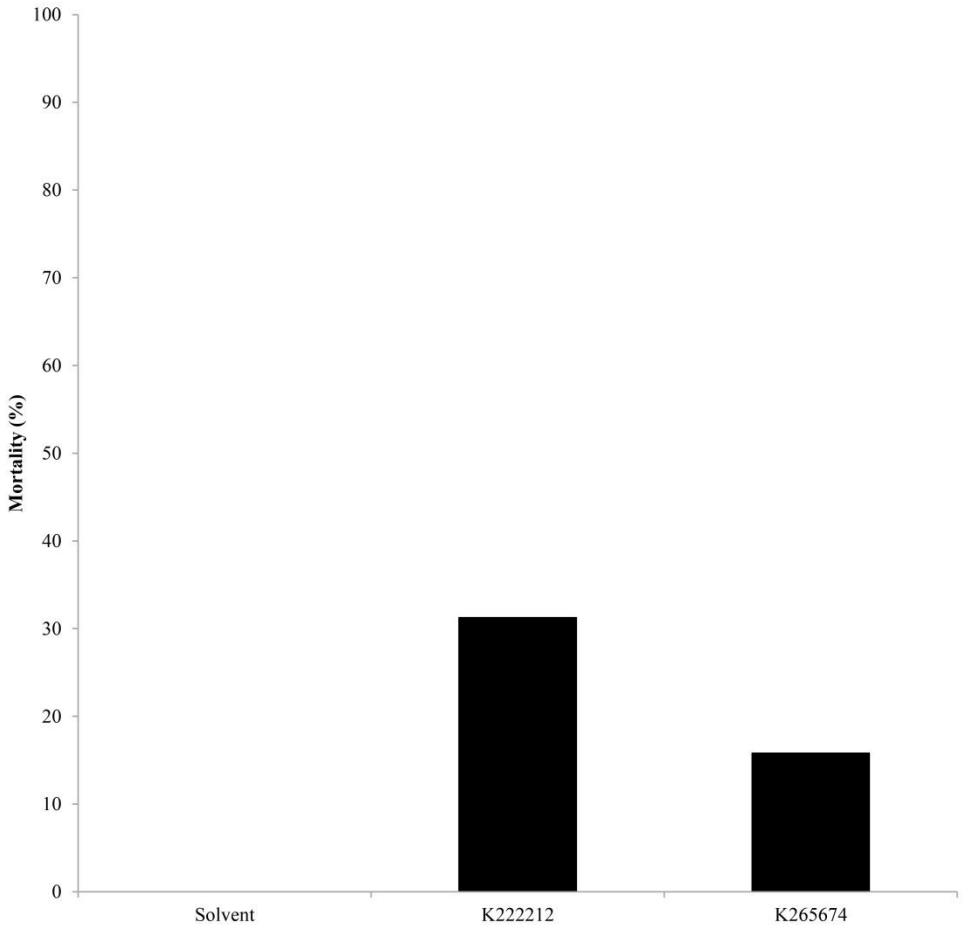


Figure 12. Insecticidal activities of chemical compounds with JHA or JHAN activity against nymphs of *L. striatellus*.

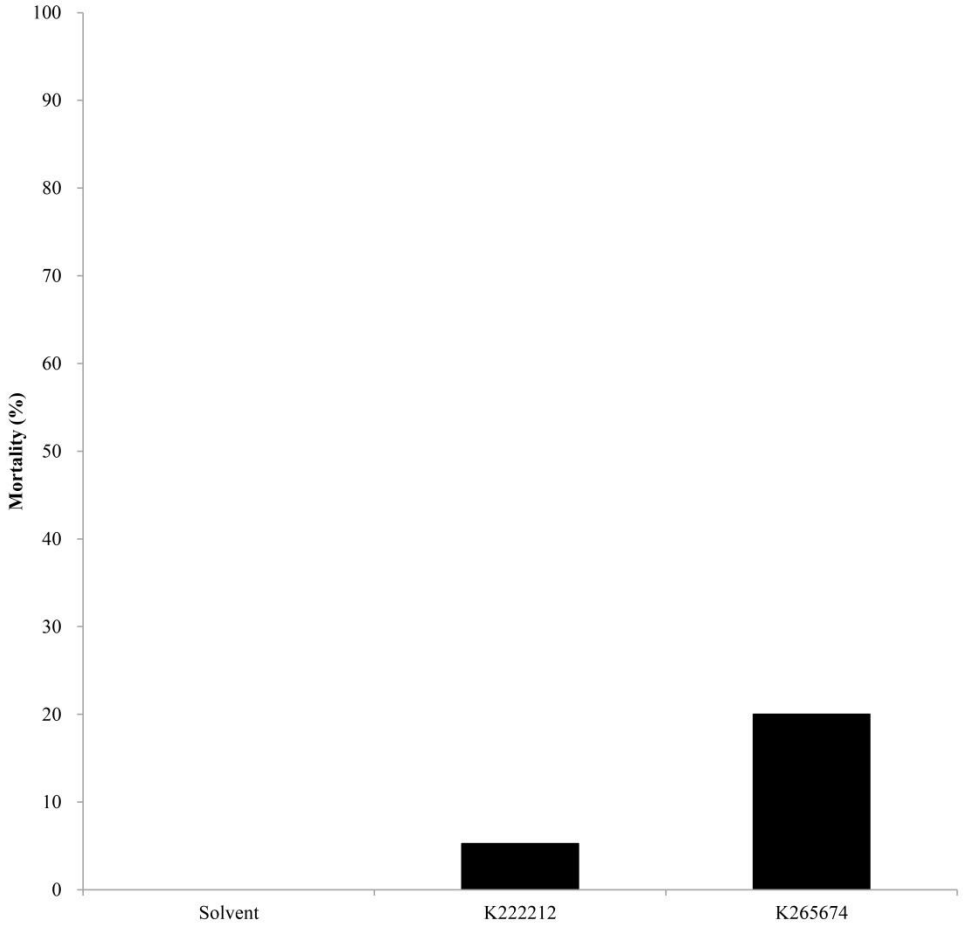


Figure 13. Insecticidal activities of chemical compounds with JHA or JHAN activity against 2nd instar larvae of *P. interpunctella*.

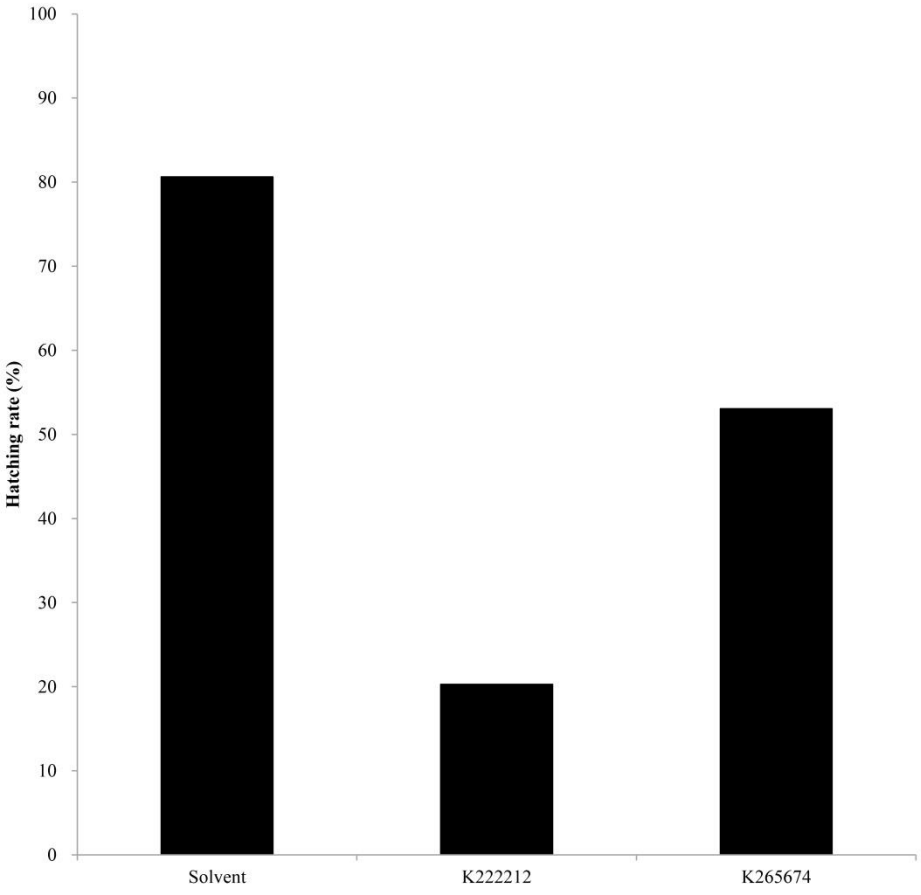


Figure 14. Embryonic lethalities of chemical compounds with JHA or JHAN activity against eggs of *D. melanogaster.*

DISCUSSION

Loreclezole hydrochloride, a compound with JHA activity, is a sedative as well as an anticonvulsant, and has been determined as the positive allosteric modulator of the mammalian type A γ-aminobutyric acid ($GABA_A$) receptor (Wingrove et al., 1994). The β_2 or β_3 subunits of the $GABA_A$ receptor have a high affinity for loreclezole hydrochloride. In insects, 100 μM of loreclezole hydrochloride enhanced the response to the *D. melanogaster* GABA receptor subunit RDL homo-oligomer and mammalian $GABA_A$ receptors containing the β_1 subunit. However, the same amount of loreclezole hydrochloride had no effect on the RDL homo-oligomer and mammalian $GABA_A$ receptors containing β_2 and β_3 subunits (Hosie and Sattelle, 1996). Penfluridol, which has JHAN activity, is a long-lasting, oral neuroleptic drug, and is a member of a group of chemicals in animals called diphenylbutylpiperidines (Janssen et al., 1970). The pharmacological profile of penfluridol is a typical antipsychotic and a dopamine receptor blocking agent (Jackson et al., 1975). In insects, dopamine is known to be an important neurotransmitter, neuromodulator, and neurohormone (Blenau and Baumann, 2001; Neckameyer and Leal, 2002; Waddell, 2010). In particular, dopamine as a neurohormone is involved in the regulation of JH synthesis (Gruntenko et al., 2005; Woodring and Hoffmann, 1994). The pharmacological and physiological effects of both loreclezole hydrochloride and penfluridol are well known in vertebrates and invertebrates, but their JHA or JHAN activity and their insecticidal activity is not well known.

Our screening system revealed that loreclezole hydrochloride mediated the formation

of the *A. aegypti* Met-FISC receptor complex, while penfluridol interfered with the formation of pyriproxyfen-mediated *A. aegypti* Met and FISC. Also, both loreclezole hydrochloride and penfluridol showed higher mosquitocidal activities against 3rd instar larvae of *A. albopictus* than pyriproxyfen. Additionally, not only hydrochloride salt formation, but also not salt formation of loreclezole hydrochloride was demonstrated similar level of JHA and larvicidal activity. It is well known that the solubility of an insecticide is major factor when the insecticide is applied in field. Therefore, this result could be an advantage in its application.

To determine the specificity of insecticidal activity of loreclezole hydrochloride and penfluridol toward mosquitoes, other agricultural pests were treated with loreclezole hydrochloride and penfluridol. Interestingly, penfluridol showed larvicidal activity against 3rd instar larvae of *P. xylostella* and neonates of *O. furnacalis.* These larvicidal activities however, were due to their antifeedant effects. However, the "activity" of JHA pesticides has generally been known to include a mixture of real JH activity in target species with a complex of pharmacological side effects, including antifeedant, antimetabolic or toxic properties, in different species and different developmental stages of the target insect (Sláma, 1999). Therefore, the antifeedant property of penfluridol against non-target insects can be considered as JHAN activity in the broad sense.

Additionally, methoprene (ZR-515), a commercial JHA, had effects on early embryogenesis in *D. melanogaster* (Smith and Arking, 1975). Loreclezole hydrochloride caused embryonic lethality when topically applied to freshly laid *D. melanogaster* eggs. These results suggested that JHA and JHAN activity and insecticidal activity of loreclezole hydrochloride and penfluridol are Diptera-specific activity.

In conclusion, we determined that loreclezole hydrochloride shows JHA activity and penfluridol displays JHAN activity, using *in vitro* yeast two-hybrid β-galactosidase assays. These compounds demonstrated Diptera-specific mosquito larvicidal activities. These results suggested that loreclezole hydrochloride and penfluridol are potential for the development of environmentally safe JH-related IGR insecticides against mosquito.

CHAPTER Ⅱ. Identification and characterization of loreclezole hydrochloride and its derivatives with juvenile hormone-related insect growth regulator activity against Asian tiger mosquito

ABSTRACT

Mosquitoes are medically important insect pests that transmit various diseases when they feed on humans. The Asian tiger mosquito, *Aedes albopictus*, is one of the most invasive vectors of various diseases including dengue fever, chikungunya, and zika virus. Chemical insecticides such as *N*,*N*-diethyl-*m*-toluamide and temephos have been commonly used to control of mosquitoes. However, due to their toxicity to environments and development of insect resistance, the demands for environmentally benign insecticides is on the rise. Insect growth regulators (IGRs) could become an effective alternative to control mosquitoes and other vector transmitted diseases because they are specific to target insects and relatively low toxic to environment.

To identify novel effective JH-related IGRs for control of Asian tiger mosquitoes, loreclezole hydrochloride (K222212) with JHA activity and its 32 derivatives were surveyed on their JH agonist (JHA) and antagonist (JHAN) activity using *in vitro* yeast two-hybrid *β*-galactosidase ligand binding assay. Among them, 3 derivatives was meditated the formation of JH receptor complex and 4 derivatives were interfered with

the formation of pyriproxyfen-mediated JH receptor complex. Loreclezole hydrochloride and K21877 (1-chloro-[(Z)-2-(phenylphenyl)ethenyl]-1,2,4-triazole) in the derivatives with JH-related IGR activity showed high level of insecticidal activity against 3rd larvae of *Aedes albopictus*. In addition, loreclezole hydrochloride and K21877 demonstrated high level of JHA and JHAN activity, respectively, median lethal dose (LC_{50}) against entire larval stages, embryonic lethality, adult toxicity against *A. albopictus*, and *in vivo* modulate JH-regulated physiological functions such as expression of JH-responsive genes and reproduction. These results suggested that loreclezole hydrochloride and K21877 could be useful for control of mosquitoes and provided a better understanding of relationship between structure and JHA or JHAN activity.

INTRODUCTION

Recent advances on mechanism of juvenile hormone (JH) action including identification of JH receptor should help in developing new more potent and safer JH agonist (JHA) for use in pest management. Especially, JHAs, such as methoprene and pyriproxyfen, were used for insect problems in urban environment and disease transmission. However, JHAs commercially used for control of mosquitoes also were reported to develop resistance (Dennehy et al., 2010; Paul et al., 2005; Silva and Mendes, 2007). There is an urgent need to develop and commercialize new, more potent and safer JH-related insect growth regulators (IGRs). Based on the basic research progress in molecular level, JH antagonists (JHANs), novel JH-related IGR, also reported and they might be more effective for the control of pests than JHAs because of the mode of action.

In the previous chapter, it was demonstrated that the novel JH-related IGRs, loreclezole hydrochloride (K222212) and penfluridol (K265674), were identified and characterized using *in vitro* yeast two-hybrid *β*-galactosidase ligand binding assay and bioassay against *A. albopictus* and other agricultural pests. Among them, loreclezole hydrochloride with JH agonist activity is a sedative and anticonvulsant, has been determined the mammalian type A γ-aminobutyric acid ($GABA_A$) receptor positive allosteric modulator (Wingrove et al., 1994). This compound belong to a 1,2,4-triazole derivatives which were reported to show broad-spectrum activities, such as fungicidal, herbicidal, anticonvulsant, and plant growth regulatory activity (Asif, 2014; Awasthi et al., 2009; Li et al., 2012; Saini and Dwivedi, 2013). That means loreclezole hydrochloride derivatives could be possibility to

find more effective compounds with JH-related IGRs activity for control of mosquitoes. Therefore, in this chapter, to identify more effective JH-related IGR compounds, loreclezole hydrochloride and its derivatives were synthesized and their biological characteristics were investigated.

MATERIALS AND METHODS

1. Yeast two-hybrid β-galactosidase assays

In this chapter, the yeast two-hybrid β-galactosidase assay was performed to identification of novel JHA and JHAN compounds from loreclezole as previously chapter.

2. Larvicidal activity tests against Asian tiger mosquito, *A. albopictus*

For screening bioassay, twenty 3rd instar larvae of *A. albopictus* in 5 ml tap water with food mixtures were treated with each 10 ppm of compound. The number of dead larvae was counted at 24 h after treatment for 3 days. To determine the median lethal dose (LC_{50}), twenty larvae of entire stage were treated with serial dilutions of each compound, respectively. The number of dead larvae was counted at every 24 h for 3 days. Experiments for determine the LC_{50} were performed in triplicates and the IBM SPSS Statistics for Windows, version 23.0 (IBM Corp., Armonk, NY, USA) was used to calculate LC_{50} via linear regression.

3. Mosquitocidal activity tests against female adult of *A. albopictus*

Twenty adults of *A. albopictus* at first day after post emergence were topically applied with 1 μg of each compound onto the abdomen. The number of dead adults was counted every 24 h for 3 days. This experiment was performed in triplicate.

4. Embryonic lethality tests

Twenty males and blood-fed females of *A. albopictus* were reared in cage by placing egg-laying cup (12 oz plastic cup) covered filter paper which was filled with tap water that was treated with each 10 ppm of compound. Three days after treatment, eggs were collected from rearing cage and incubated in breeding chambers at 28℃ and 70% relative humidity in sample-treated tap water. Embryonic lethality was determined by counting the number of unhatched eggs 120 h after treatment and the experiment was performed in triplicate.

5. RNA preparation and quantitative PCR (qPCR)

At circadian time 0 (time that dark switch to light) of 1 day post eclosion (PE), each 0.5 μg of compound in 0.5 μl of acetone was applied topically onto the abdomen of female *A. albopictus* within 1 h after eclosion. Total RNA from treated mosquitos was prepared using Qiazol (QIAGEN, Hilden, Germany) 8 h after treatment. Single-stranded cDNA was synthesized from the total RNA using QuantiTect Reverse Transcription Kits (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. The qPCR was conducted with the EvaGreen qPCR kit (Applied Biological Materials, Richmond, Canada) and the CFX96TM Real-Time System (BIO-RAD, Hercules, CA, USA). The cycling profile used for qPCR was as follows: a preheating step for enzyme activation at 95℃ for 10 min, followed by 40 cycles of 95℃ for 15 s and 55℃ for 60 s. The 40S ribosomal protein S7 (RPS7) was used as a reference gene for the calculation of fold change. The relative transcription level (RTL) was calculated by using $2^{-\Delta Ct}$ method (Pfaffl, 2001). Oligonucleotides specific to Hairy, Hairy-Fw (5'-

TGACCGTGAAACATTTGGAA-3') and Hairy-Re (5'-CGGTCTCCAAGGTTTGTCAT-3'), were used for qPCR. All assays were performed in triplicates and at least 10 female mosquitos were used for qPCR at each time points.

6. Ovary Dissection

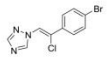
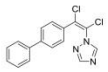
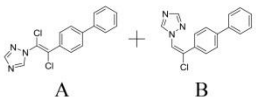
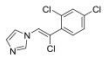
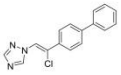
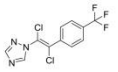
Half a microgram of each compound in 0.5 µl of acetone was applied topically onto the abdomen of female *A. albopictus* immediately post emergence or blood meal (PE or PBM), respectively. Mosquitoes were allowed blood meals on lab mice for 6 h along with a diet of 10% sucrose solution. Blood-fed mosquito was identified with the blood swelling in its abdomen and mosquitos that did not fed on blood were discarded. Two days after blood meal, the mosquitos were anesthetized in the freezer (-20℃) for 5-10 min and the ovaries were dissected in a drop of PBS buffer by pulling out the last segment of the abdomen. At least 10 female *A. albopictus* were treated for each compound. Mean follicle length for each female was calculated from measurement of 5 follicles, and data were collected from 10 individuals. An unpaired t-test was used to distinguish difference in follicle size among chemical compounds treated- and solvent treated-mosquitoes.

RESULTS

1. Screening of JHA or JHAN from loreclezole hydrochloride derivatives

To investigate the relationship between JHA/JHAN activity and chemical structure, 32 loreclezole hydrochloride derivatives were synthesized (Table 5). Loreclezole hydrochloride and its derivatives were tested whether the binding of *A. aegypti* Met-FISC could be simulated or the pyriproxyfen-mediated binding of *A. aegypti* Met-FISC could be disrupted in the yeast two-hybrid β-galactosidase assays. Three compounds, including, K21861, K21871 and K21884, showed high level of JHA activity (JHA activity > 0.5) (Fig. 15). In contrast, four compounds, including K21873, K21875, K21876 and K21877, showed relatively high JHAN activity (JHAN activity > 0.5) (Fig. 16).

Table 5. Structure of K222212 derivatives.

Chemical	Structure	Chemical	Structure	Chemical	Structure
K21819		K21871	Confidential	K21881	Confidential
K21861	Confidential	K21872	Confidential	K21882	
K21862	Confidential	K21873	Confidential	K21883	Confidential
K21863	Confidential	K21874		K21884	Confidential
K21864	Confidential	K21875		K21885	Confidential
K21865	Confidential	K21876	 A B A:B = 1:1 mixture	K21886	
K21866	Confidential			K21887	
K21867	Confidential	K21877		K21909	Confidential
K21868	Confidential	K21878		K21910	Confidential
K21869	Confidential	K21879		K21911	Confidential
K21870	Confidential	K21880	Confidential	K21912	

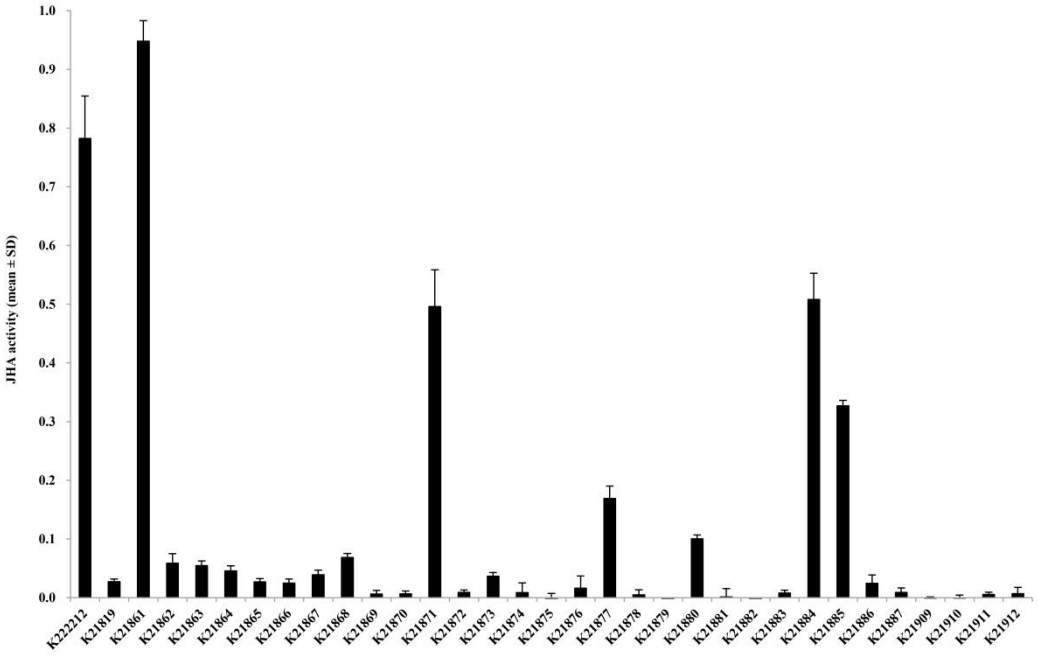


Figure 15. Screening of the K222212 derivatives with JHA activity. The Met-FISC binding triggered by K222212 derivatives was simulated by β-galactosidase activity in the yeast two-hybrid system. Each chemical compounds was added to the yeast culture at a concentration of 10 ppm.

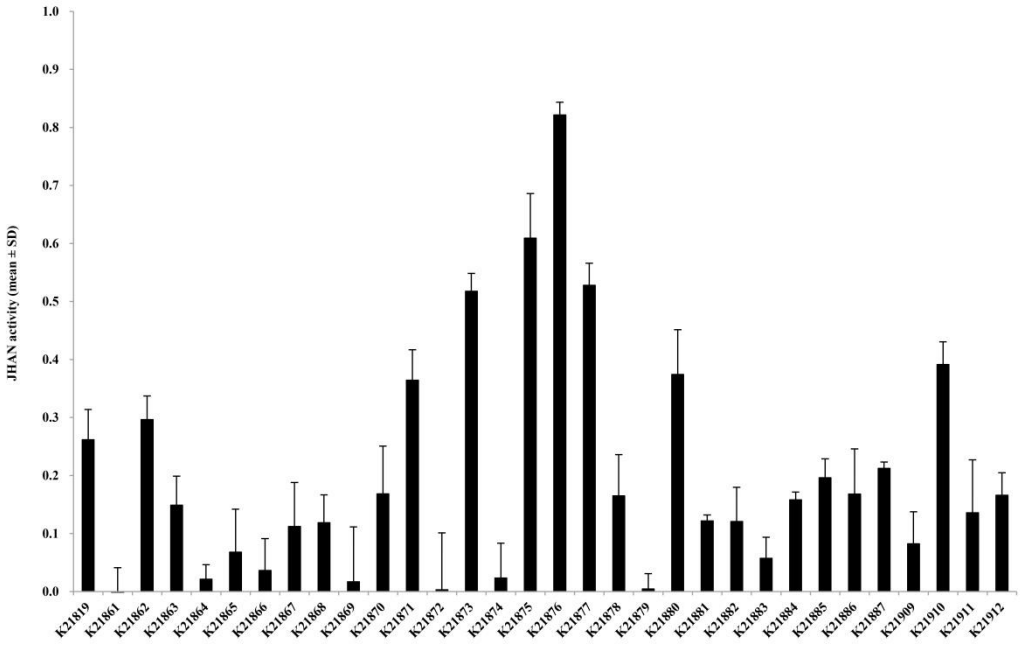


Figure 16. Screening of the K222212 derivatives with JHAN activity. The Met-FISC binding triggered by chemical compound was simulated by β-galactosidase activity in the yeast two-hybrid system. Each chemical compounds was added to the yeast culture at a concentration of 10 ppm.

2. Screening larvicidal activity of against *A. albopictus*

To evaluate the mosquito larvicidal activities of loreclezole hydrochloride and its derivatives with high JHA or JHAN activities, the mortality of 3rd instar larvae of *A. albopictus* treated with these compounds was determined (Fig. 17). Among these compounds, loreclezole hydrochloride and K21877 (1-chloro-[(Z)-2-(phenylphenyl)ethenyl]-1,2,4-triazole) caused 100% mortalities at 10 ppm.

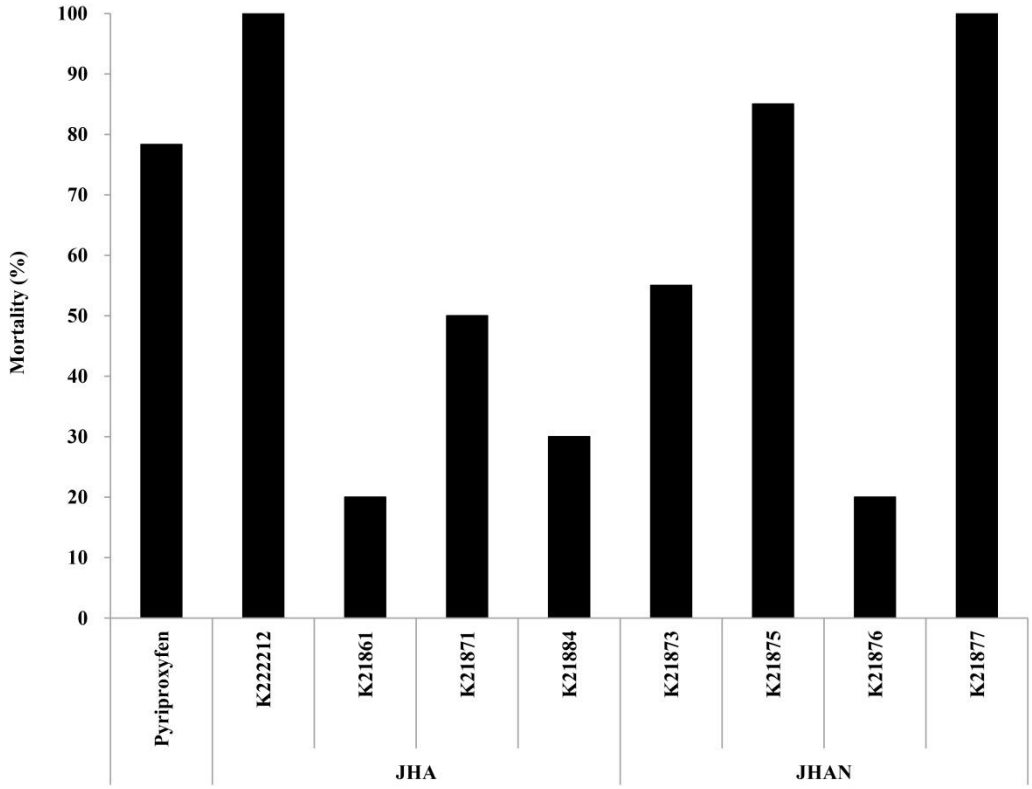


Figure 17. Mosquitocidal activities of K222212 derivatives with JHA or JHAN activity. Third instar larvae of *A. albopictus* were treated with 10 ppm of corresponding compounds and the mortality was calculated at 72 h after treatment.

3. Median lethal concentrations (LC_{50}) of loreclezole hydrochloride and its derivative against *A. albopictus* larvae

To further examine the mosquitocidal activities of loreclezole hydrochloride and K21877, the median lethal concentrations (LC_{50}) against all stage of *A. albopictus* larvae were determined (Table 6). Against 2nd and 3rd instar larvae, both loreclezole hydrochloride and K21877 showed high levels of larvicidal activities compared to those of pyriproxyfen with approximately 1.34-3.21 and 1.67-2.47 times lower LC_{50} values, respectively. Especially, whereas loreclezole hydrochloride with JHA activity was more effective than other compounds against last larval stage, K21877 with JHAN activity was more effective at early stages.

Table 6. Median lethal concentration (LC_{50}) of K222212 and K21877 with JHA or JHAN activity against entire larva stages of *A. albopictus.*

Sample	Stage	LC_{50}	95 % Fiducial limits	Slope	Intercept	SE	R^2	χ^2 sig.	df
Pyriproxyfen	1st instar	1.685	1.176-2.414	1.995	4.559	0.080	0.890	0.989	5
	2nd instar	2.301	1.832-2.889	4.250	3.477	0.050	0.886	0.754	3
	3rd instar	6.347	5.246-7.680	5.815	0.336	0.042	0.982	0.945	2
	4th instar	10.618	9.551-11.803	8.710	-3.937	0.023	0.944	0.917	2
K222212	1st instar	2.169	1.547-2.982	2.924	4.017	0.071	0.992	0.522	2
	2nd instar	1.714	1.360-2.161	4.751	3.891	0.051	0.978	0.745	1
	3rd instar	1.974	1.589-2.453	5.195	3.472	0.048	0.963	0.801	2
	4th instar	3.112	2.235-4.335	2.510	3.858	0.073	0.721	0.640	4
K21877	1st instar	1.277	0.857-1.903	2.223	4.762	0.088	0.917	0.736	3
	2nd instar	1.372	1.058-1.781	4.193	4.421	0.058	0.971	0.767	2
	3rd instar	2.562	1.957-3.354	2.845	3.837	0.060	0.960	0.983	4
	4th instar	9.026	8.041-10.131	6.557	-1.263	0.026	0.890	0.935	5

4. Hatching rate of mosquito eggs and mosquitocidal activities against adults treated loreclezole hydrochloride and its derivative

When loreclezole hydrochloride and K21877 were topically applied to eggs of *A. albopictus*, respectively, to investigate the effect of these compounds on embryogenesis, both loreclezole hydrochloride and K21877 showed high levels of embryonic lethality (Tukey-HSD, $F = 4677.549$; $df = 2, 6$; $P < 0.001$) (Fig. 18A). In contrast, loreclezole hydrochloride caused much more detrimental effect against adult mosquitoes (Tukey-HSD, $F = 1225.752$; $df = 2, 6$; $P < 0.001$) (Fig. 18B). These results suggested that JHANs might be effective for control of mosquitoes in larval stage whereas JHAs might be more effective for control of adult mosquitoes.

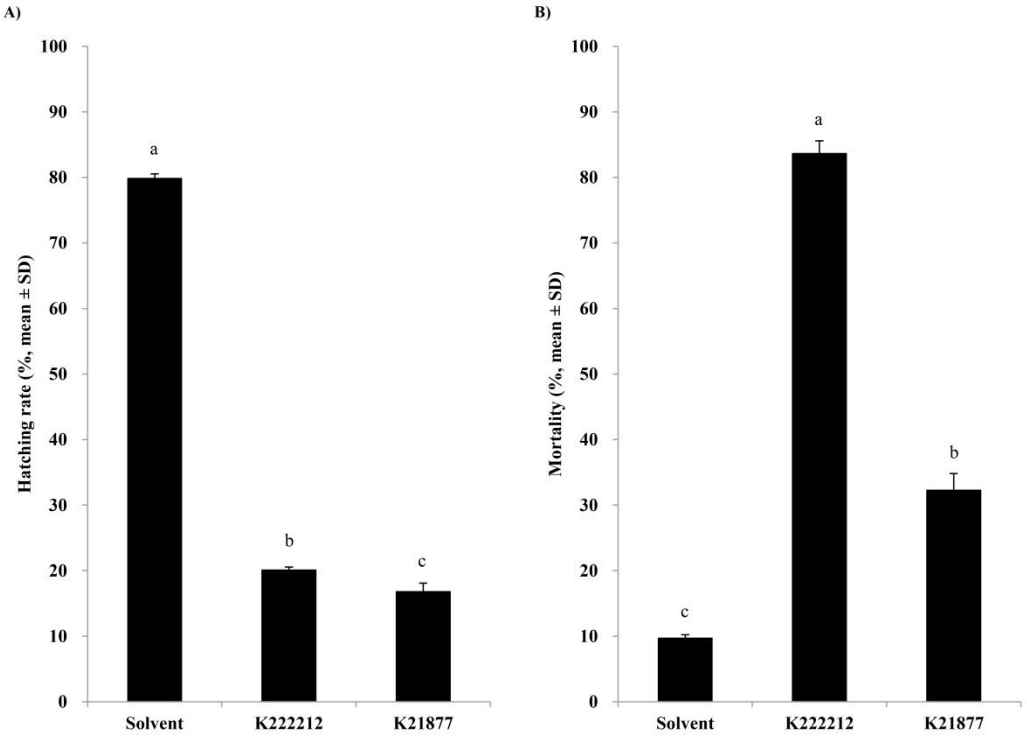


Figure 18. Embryonic lethalities (A) and mosquitocidal activities against adults (B) of K222212 and K21877.

5. Expression of the *Hairy* gene treated loreclezole hydrochloride and its derivative

To determine the effects on the expression of JH-induced gene, qPCR analyses of hairy gene expression were tested with female *A. albopictus* adults (Tukey HSD, $F = 76.880$; $df = 22, 46$; $P < 0.001$) (Fig. 19). Topical application of loreclezole hydrochloride with JHA activity enhanced the expression of this gene compared to that of solvent-treated mosquitoes. In contrast, expression of the hairy gene was significantly reduced in the mosquitoes treated with K21877, suggesting that K21877 disrupt JH-dependent regulation of previtellogenic development by interfering the binding of the JH receptor complex.

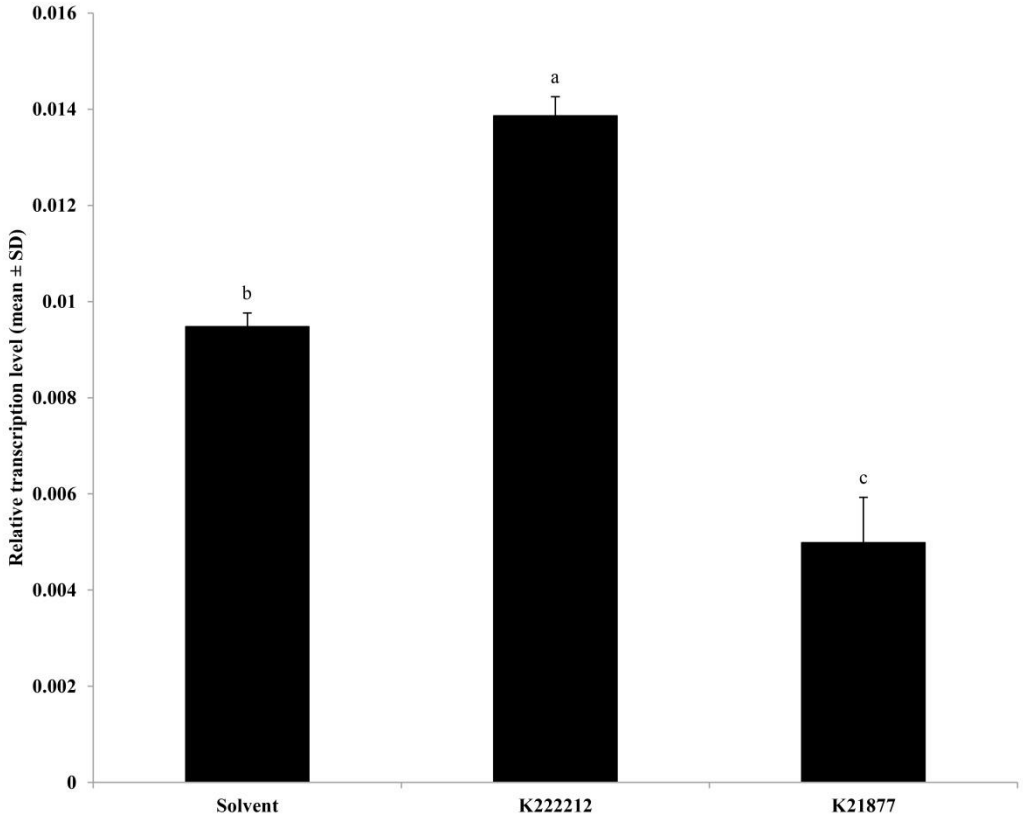


Figure 19. Relative transcription level of the Hairy gene. The start of the light phase in a 12 h dark/12 h light photoperiod is circadian time 0. Female *A. albopictus* within after emergence were collected at CT0 and topically treated with the corresponding compound. Total RNA was extracted 8 h after treatment and subjected to qPCR for the analysis of the relative transcription levels of the *Hairy* gene.

6. Effect of loreclezole hydrochloride and its derivative on the ovary development of female *A. albopictus*

JH removal or Met RNA interference silencing resulted in arrest of ovarian development with a significant reduction in the size of ovarian follicles (Zou et al., 2013). Topical application of loreclezole hydrochloride and K21877 induced severe retardation of ovarian growth after post emergence and post blood meal (Fig. 20 and 21). When treated immediately after emergence, K21877 with JHAN activity caused detrimental effect on ovary development (follicle length of K21877 treatment: 0.244 mm ± 0.009; follicle length of control: 0.365 ± 0.033, $p < 0.01$) (mean ± SD). In contrast, when treated immediately after blood meal, loreclezole hydrochloride with JHA activity caused detrimental effect on ovary development (follicle length of loreclezole hydrochloride treatment: 0.195 mm ± 0.022; follicle length of control: 0.338 ± 0.014, $p < 0.01$) (mean ± SD). The ovaries of females treated with acetone solvent normally matured irrespective of treated time. The eggs changed shape from circular to elliptical, and the entire ovary became large size. However, the female ovaries retarded by treatment of loreclezole hydrochloride and K21877 maintained immature, circular eggs and significantly small ovary sizes.

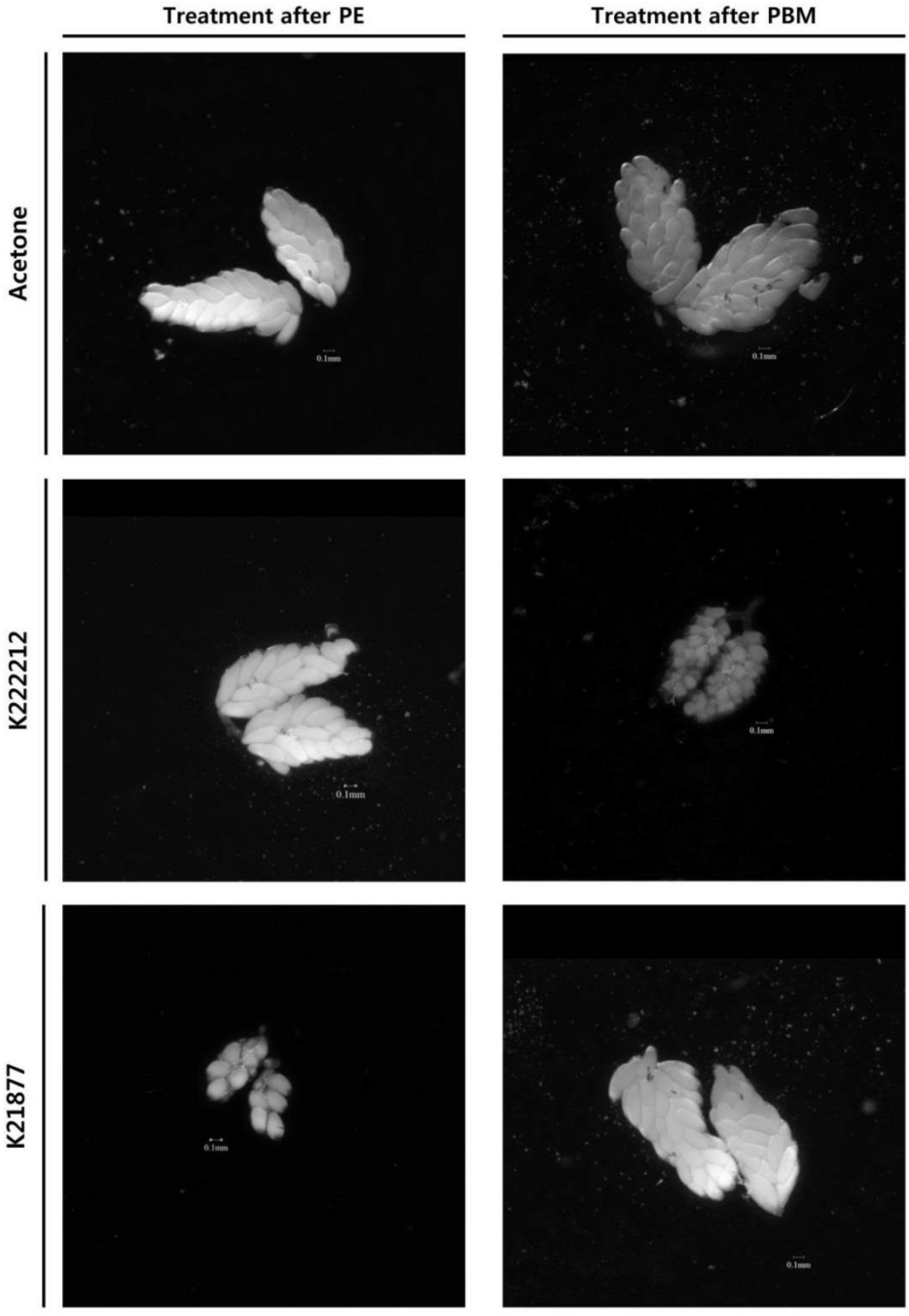


Figure 20. Effects of K222212 and K21877 on the ovary development of female *A. albopictus*. Female mosquitos were treated with the corresponding compound immediately post eclosion (PE) and post blood meal (PBM), respectively. The ovaries were dissected 2 days after the blood meal on white lab mice.

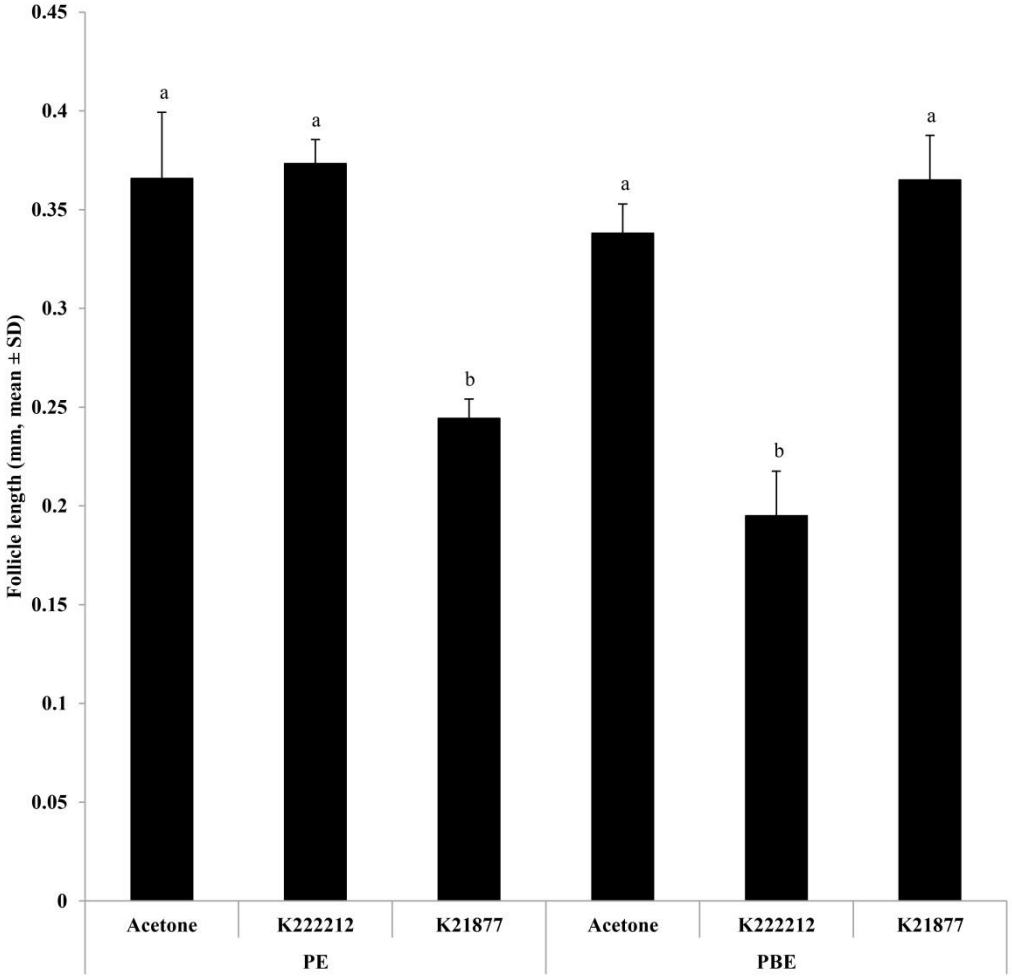


Figure 21. Effects of K222212 and K21877 on previtellogenic and vitellogenic follicle development of female *A. albopictus*. To determine effects of K222212 and K21877 on previtellogenic and vitellogenic follicle development, female of *A. albopictus* were treated with JHA and JHAN after post emergence (PE) and post blood meal (PBE).

DISCUSSION

Triazoles are heterocyclic compounds that have a penta-cyclic ring of two carbon atoms and three nitrogen atoms. 1,2,4-Triazole is one set of isomeric compounds of triazoles. 1,2,4-Trizole derivatives have received considerable attention recently because of their involvement in various biological processes including anti-bacterial, anti-fungal, anti-viral, anti-inflammatory, anti-tubercular, anticonvulsant, anti-cancer, and anti-malarial functions (Asif, 2014; Awasthi et al., 2009; Li et al., 2012; Saini and Dwivedi, 2013). Loreclezole hydrochloride is a 1,2,4-triazole derivative with anticonvulsant properties which has been determined as the positive allosteric modulator of the mammalian type A γ-aminobutyric acid ($GABA_A$) receptor (Wauquier et al., 1990; Wingrove et al., 1994).

In the previous chapter, loreclezole hydrochloride was identified as a JH-related IGR compound with JHA activity. In this chapter, we tested the effect of loreclezole hydrochloride derivatives (Korea Research Institute of Chemical Technology, Daejeon, Korea) on their interaction with JH receptor complex of *A. aegypti* using *in vitro* yeast two-hybrid *β*-galactosidase assays.

From the assays, 3 compounds displayed high levels of JHA activities (JHA activity > 0.5). Even K21861 showed the highest JHA activity. However, these compounds showed lower mosquito larvicidal activities than the parent compound, loreclezole hydrochloride. A total of 4 compounds also showed high levels of JHAN activity (JHAN activity > 0.5). Among them, K21877 showed the highest larvicidal activity against *A. albopictus* larvae.

These results suggested that the measured JHA or JHAN activity using the *in vitro* screening assay does not guarantee insecticidal activity. This is because insecticidal activity involves numerous other factors such as the rate of penetration through the cuticle, or the metabolic detoxification capabilities and resistance of the target insects.

The larvicidal activities of loreclezole hydrochloride and K21877 were determined for all stages of *A. albopictus* development. For all larval stages, loreclezole hydrochloride and K21877 showed similar (1st instar and 4th instar) or higher (2nd instar and 3rd instar) levels of insecticidal activities compared to pyriproxyfen. The *difference in mosquito larvicidal activities* between JHA and JHAN against early larval stages (1st instar) and late larval stages (4th instar) were quite significant. Against early larval stages, K21877 (JHAN) showed high larvicidal activity. In contrast, loreclezole hydrochloride (JHA) was more effective than K21877 against late larval stages. In Diptera, significant embryonic sensitivity to JH has already been reported (Spielman and Williams, 1966). Exogenous JHA such as methoprene caused embryonic lethality when topical applied to freshly laid *Drosophila melanogaster* eggs (Smith and Arking, 1975). Loreclezole hydrochloride and K21877 also caused embryonic lethality against freshly laid eggs of *A. albopictus*. Like the larvae, freshly laid eggs of *A. albopictus* were much more susceptible to K21877 (JHAN), than to loreclezole hydrochloride (JHA). In contrast, JHA was more effective than JHAN when topically applied onto the abdomen of adult *A. albopictus* females. These results suggested that JHANs might be more effective at controlling target pests at early life stages by interfering with the normal action of JHs.

The application of exogenous JH or JHAs resulted in enhanced expression of a JH-responsive gene, *Hairy*, which was mediated by the formation of the JH receptor complex

(Shin et al., 2012; Zou et al., 2013). On the other hand, the application of JHAN compounds resulted in repression of *Hairy* (Lee et al., 2015). Loreclezole hydrochloride (JHA) greatly enhanced expression of *Hairy*. In contrast, the expression of this gene was significantly reduced in female adults of *A. albopictus* treated with K21877. In *A. aegypti*, JH titer in hemolymph has been associated with stages of female reproduction (Zhu et al., 2010). The development of the ovaries during a gonotrophic cycle can be divided into three major periods in *A. aegypti*: previtellogenesis, ovarian resting stage and vitellogenesis (Klowden, 1997). The rise of JH titer in hemolymph after emergence is driven by a major nutritionally dependent increase in JH synthesis around 12 hours post emergence. This increase in JH synthesis stimulates the activation of previtellogenic maturation. After previtellogenic maturation has been completed (48-72 h), female mosquitoes enter the ovarian resting stage. At this stage, the suppression of JH synthesis due to blood feeding, and the resulting reduction of JH titer in the hemolymph stimulates development of follicle quality and completes the vitellogenesis phase. Loreclezole hydrochloride (JHA) caused retardation of follicle development after blood feeding. K21877 (JHAN) also caused the retardation of follicle development after emergence. Both JHA and JHAN compounds significantly slowed down ovary development, causing a reduction in the length of the ovaries and the size of follicles in *A. albopictus* females. These results indicate that both JHA and JHAN (loreclezole hydrochloride and K21877), can modulate *in vivo* JH-regulated physiological functions such as the expression of JH-responsive genes and reproduction.

Before 1975, compounds with JH activity have been used as novel insecticides, which were identified by official registration of JH-active compounds such as hydroprene and

methoprene, for control of mosquitoes, ants and flies. Methoprene in particular, was known for being a biodegradable, environmentally safe compound with high target specificity. Although methoprene and other compounds with JH activity are relatively nontoxic to the environment and have high target specificity for insects, they have a lot of disadvantages in the field. The first is that they do not cause immediate knockdown. Also, they require professional knowledge and skill for their application. To avoid these disadvantages, novel JHAs have been investigated. These JHAs incorporated bicyclic, 4-phenoxyphenyl groups into the JHA molecule. The bicyclic, 4-phenoxyphenyl groups were used for the synthesis of highly toxic pyrethroids. Some of these pyrethroids like the 4-phenoxyphenyl JHAs (fenoxycarb and pyriproxyfen), showed high JH activity and immediate insecticidal activity. However, it was difficult to determine whether the insecticidal activities occurred due to hormonal activity (JHA activity) or because of direct neurotoxicity from the pyrethroids. Loreclezole hydrochloride (JHA) and K21877 are 1,2,4-Trizole derivatives, which are JHAs that are different in structure. Because they showed high hormonal activities like conventional JHAs and high and immediate mosquito larvicidal activities like 4-phenoxyphenyl JHAs, they have advantages such as a novel mode of action, high hormonal activities and effective mosquitocidal activities

In conclusion, we identified novel JH-related IGR insecticides from loreclezole hydrochloride derivatives. Loreclezole hydrochloride and K21877 demonstrated high levels of JHA and JHAN activity *in vitro*, respectively. They also displayed high larvicidal activities, embryonic lethality and adult toxicity against *A. albopictus*, and were shown to modulate JH-regulated physiological functions such as expression of JH-responsive genes and reproduction. These results suggested that loreclezole hydrochloride

and K21877 could be useful for effective control of mosquitoes and provide a better understanding of the relationship between compound structure and JHA or JHAN activity.

CHAPTER Ⅲ. The transcriptional responses of Asian tiger mosquito treated with juvenile hormone-related insect growth regulators

ABSTRACT

Insect growth regulators (IGRs) are insecticides that disrupt the normal development of target insects. Among the IGR insecticides, juvenile hormone (JH)-related IGRs are of particular interest because they stimulate or interfere with the formation of JH receptor complex. In the previous chapters, novel JH-related IGRs loreclezole hydrochloride (K222212) and 1-chloro-[(Z)-2-(phenylphenyl)ethenyl]-1,2,4-triazole (K21877) with JH agonist (JHA) and JH antagonist (JHAN) activity were identified by using yeast two-hybrid system transformed with the *Aedes aegypti* JH receptor complex, respectively. In this chapter, the transcriptomic responses of *Aedes albopictus* were investigated upon loreclezole hydrochloride and K21877 treatment, respectively. Loreclezole hydrochloride and K21877 were applied topically onto abdomen of *A. albopictus* female adults and each total RNA sample was extracted for RNA-seq by using Illumina sequencing. To identify the genes which altered their transcription level as a response to JHA and JHAN, the short reads of each samples were mapped to the transcripts of *A. aegypti* and then subjected to gene ontology (GO) enrichment and the Kyoto encyclopedia of genes and genomes (KEGG) pathway analysis. Total of 53 GO terms and the genes related to translation

pathway were significantly enriched by loreclezole hydrochloride and depleted by K21877 treatment. These results will provide important information about understanding of impact of JH-related IGRs in transcription level.

INTRODUCTION

The 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. JHs play key roles in various physiological functions including development and reproduction of insect. Although JHs are very important for insect physiology, their regulatory mechanisms have remained elusive (Riddiford, 2008).

JH III is found to be the predominant JH in mosquito (Baker et al., 1983), and plays various roles in regulating larvae and egg development, reproduction, vitellogenesis, immune responses, lipid metabolism, diapause, and emergence (Clements, 1992; Raikhel et al., 2005; Spielman, 1974; Suman et al., 2015; Van Ekert et al., 2014; Wang et al., 2017a; Wang et al., 2017b). Because of these important roles of JH in mosquito, JH-related insect growth regulators (IGRs) fatally affect the physiological regulation in mosquito and are effective for control of the targets (Lee et al., 2015; Slama, 1971).

However, although JH is physiologically very important in mosquito and JH-related IGRs are effective for pest control, little is known about the transcriptional responses of the insects treated with the compound modulating JH. In the previous chapter, it was demonstrated that the novel JH-related IGRs loreclezole hydrochloride (K222212) and its derivative, 1-chloro-[(Z)-2-(phenylphenyl)ethenyl]-1,2,4-triazole (K21877) with JH agonist (JHA) and JH antagonist (JHAN) activity, respectively, were identified and characterized by using *in vitro* yeast two-hybrid *β*-galactosidase ligand binding assay and

bioassay against *A. albopictus*. Because similarity of molecular structure between K22212 and K21877, these compounds are suitable to investigate the transcriptional responses of mosquito treated with JHA and JHAN.

In this chapter, to investigate the transcriptional responses of JHA and JHAN in the mosquitoes, the two compounds showing similar chemical structure, loreclezole hydrochloride and its derivatives, K21877 with JHA activity and JHAN activity, respectively, were treated to mosquitoes and their transcriptome was analyzed by RNA-seq. Through the analysis of differentially expressed genes and enrichment based on the association of Gene Ontology (GO) terms and Kyoto Encyclopedia of Genes and Genomes (KEGG) database, the transcriptional responses of mosquito treated with JHA and JHAN were identified and the JHA and JHAN-responsive genes were verified by qPCR.

MATERIALS AND METHODS

1. Chemical treatment and RNA extraction

Half-microgram of loreclezole hydrochloride and K21877 in 0.5 μl of acetone was applied topically onto the abdomen of the female adults of *A. albopictus* within 1 h after eclosion, respectively. Total RNA from control and treated mosquitoes were isolated by using Qiazol lysis reagent (Qiagen, Hilden, Germany) according to the manufacturer's protocol at 8 h post treatment. Ten of chemical treated individuals were pooled and homogenized in 1 ml of Qiazol reagent, and added 0.2 ml of chloroform. After vortexing, the samples were centrifuged at 12,000 g, the supernatant was transferred to a new tube and added 0.5 ml of isopropanol for RNA precipitation. The RNA was washed with 75% ethanol and resolved in nuclease-free water, and stored -80℃ for qPCR and sequencing.

2. Illunmina sequencing

To investigate the impact of JHA and JHAN in molecular level, mRNA expression profiling of *A. albopictus* treated with loreclezole hydrochloride and K21877 was analyzed using the Illumina sequencing. The cDNA libraries from total RNA was constructed using TruSeq RNA sample preparation kit (Illumina, San Diego, USA).

Firstly, total mRNA were purified using poly-T oligo attached magnetic beads followed by fragmentation. Single strand cDNA were made from the cleaved fragments using reverse transcriptase with random primers. After then, the single strand cDNA were ligated sequencing primers to second strand cDNA synthesis, and enriched with PCR to

create the final cDNA library. The cDNA library of 250-300bp insert was subjected to Illumina HiSeq 2000 sequencing platform for RNA-seq to obtain 101 bases of paired-end sequences.

3. Sequence analysis

The short reads of each sample were mapped to the reference gene set version 3.4 of *Aedes aegypti* (https://www.vectorbase.org/download/aedes-aegypti-liverpooltranscriptsaaegl34fagz, vectorbase) by using Kallisto program (Bray et al., 2016) to compare the differential expression of each gene between control and treated samples.

For gene ontology (GO) analysis, the cDNA sequences were searched by Blast2GO (Conesa et al., 2005). Blast2GO assigned the GO annotation to the mapped gene. Then these GO annotated sequences were performed enrichment analysis by g:GOSt program (Reimand et al., 2007) for statistical approached to identify significantly enriched or depleted groups of genes. Because evidence codes of g:GOSt program not only used GO terms but also Kyoto Encyclopedia of Genes and Genomes (KEGG) database, the mapped genes were analyzed by using g:GOSt against KEGG database to determine the over-representation of biological pathway. This multiple testing was corrected by using the false discovery rate (FDR < 0.05). In addition, immune-related genes were annotated by using ImmunoDB (http://cegg.unige.ch/Insecta /immunodb) dataset.

4. Validation of the sequencing results by qPCR

To validate the RNA-seq data, the expression levels of genes with varying expression pattern by treatment of loreclezole hydrochloride and K21877 were calculated by qPCR,

and compared with those of Illumina sequencing results in TPM value. qPCR was conducted by using ReverTra Ace® qPCR RT Kit (TOYOBO, Osaka, Japan), THUNDERBIRD® SYBR® qPCR Mix (TOYOBO, Osaka, Japan), and CFX96TM Real-Time PCR system (Bio-Rad, Hercules, CA, USA) according to the manufacturers' instructions. The amplification condition of qPCR was 95℃ for 60 sec (pre-denaturation), followed by 40 cycles of 95℃ for 15 sec (denaturation) and 60℃ for 60 sec (extension). The 40S ribosomal protein S7 (RPS7) was used as a reference gene for the calculation of fold change. The relative transcription level (RTL) was calculated by using $2^{-\Delta Ct}$ method (Pfaffl, 2001). The qPCR primer sequences of the genes are described in Table 7.

5. Screening of potential target genes for RNAi mediated mosquito control

To control mosquitoes by using the data from analysis of transcriptome, genes that were essential and down-regulated by both loreclezole hydrochloride and K21877 treatment were selected for RNAi target. The expression levels of these genes were identified using qPCR.

Table 7. Oligonucleotides sequences used for quantitative real-time PCR.

Seq name[a]	Primer name	Primer sequence (5' → 3')	Annotation	Reference number
AAEL000321-RA	AAEL321_F	GGAAGCGTGGATGTGGTACT	acetyl-coenzyme A synthetase	K01895[b]
	AAEL321_R	ATGGGAAGGAAGCTGATCCT		
AAEL000495-RA	AAEL495_F	ACTCCGCGTGTGAGAAAGTT	glutathione peroxidase	5578481[c]
	AAEL495_R	TGCGAGCATGGAAACAGTAG		
AAEL000621-RA	AAEL621_F	AAGCTGTTCGCAATCGTTCT	cecropin	K20696[b]
	AAEL621_R	GAAGACTCGTTTGCCGACTC		
AAEL001506-RA	AAEL1506_F	GCAGGAGAAGCTACTGAAGA	U3 small nucleolar ribonucleoprotein protein mpp 10	K14559[b]
	AAEL1506_R	ACTGACTTCATCCTGCAACT		
AAEL002185-RA	AAEL2185_F	TGTTTCCCCCACTTTGGATA	adult cuticle protein 1	5573903[c]
	AAEL2185_R	AGATGACTGCAGCGATGATG		
AAEL002191-RA	AAEL2191_F	TGGCCTATTCCGCTTATCAC	adult cuticle protein 1	5573913[c]
	AAEL2191_R	ACAACGGATGACTGGTAGGC		
AAEL003396-RA	AAEL3396_F	CTTATAAGCCCAAAATCGTC	60S ribosomal protein L32	K02912[b]
	AAEL3396_R	TCGGTTATCAATACCTTTCG		
AAEL003857-RA	AAEL3857_F	TTGCACAAACTCGTTCAAGC	defensin	5579095[c]
	AAEL3857_R	TGCACATAGCCAGGAAACAA		
AAEL004042-RB	AAEL4042_F	CCCTGGATTGCAAGGATAAA	probable glucosamine 6-phosphate N-acetyltransferase	K00621[b]
	AAEL4042_R	AGGGAGTTGCTTTGCTCGTA		
AAEL005416-RA	AAEL5416_F	CCCTGCAACCTCACGTATTT	oxidase/peroxidase	XP_021693132.1[d]
	AAEL5416_R	GTCCATGGTTCAAGGTCGAT		
AAEL006798-RA	AAEL6798_F	TACGATGACGGAAAGGGAAC	cytochrome p450 CYP9J10 v1	K15003[b]
	AAEL6798_R	CAAACTTTTCCGGGTTTTCA		
AAEL007653-RA	AAEL7653_F	CCGTACATGGATCGGAAGTT	allantoinase	K01466[b]
	AAEL7653_R	TTCTGCCACCCTTCTTCATC		
AAEL007697-RA	AAEL7697_F	CTGGAAAAGCTAGGTCAAAA	rRNA-processing protein FCF1	K14566[b]
	AAEL7697_R	GGTGGCCACAATATAACACT		
AAEL007745-RA	AAEL7745_F	CACCGGGACAAACTGAAACT	blood vessel epicardial	K21108[b]
	AAEL7745_R	TCACCTGCATCAGCTTCTTG		
AAEL012001-RA	AAEL12001_F	CCCCCTGCACATCAGTATTC	*Anopheles gambiae* PEST galectin	1275208[c]
	AAEL12001_R	CGAAGTAGGATCGCTTCTGG		
AAEL012185-RA	AAEL12185_F	GAAACGGAACATGGAAATAG	ribosome biogenesis regulatory protein	K14852[b]
	AAEL12185_R	ACCCTTACCTTTCTTCTTGC		
AAEL013139-RG	AAEL13139_F	CAGAAACGATCCTCACACGA	cytokinesis actomyosin contractile ring assembly	K04513[b]
	AAEL13139_R	TAGGCGAAAGCGTTGATCTT		
AAEL013789-RA	AAEL13789_F	CACCATGAGGCTCACTACGA	cuticle protein 5	5598638[c]
	AAEL13789_R	ACTCCGTTGTGGATCTTTGG		
AAEL014506-RA	AAEL14506_F	GTTGGAGCCAGTTGTAAAAG	Midasin	K14572[b]
	AAEL14506_R	TCCAATGCTTTAATGGAATC		
AAEL014607-RA	AAEL14607_F	ACGATCGATAAGGGAACGTG	cytochrome p450 CYP9J27 v2	K15003[b]
	AAEL14607_R	GTTGATGTCCCGCTTGTTTT		

[a] Transcript ID of VectorBase (geneset *Aedes aegypti* AaegL3.4 transcripts).

[b] K number of KEGG Orthology

[c] Genbank gene ID

[d] Number of NCBI Reference sequence

RESULTS

1. Overview of the Illumina paired-end sequencing

The loreclezole hydrochloride, K21877 and solvent treated female adults were collected at 8 h post treatment. The total RNA samples were isolated, and subjected to Illumina HiSeq 2000 sequencer to obtain 60,524,990, 70,538,016 and 60,553,046 of raw reads, respectively. The detailed Illumina sequencing results of each sample are summarized in Table 8.

2. Mapping of the short reads to the reference gene set of *A. aegypti* and analysis of differentially expressed genes

For characterization each transcriptome and analysis of differentially expressed genes upon IGR treatments, total raw reads were mapped to the reference gene set of *A. aegypti* which contained 13,817 transcripts by the Kallisto program to obtain the level of gene expression to TPM (transcripts per million) value. Total of 9,585 *A. aegypti* genes were mapped (Fig. 22), and analysis of differentially expressed gene upon IGR treatment showed 544 and 277 of up-regulated, and 418 and 675 of down-regulated over two-fold in the loreclezole hydrochloride and K21877 treated samples, respectively. Also, 175 of up-regulated over two-fold, and 158 of down-regulated over two-fold in both IGR treatments were identified (Fig. 23).

Table 8. Summary of the Illumina sequencing.

	Control	K222212	K21877
Total paired-end sequence read bases (bp)	6,113,023,990	7,124,339,616	6,115,857,646
Total paired-end sequence reads	60,524,990	70,538,016	60,553,046
GC% of paired-end sequence reads	49.124	48.730	48.585
Q20% of paired-end sequence reads	97.031	97.017	97.105
Q30% of paired-end sequence reads	94.880	95.022	94.986

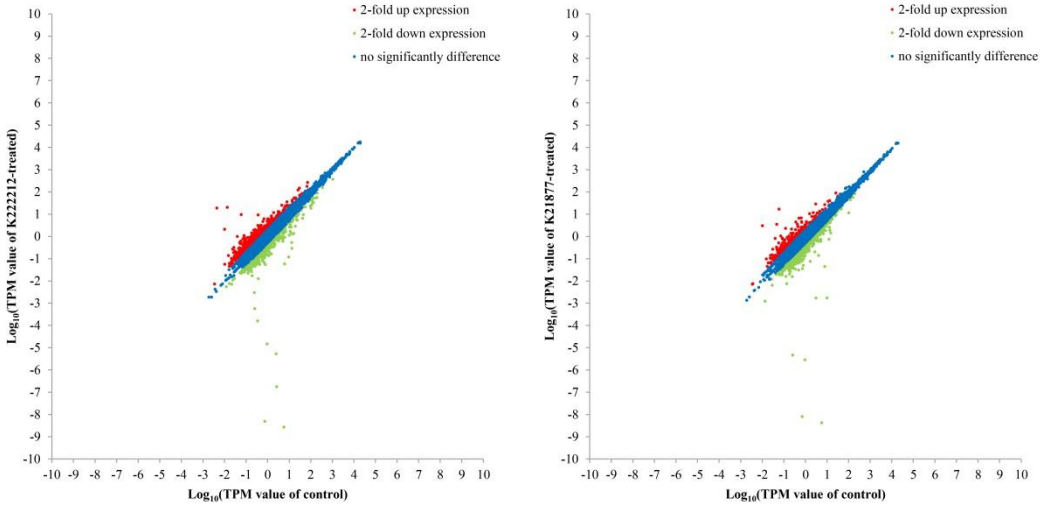


Figure 22. Analysis of differentially expressed genes upon IGR-treatments. All genes were mapped to the reference sequence of *A. aegypti*. The X-axis represents control log10 value of expression and Y-axis represents K222212 (left) and K21877 (right) log10 value of expression, respectively. Red (up) and green (down) dots denote that the expression was 2-fold difference.

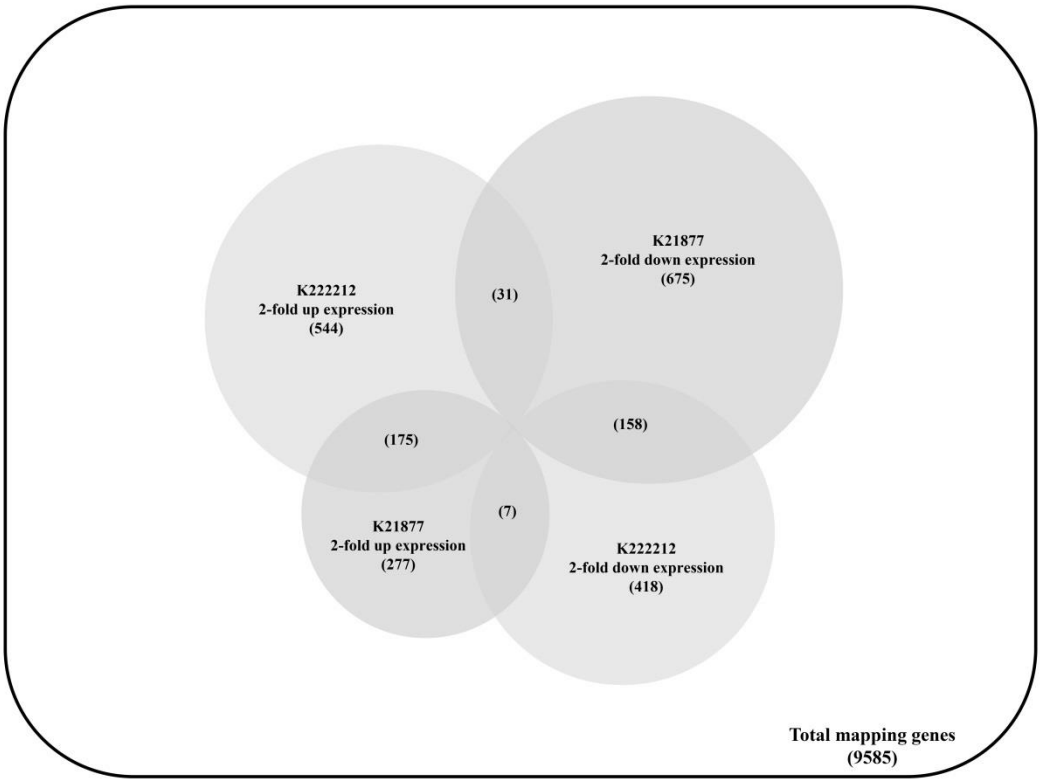


Figure 23. The diagram of analysis of differentially expressed genes upon IGR treatments. The number in bracket represent the number of genes.

3. Gene ontology enrichment analysis

For gene ontology annotation, total of 9,585 *A. aegypti* genes which were mapped with the Illumina short reads were categorized based on GO annotation terms using Blast2GO software. Total of 8,069 genes were categorized into GO level 2 which included Biological Process (GO:0008150), Cellular Component (GO:0005575) and Molecular Function (GO:0003674) (Fig. 24). Immune-related genes were annotated using *A. aegypti* immune-related gene dataset in ImmunoDB. Total of 275 genes were categorized into 27 immune-related gene families (Fig. 25).

The expression level of each gene annotated in terms of GO level 3 was influenced by treatment of loreclezole hydrochloride and K21877. Overall, the gene expression patterns in GO level 3 were enriched by loreclezole hydrochloride and depleted by K21877 treated (Fig. 26, 27, and 28).

For statistical analysis, the genes mapped with the Illumina short reads were subjected to GO enrichment analysis by g:GOSt program. To account for multiple testing, *p*-values were calculated by cumulative hypergeometric probability and adjusted by the Benjamini and Hochberg method. A false discovery rate (FDR) < 0.05 was set as the cutoff value. These results also were categorized into GO terms Biological Process, Cellular Component, and Molecular Function (Fig. 29).

In Biological Process, 35 GO terms were significantly enriched by loreclezole hydrochloride and depleted by K21877 treatment; Cellular component organization or biogenesis (GO:0071840), Biosynthetic process (GO:0009058), Cellular component organization (GO:0016043), Regulation of metabolic process (GO:0019222), Cellular metabolic process (GO:0044237), Nucleobase-containing compound metabolic process

(GO:0006139), Cellular aromatic compound metabolic process (GO:0006725), Regulation of biosynthetic process (GO:0009889), Regulation of cellular metabolic process (GO:0031323), Cellular nitrogen compound metabolic process (GO:0034641), Cellular biosynthetic process (GO:0044249), Cellular macromolecule metabolic process (GO:0044260), Heterocycle metabolic process (GO:0046483), Regulation of nitrogen compound metabolic process (GO:0051171), Regulation of macromolecule metabolic process (GO:0060255), Regulation of primary metabolic process (GO:0080090), Organic cyclic compound metabolic process (GO:1901360), Organic substance biosynthetic process (GO:1901576), Macromolecule biosynthetic process (GO:0009059), Gene expression (GO:0010467), Regulation of gene expression (GO:0010556), Regulation of macromolecule biosynthetic process (GO:0010556), RNA metabolic process (GO:0016070), Regulation of nucleobase-containing compound metabolic process (GO:0019219), Regulation of cellular biosynthetic process (GO:0031326), Cellular macromolecule biosynthetic process (GO:0034645), Cellular protein metabolic process (GO:0044267), Cellular nitrogen compound biosynthetic process (GO:0044271), Nucleic acid metabolic process (GO:0090304), Regulation of cellular macromolecule biosynthetic process (GO:2000112), Regulation of RNA biosynthetic process (GO:2001141), Regulation of nucleic acid-templated transcription (GO:1903506), Regulation of transcription, DNA-templated (GO:0006355), Intracellular signal transduction (GO:0035556) and Nitrogen compound transport (GO:0071705). Also, nine GO terms were significantly depleted by both loreclezole hydrochloride and K21877; Signaling (GO:0023052), Cellular response to stimulus (GO:0051716), Single-organism cellular process (GO:0044763), Single organism signaling (GO:0044700), Signal transduction

(GO:0007165), Cell communication (GO:0007154), Macromolecule modification (GO:0043412), Protein modification process (GO:0036211) and Cellular protein modification process (GO:0006464). Total of six GO terms were significantly enriched by loreclezole hydrochloride and depleted by K21877 treatment in Cellular Component; Cell (GO:0005623), Macromolecular complex (GO:0032991), Protein complex (GO:0043234), Intracellular (GO:0005622), Intracellular part (GO:0044424) and Cytoplasm (GO:0005737). In Molecular Function group, 12 GO terms were significantly enriched by loreclezole hydrochloride and depleted by K21877 treated; Carbohydrate derivative binding (GO:0097367), Ribonucleotide binding (GO:0032553), Purine ribonucleoside triphosphate binding (GO:0035639), Purine ribonucleotide binding (GO:0032555), Purine nucleotide binding (GO:0017076), ATP binding (GO:0005524), Adenyl nucleotide binding (GO:0030554), Adenyl ribonucleotide binding (GO:0032559), Hydrolase activity, acting on acid anhydrides (GO:0016817), Hydrolase activity, acting on acid anhydrides, in phosphorus-containing anhydrides (GO:0016818), Pyrophosphatase activity (GO:0016462) and Nucleoside-triphosphatase activity (GO:0017111). Most of significantly changed GO terms were up-regulated by loreclezole hydrochloride and down-regulated by K21877. The other GO terms were significantly affected independently by loreclezole hydrochloride and K21877, respectively.

Especially, low level GO terms of Regulation of metabolic process term in Biological process terms such as Regulation of macromolecule metabolic, Regulation of nitrogen compound, Regulation of primary metabolic, Regulation of cellular metabolic, Regulation of biosynthetic process were regulated by JHA and JHAN. These results supported the roles of JH as regulations of physiological functions. In Molecular function,

low level GO terms of Nucleotide binding (GO:0000166) terms, including Purine nucleotide binding, Ribonucleotide binding, Adenyl nucleotide binding, Purine ribonucleotide binding, and Adenyl ribonucleotide binding, and its related to the GO terms were up- and down-regulated by loreclezole hydrochloride and K21877, respectively. Among GO level 3 terms in Cellular component, Cell and Macromolecular complex terms were up- and down-regulated by loreclezole hydrochloride and K21877, respectively. These results of analysis suggested that the genes related to Cell and its low level GO terms are regulated by JH in Cellular component. However, other GO level 3 terms in Cellular component such as Organelle (GO:0043226) and Membrane (GO:0016020) did not show significant difference.

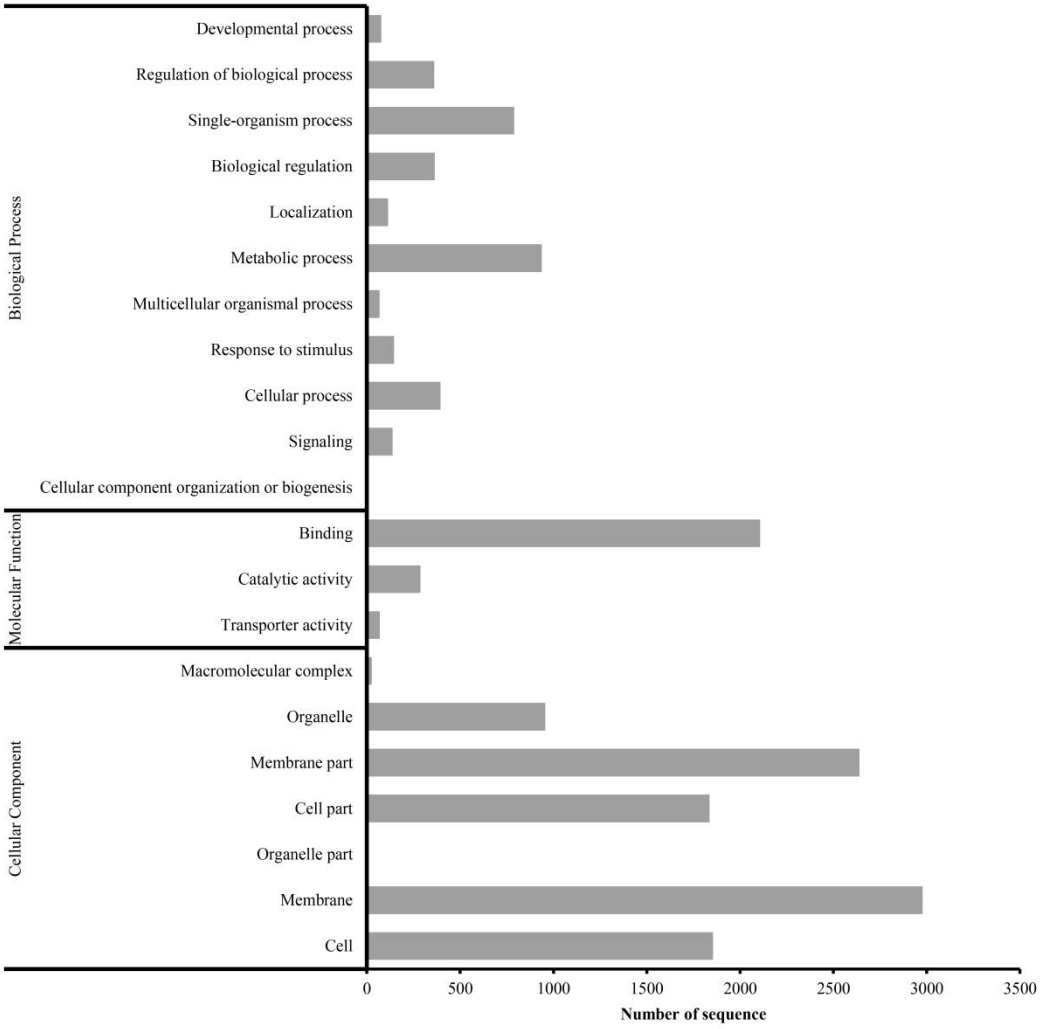


Figure 24. Gene ontology classification of *A. albopictus* cDNA library. Total of 8,069 genes were categorized into functional GO terms.

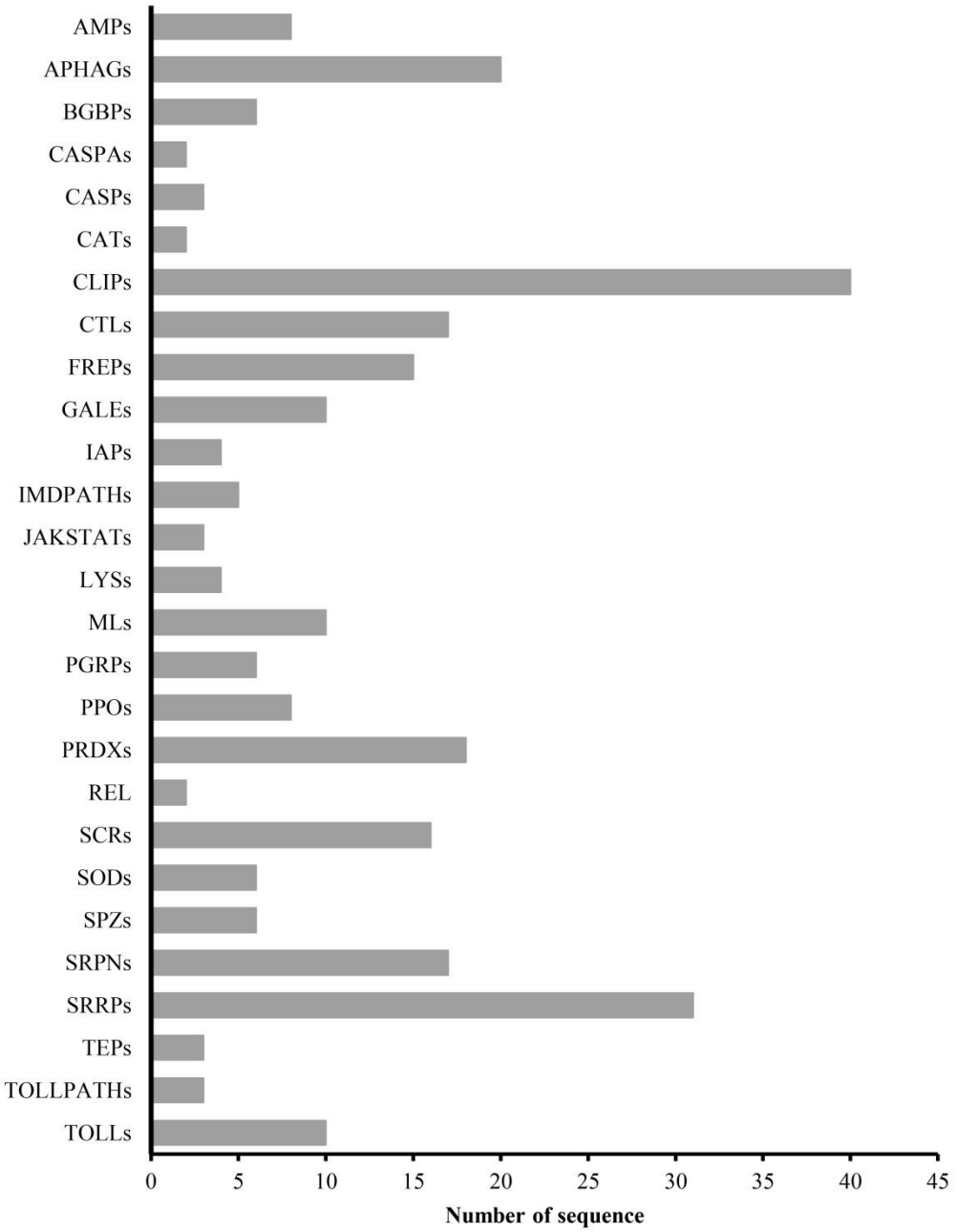


Figure 25. Gene ontology classification of immune-related genes. Total of 275 genes were categorized into 27 immune-related gene families.

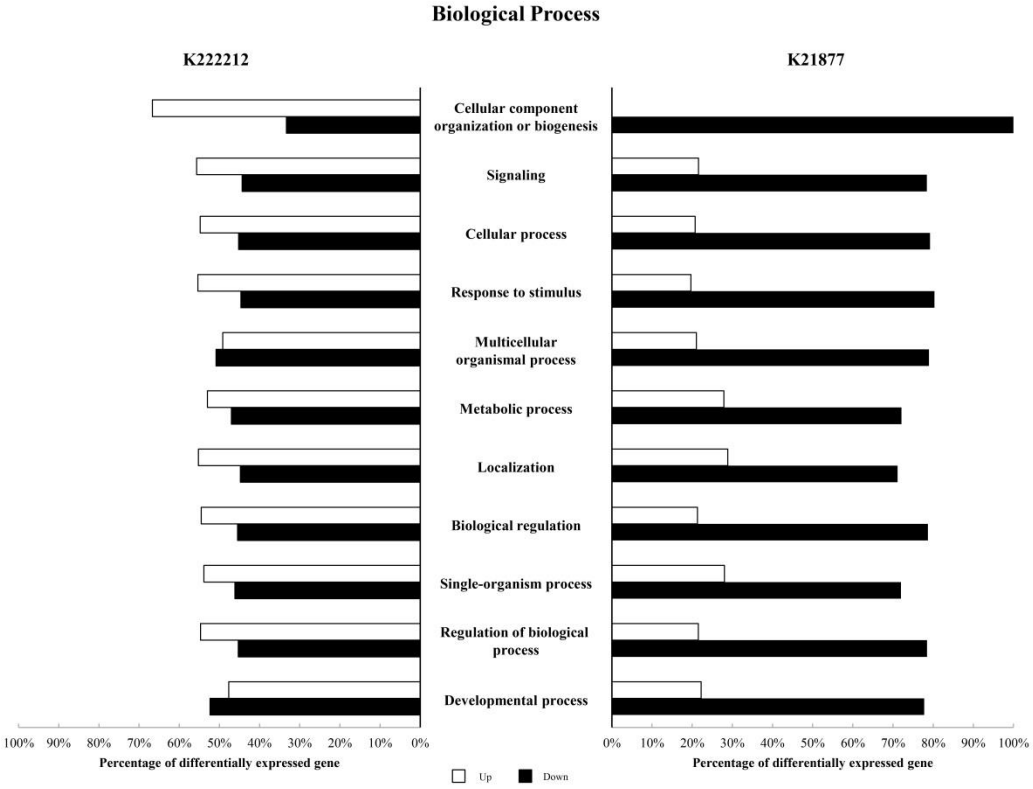


Figure 26. Analysis of differentially expressed genes upon IGR-treatments within Biological process GO term. White bar represents the percentage of up-regulated genes and black bar presents the percentage of down-regulated genes by corresponding compounds.

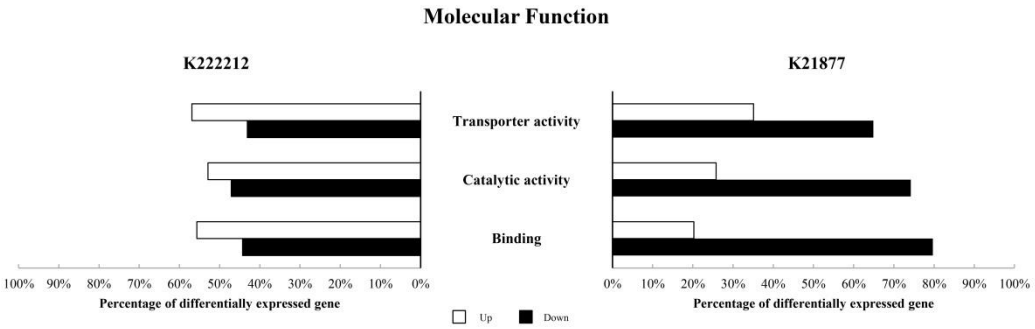


Figure 27. Analysis of differentially expressed genes upon IGR-treatments within Molecular function GO term. White bar represents the percentage of up-regulated genes and black bar presents the percentage of down-regulated genes by corresponding compounds.

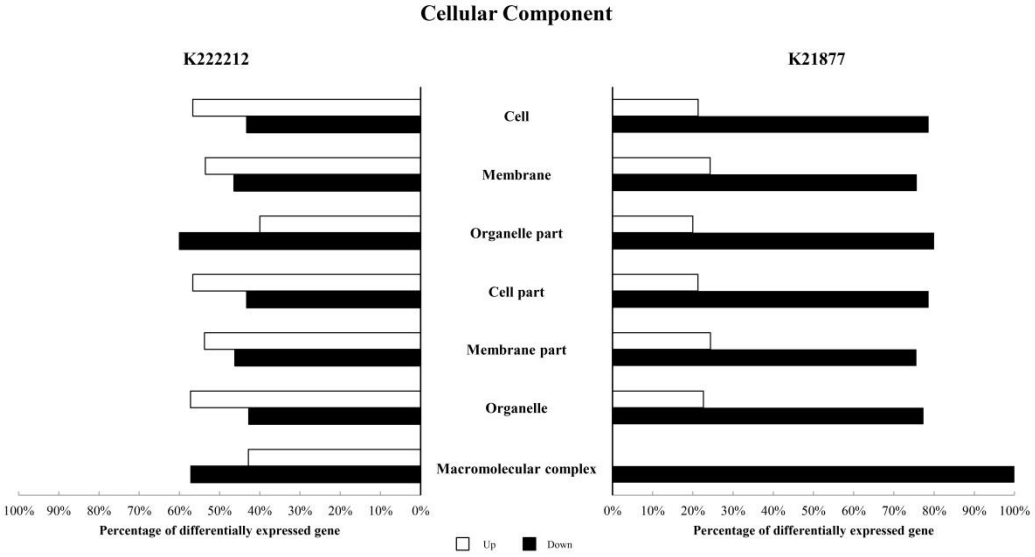


Figure 28. Analysis of differentially expressed genes upon IGR-treatments within Cellular component GO term. White bar represents the percentage of up-regulated genes and black bar presents the percentage of down-regulated genes by corresponding compounds.

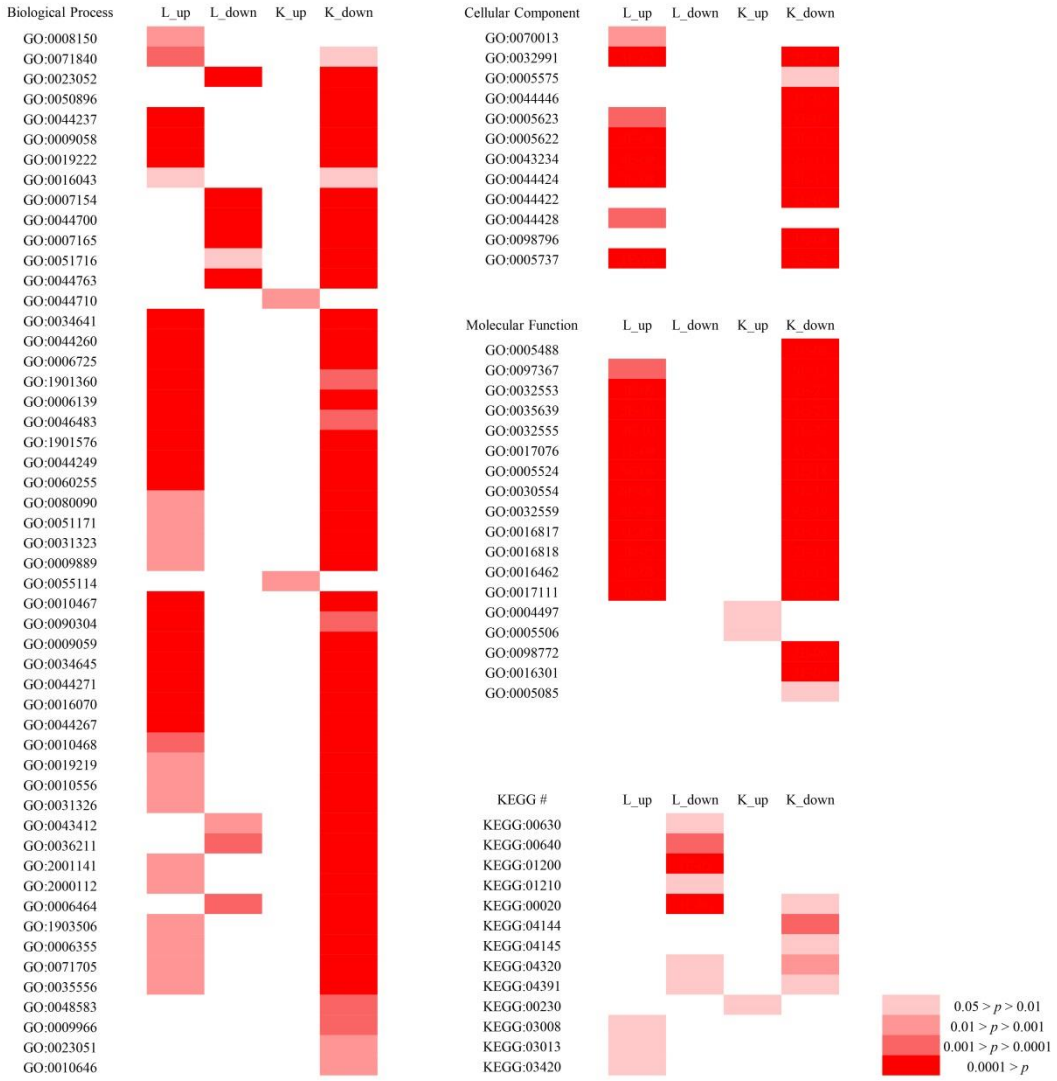


Figure 29. Gene ontology enrichment analysis of differentially expressed genes upon IGR-treatments. These results were categorized into the three main GO terms (Biological Process, Cellular Component, and Molecular Function) and KEGG reference pathway. L_up and K_up represent the up-regulated genes by K222212 (L) and K21877 (K), respectively. L_down and K_down also represent the down-regulated genes by K222212 (L) and K21877 (K), respectively.

4. Analysis of differentially expressed genes related detoxification

In *A. aegypti*, detoxification genes differentially expressed in pyrethroids resistant population relative to the susceptible strain were reported (Bariami et al., 2012). Among detoxification genes in response to pyrethroids, total of 17 and 7 genes were up- and down-regulated by loreclezole hydrochloride and K21877 treatment, respectively (Fig. 30 and 31). To validate the analysis of differentially expressed genes related detoxification from the RNA-seq data, the expression level of two genes, AAEL014607-RA and AAEL006798-RA, were selected for qPCR analysis (Fig. 32). The qPCR result demonstrated that the transcription level of genes related to detoxification were in good accordance with the sequence analysis result.

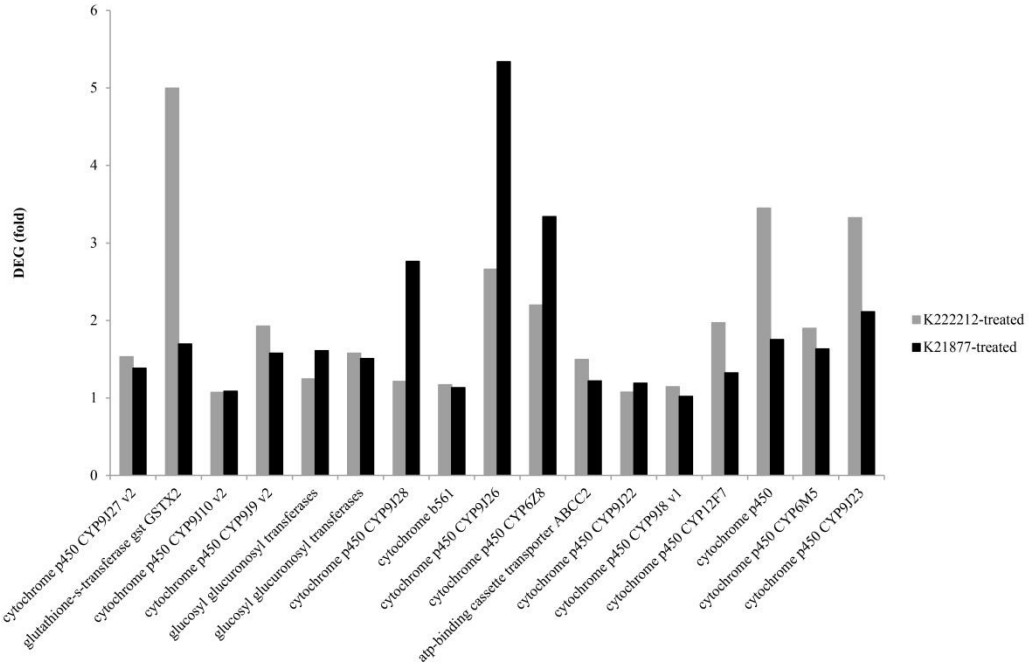


Figure 30. Analysis of differentially expressed genes related detoxification upon IGR-treatment. Total of 17 genes were up-regulated by K222212 and K21877 treatment.

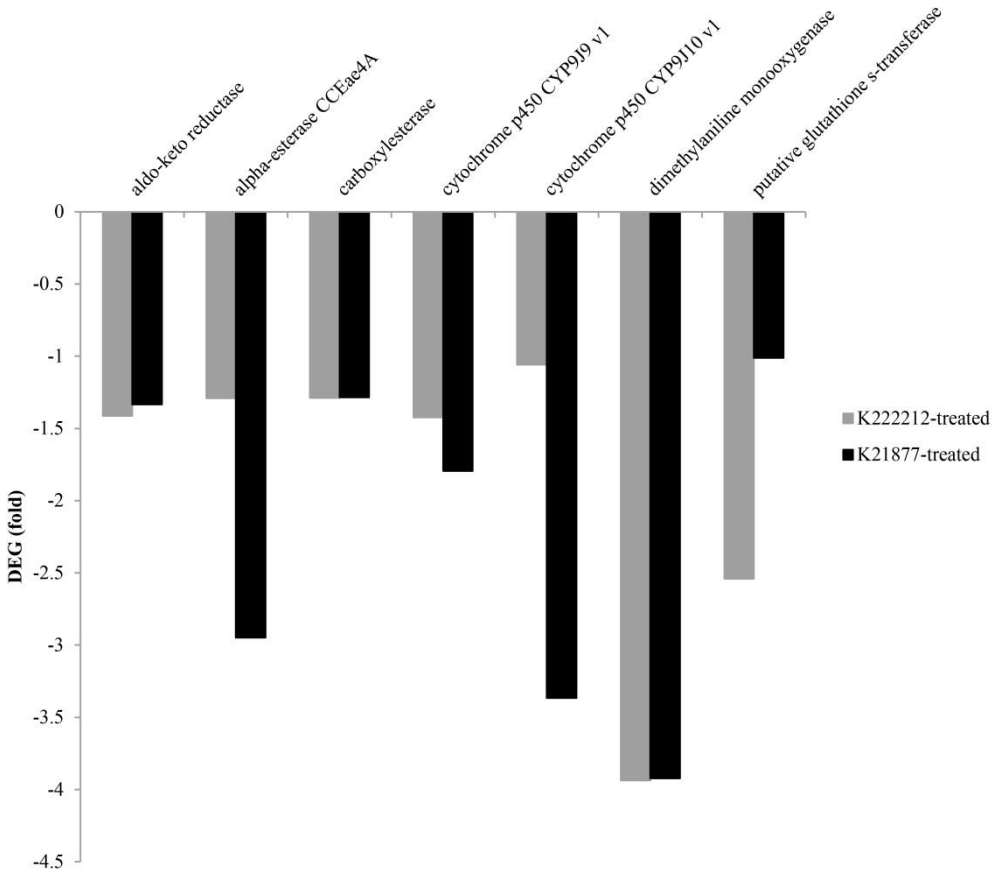


Figure 31. Analysis of differentially expressed genes related detoxification upon IGR-treatment. Total of 7 genes were down-regulated by K222212 and K21877 treatment.

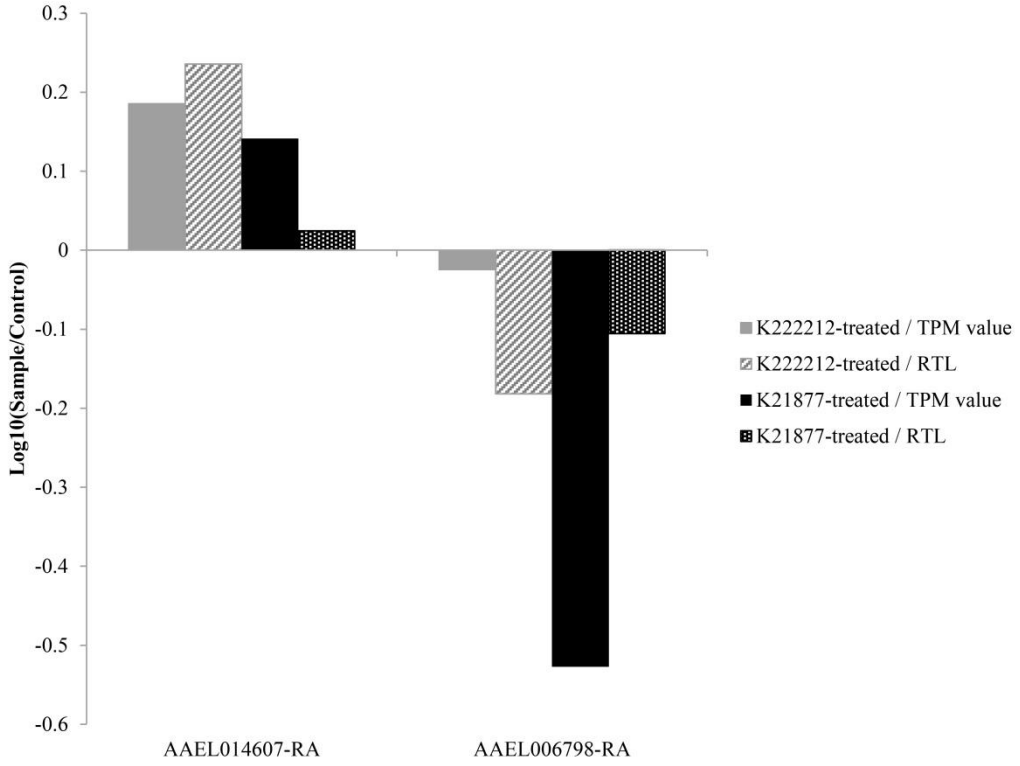


Figure 32. Validation of the Illumina sequencing result of detoxification-related genes by qPCR. The relative transcription levels were demonstrated in log10 scale of IGR-treated to control sample.

5. Screening for potential target genes for RNAi mediated mosquito control by qPCR

To control mosquitoes by using the data from analysis of transcriptome, total of nine potential RNAi targets genes were selected because these genes showed depleted by both loreclezole hydrochloride and K21877 treatment. To validate the analysis of differentially expressed genes related potential RNAi targets from the RNA-seq data, the expression level of these genes were calculated by qPCR (Fig. 33 and 34). As shown in the Figures, the qPCR results confirmed the RNA-seq data, with all analyzed genes down-regulated by loreclezole hydrochloride and K21877 treated.

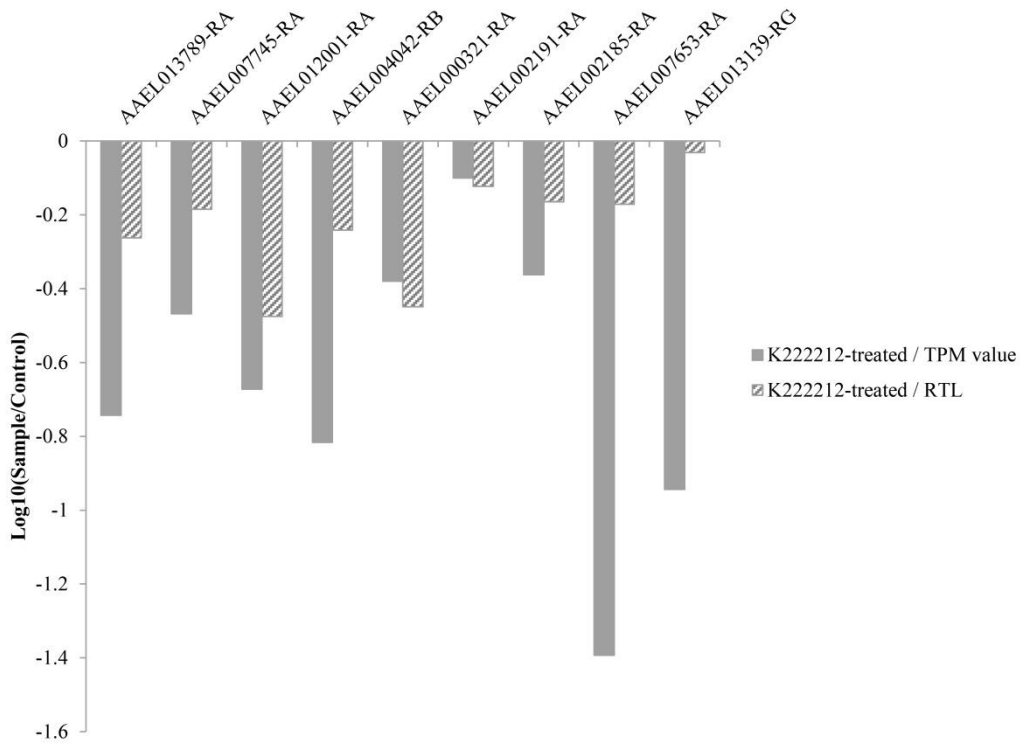


Figure 33. Validation of the Illumina sequencing result of the potential target genes for RNAi by qPCR. The relative transcription levels were demonstrated in log10 scale of K222212-treated to control sample.

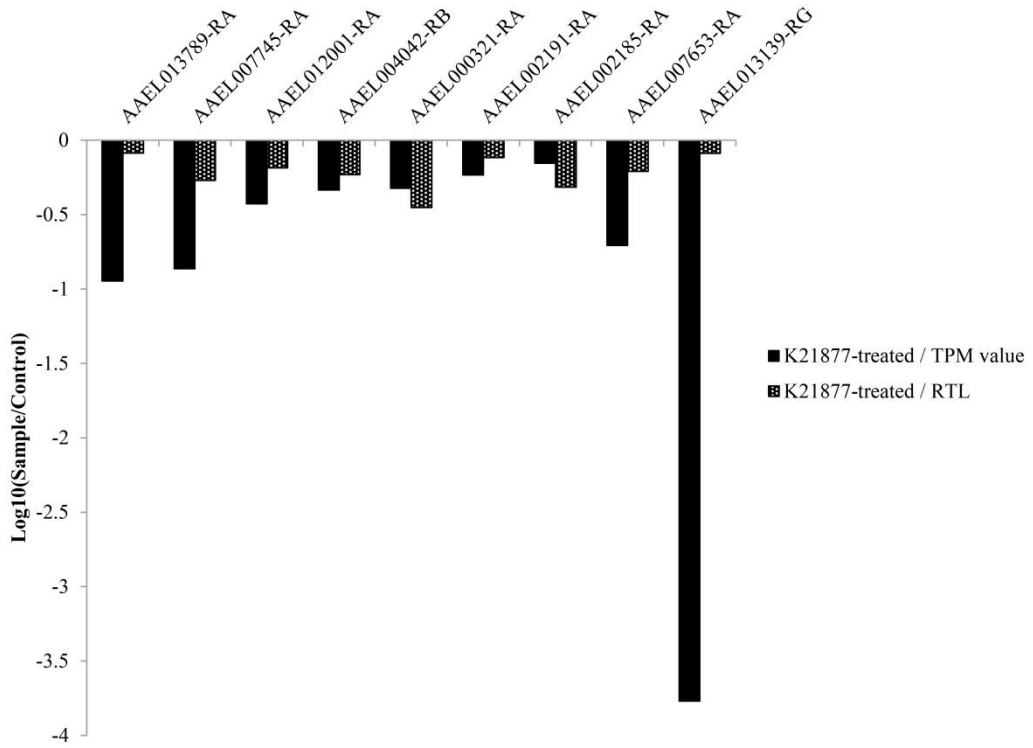


Figure 34. Validation of the Illumina sequencing result of the potential target genes for RNAi by qPCR.
The relative transcription levels were demonstrated in log10 scale of K21877-treated to control sample.

DISCUSSION

Technological advancement of sequencing could have allowed large-scale comparative studies to be performed that were unimaginable in the past. Sequencing technologies are various methods that are grouped broadly as template preparation, sequencing and imaging, and data analysis. The variety of sequencing platforms is produced by the combination of sequencing methods (Metzker, 2010; Simon et al., 2009). In this chapter, the transcript level responses of *A. albopictus* treated with JHA or JHAN were analyzed by Illumina sequencing and bioinformatics methods because the Illumina sequencing is one of most widely used.

Although the transcripts of *A. albopictus* are available, the transcripts of *A. aegypti* are used by the reference gene sequences to analyze the response to JH-related IGRs in *A. albopictus* because not only genomics and transcriptomics studies, but also endocrinological studies including JH are better studied in *A. aegypti* than that of *A. albopictus*.

In 1996, the genome of *Saccharomyces cerevisiae* was completely sequenced. After then, the complete genomic sequences of higher model organism including human were announced. The comparison between complete eukaryotic genomes revealed that biological roles of numerous genes and proteins could be inferred from their similarity to their putative orthologues. This high degree of sequence and functional conservation among eukaryotes has led to unification of biological annotations. The Gene Ontology (GO) Consortium was established to maintain and develop a controlled and organism-

independent vocabulary for describing the roles of genes and gene products. GO maintains vocabularies of three domains, Biological process (GO:0008150, BP or P), Molecular function (GO:0008639, MF or F), and Cellular component (GO:0005575, CC or C). These three categories of GO represents information sets that are common to all eukaryotes. Then, each vocabulary is no parent-child relations and has one root term (Ashburner et al., 2000). This GO analysis has become a commonly used approach for functional studies enormous set of data such as genomic and transcriptomic data. Although the genes affected by JHA or JHAN treatment were identified by analysis through GO annotation, however, GO analysis does not provide any statistical significance to over/under-represented GO terms between groups. Therefore, the RNA-seq data which mapped to the transcripts of *A. aegypti* were performed GO enrichment analysis in order to identify GO terms affected by JHA or JHAN treatments. The GO enrichment analysis showed that 35, 6, and 12 GO terms were significantly enriched by loreclezole hydrochloride and depleted by K21877 treated in Biological process, Molecular function, and Cellular component, respectively.

JH is highly versatile hormone and immune responses are also regulated by JH. 20-hydroxy-ecdysone (20E) is commonly known to induce expression of AMP genes, such as *diptericin* and *drosomycin*, and JH strongly interfere with 20E-dependent immune response in *Drosophila melanogaster* (Dimarcq et al., 1997; Flatt et al., 2008; Meister and Richards, 1996; Silverman et al., 2000). In contrast, several AMP genes, including *drosocin* and *defensin*, were down regulated by 20E in *EcR*-dependent manners (Beckstead et al., 2005). The conflicting reports in *Drosophila* imply that JH and 20E regulate expression of AMP genes in a complex manner. However, how these hormones

regulate the immune responses remains unclear. Especially, there is a lack of understanding about the roles of JH or JH-dependent interaction of Met in immune responses. In this study, total of 275 genes were categorized into 27 immune-related gene families. Among them, differential expressions of two AMP genes, *defensin* (AAEL003857-RA) and *cecropin* (AAEL000621-RA), were noteworthy (Fig. 35). *Defensin* was up and down-regulated by loreclezole hydrochloride (JHA) and K21877 (JHAN), respectively, while *cecropin* was regulated vice-versa. These results need to consider aspect of the spectrum of AMPs. AMPs are an important response of the humoral immunity and seven AMP families exist in *Drosophila* (Lemaitre and Hoffmann, 2007). However, only three of them were identified in mosquitoes, including defensins, cecropins and attacins. Among them, AMPs of mosquito are mainly represented by defensins and cecropins (Waterhouse et al., 2007). Generally, mosquito defensins are active against Gram-positive bacteria and cecropins have a broad spectrum of antimicrobial activity (Kokoza et al., 2010; Lowenberger et al., 1995; Lowenberger et al., 1999; Vizioli et al., 2000). Consequently, these results suggested that AMPs that have a broad-spectrum could be expressed by low JH titer in hemolymph because JH titer is generally being maintained at low level and, in contrast, expression of several AMPs could be regulated by JH against type of pathogenic bacteria.

All aerobic organisms need to respire in order to use molecular oxygen, and toxic reactive oxygen species (ROS) such as superoxide radical ($O\cdot_2^-$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($\cdot OH$) are generated during the reduction of molecular oxygen to water (Halliwell and Gutteridge, 2015). To detoxify these reactive free radicals, various defense systems are used (Hogg and Kalyanaraman, 1999). Peroxidases-related

immune response is important to detoxify ROS, and the peroxidases-related genes are regulated by JH and 20E (Tarhan et al., 2013). In this study, RNA-seq data and qPCR result showed that peroxidases-related genes, AAEL000795-RA (glutathione peroxidase) and AAEL005416-RA (oxidase/peroxidase), were up-regulated by loreclezole hydrochloride and down-regulated by K21877 (Fig. 35). In these results, expression of AMP genes and peroxidases-related genes are not only related with JH activity but also JH-dependent interaction of Met. These results demonstrated that hormonal regulation, including JH, of immune response is a complicated process.

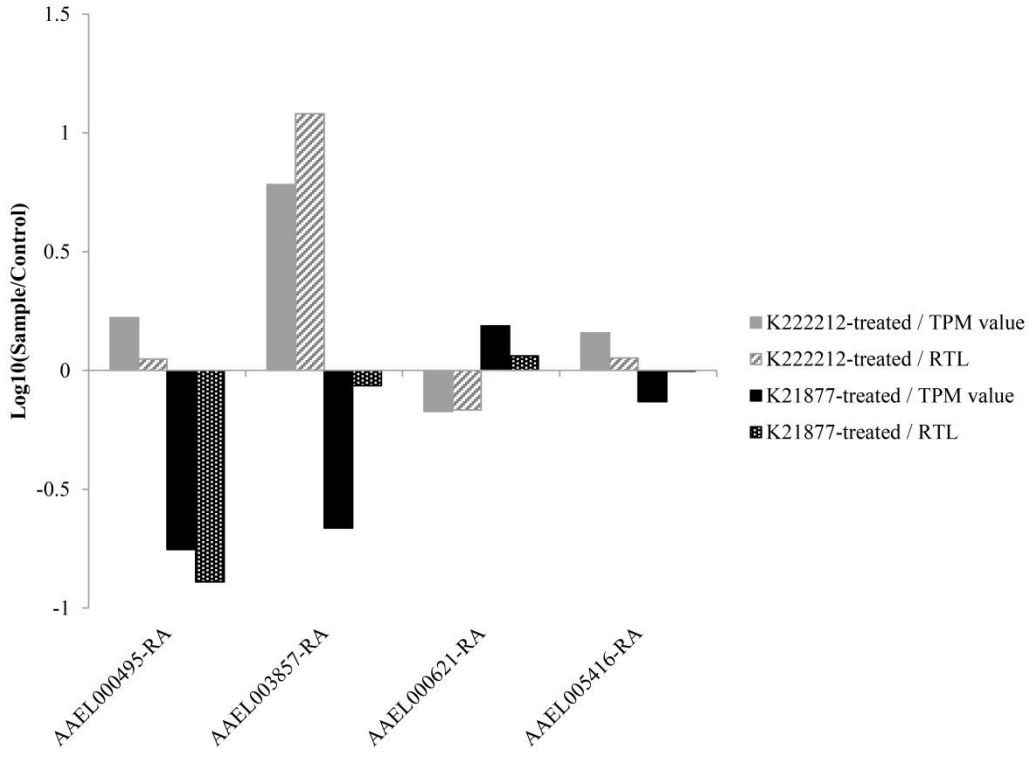


Figure 35. Validation of the Illumina sequencing result of immune-related genes by qPCR. The relative transcription levels were demonstrated in log10 scale of IGR-treated to control sample.

Among JH-regulated biological activities in insects, the role of transcription factors is most important for regulating various physiological functions. Through the regulation of genes acting downstream of JH, JH could play a key role in insects. It have been reported that JH-dependent interaction of Met promoted ribosomal biogenesis through expression of gene encoding the regulator of ribosomal synthesis 1 (RRS1) and six ribosomal protein in *A. aegypti* (Wang et al., 2017a). This result suggested that JH controls the proliferation of ribosomes to sustain an enhanced translation rate. In this study, Ribosome biogenesis in eukaryotes (KEGG reference pathway: map03008) and RNA transport (KEGG reference pathway: map03013) related genes significantly enriched by loreclezole hydrochloride -treatment. Among 90 reference genes of *A. aegypti* related to the Ribosome biogenesis in eukaryotes pathway, 55 genes were identified in the RNA-seq results. Among them, total of 32 genes (58%) were enriched and depleted by loreclezole hydrochloride and K21877 treatment, respectively (Fig. 36). And then, among 146 reference genes of *A. aegypti* related to the RNA transport pathway, 106 genes were identified in the RNA-seq results. Likewise, total of 61 genes (57%) were enriched and depleted by loreclezole hydrochloride and K21877 treatment, respectively (Fig. 37). As shown in the Fig. 38, the qPCR results were in good accordance with the RNA-seq results (Fig. 38). These results suggested that JH is critical in translation for inducing the expression of genes that related to Ribosome biogenesis in eukaryotes and RNA transport pathway, and the genes related to these pathways were enriched and depleted by loreclezole hydrochloride and K21877 treatment, respectively. The overall results of differentially expressed gene analysis based on GO annotation showed that transcription levels were enriched by JHA and JHAN compound treatment, respectively. These results

can be inferred from relationship between JH-dependent interaction of Met and the translation.

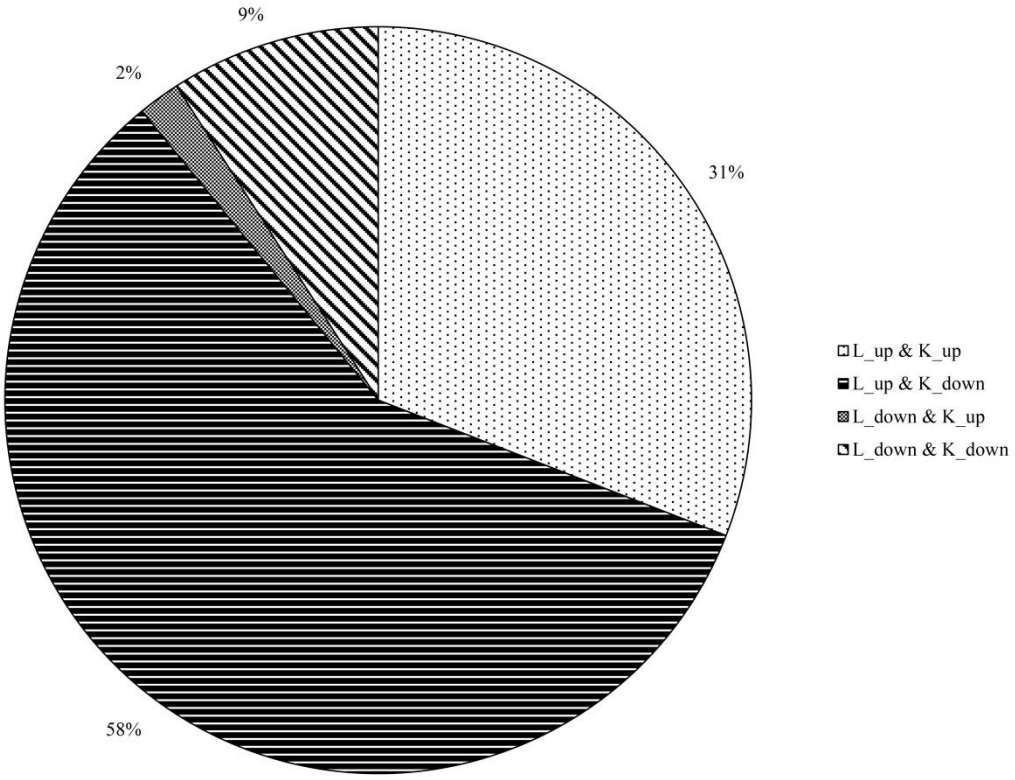


Figure 36. Analysis of differentially expressed genes related to the Ribosome biogenesis in eukaryotes pathway. 55 genes related to the pathway were assigned based on the expression pattern by treatment of K222212 and K21877. L_up and K_up represent the up-regulated genes by K222212 (L) and K21877 (K), respectively. L_down and K_down also represent the down-regulated genes by K222212 (L) and K21877 (K), respectively.

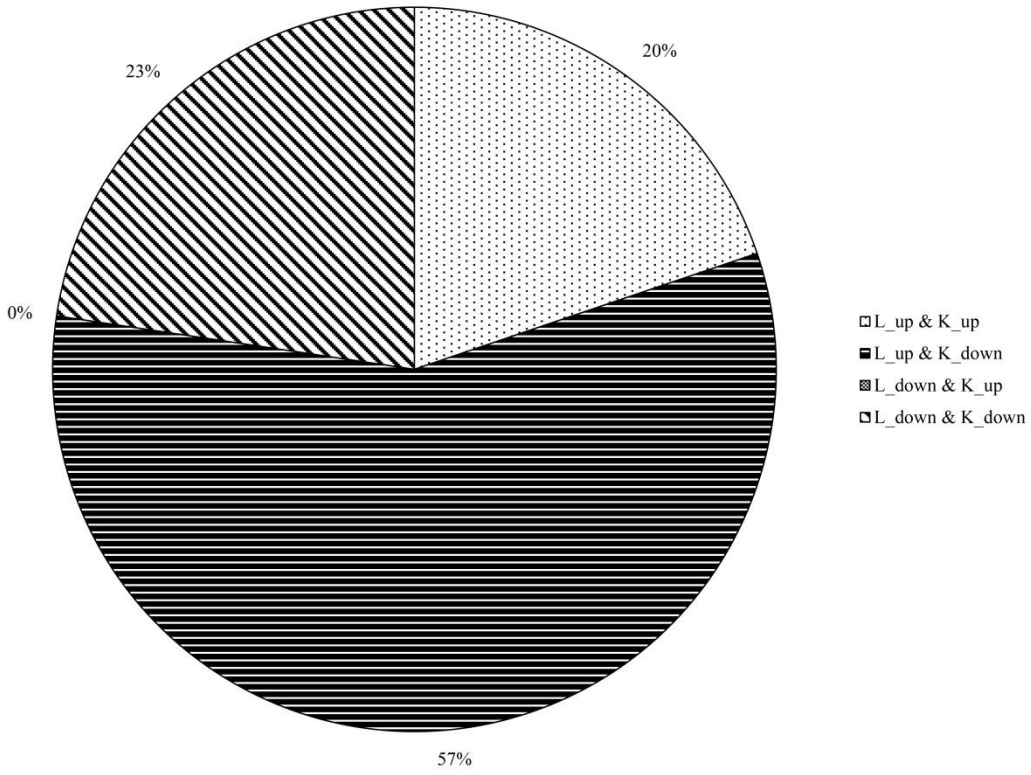


Figure 37. Analysis of differentially expressed genes related to the RNA transport pathway. 106 genes related to the pathway were assigned based on the expression pattern by treatment of K222212 and K21877. L_up and K_up represent the up-regulated genes by K222212 (L) and K21877 (K), respectively. L_down and K_down also represent the down-regulated genes by K222212 (L) and K21877 (K), respectively.

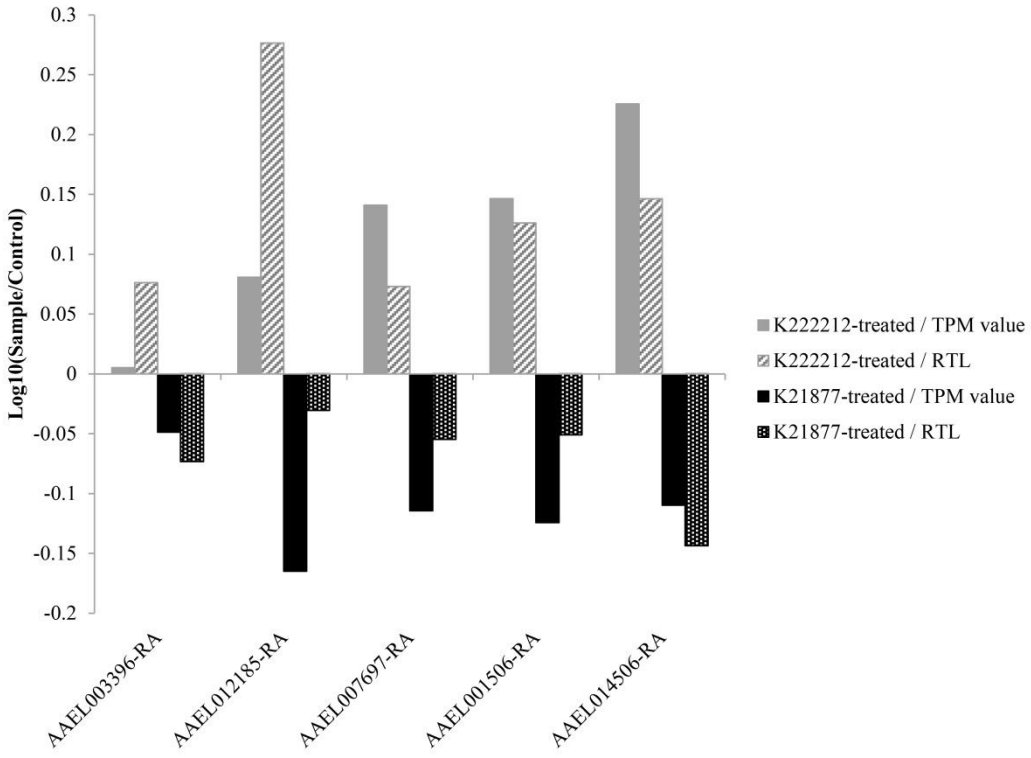


Figure 38. Validation of the Illumina sequencing result of translation-related genes by qPCR. The relative transcription levels were demonstrated in log10 scale of IGR-treated to control sample.

Aedes mosquitoes have shown a remarkable ability to develop resistance to insecticides. Therefore, resistance to commercially available insecticides, such as pyrethroids, is widespread in *Aedes* mosquitoes (Brown, 1986). In this study, novel JHA and JHAN compounds were identified by using yeast two-hybrid *β*-galactosidase binding assay and these compounds were characterized as *in vivo* modulation of JH-regulated physiological functions such as expression of JH-responsive genes and vitellogenesis. However, it could be possible that the JHA and/or JHAN compounds may also have cytotoxicity to *Aedes* mosquitoes. Therefore, the differential expressions of the genes which are related to detoxification of pyrethroids, resistance strain of *A. aegypti*, were selected. In RNA-seq data, 17 and seven of those genes were up- and down-regulated by loreclezole hydrochloride and K21877 treatment, respectively. Especially, *cytochrome p450 CYP9J27 v2* (AAEL014607-RA) gene is the most typical gene in CYP9 family of Cytochrome P450 genes that over-expressed in pyrethroids resistance strain of *A. aegypti* (Ishak et al., 2017) and this gene was validated by qPCR. These results demonstrated that both JHA and JHAN have cytotoxicity to *A. albopictus* and this cytotoxicity induces the genes related to detoxification of pyrethroids in *Aedes* mosquito. This cytotoxicity is important advantages to control mosquito. The first advantage is that both loreclezole hydrochloride and K21877 have not only general advantage of JH-related IGR, but also comparatively rapid mosquitocidal activity. This is important to overcome the disadvantage that JH-related IGR do not cause immediate knockdown. Another advantage is the possibility that those compounds could use for control of resistance mosquitoes to commonly used insecticides.

Nearly 20 years ago, the application of exogenous dsRNA to interfere with translation

of the homolog endogenous mRNA was reported and called this process RNA interference (RNAi) (Fire et al., 1998). This technique was described as 'post-transcriptional gene silencing' (Fire, 2007). RNAi soon proved to be useful in several research fields such as gene function determination in genomics. In agriculture, it shows great potential for pest control because of its high specificity (Gordon and Waterhouse, 2007; Price and Gatehouse, 2008). A new approach to control mosquitoes using the RNA-seq data from *A. albopictus* treated with exogenous JHA or JHAN was tried. If the candidate genes for RNAi target are selected, the genes could be essential for survival of the mosquito. For a new approach, nine genes were selected, and the proteins that coded by the candidate genes have essential functions such as metabolism-related (AAEL004042-RB; probable glucosamine 6-phosphate N-acetyltransferase, AAEL000321-RA; acetyl-coenzyme A synthetase, AAEL007653-RA; allantoinase), signal transduction-related (AAEL012001-RA; *Anopheles gambiae* PEST galectin) and structure-related (AAEL013789-RA; cuticle-like, AAEL007745-RA; blood vessel epicardial, AAEL002191-RA; adult cuticle 1-like, AAEL002185-RA; cuticle, AAEL013139-RG; cytokinesis actomyosin contractile ring assembly). To identify availability of the candidate genes for RNAi, expression level of those genes were calculated by qPCR. The qPCR result demonstrated that the transcription levels of those genes were down-regulated by exogenous JHA and JHAN, and the results were in good accordance with the sequence analysis result. If mosquitocidal activities of dsRNAs of those genes are proven through further studies, the processing of dsRNA for silencing of the genes could be as effective as the processing of loreclezole hydrochloride or K21877 in mosquito. These results could provide important genetic information about the RNAi

for mosquito control.

In conclusion, to investigate the transcriptional responses of the mosquito treated with JHA and JHAN, comprehensive transcriptome sequencing of adult female of *A. albopictus* topically applied with loreclezole hydrochloride and K21877 was performed. Total of 56 GO terms were up- and down-regulated by loreclezole hydrochloride and K21877 treatment, respectively. Also, through the modulation of translation-related genes, such as the genes that related to Ribosome biogenesis in eukaryotes and RNA transport pathway, the relationship between JH-dependent interaction of Met and the translation were identified. The results from investigation of the effects of JH on differential expression of immune-related genes were that the genes related to several AMPs and peroxidases were regulated by JHA and JHAN. Furthermore, new approaches to identify cytotoxicity of loreclezole hydrochloride and K21877 and to select candidate genes for mosquito control using dsRNA-based JH-responsive genes provided a clue to overcome the disadvantage that JH-related IGR and information for mosquito control used dsRNA. Therefore, these results will provide important information for the transcriptional responses treated with JHA and JHAN in mosquito.

GENERAL DISCUSSION

Among various insects, several insects need to be controlled because they not only cause severe economic damage to agricultural products but also act as vectors of human diseases (Boyer et al., 2012; Hill et al., 2005). Chemical insecticides have been commonly used to minimize the economic losses and to protect public health. Especially, among the commonly used chemical insecticides, neuroactive insecticides are effective to protect crops, agricultural products, livestock, and people. The major nerve targeting insecticides such as organophosphates, methylcarbamates, neonicotinoids, and pyrethroids act rapidly to stop crop and agricultural product damage and disease transmission. However due to their toxicity to the environment, safety issues to non-target organisms, and the development of resistance, sustained use of these insecticides are limited (Casida and Durkin, 2013). Actually, they have a limited scope of application when insecticides are used to control of urban pests, household pests or their resistant pests. Therefore, there are growing demands for novel insecticides which are more effective and safe than currently used insecticides.

IGRs can be highly potent, effective, and selective insecticides and are enough alternatives to overcome the limitation of using chemical insecticides for pest control. Among types of IGRs, based on non-target insect and safety tests conducted in industry, government, and academic laboratories, JHAs appear to be safer insecticides when compared to the neurotoxic insecticides used in pest management. Various JHAs such as methoprene, fenoxycarb, and pyriproxyfen have been used for control of several insects

in field. However, use and development of JHA insecticides also were limited because there are several recent reports about the resistance to JHAs and the lack of understanding of mode of actions of JHAs at the molecular level.

Therefore, there is an obvious need for more studies about identification and development of novel JH-related IGR insecticides which have a new structure and a new mode of action of JH-related IGRs. For these studies, *Aedes* mosquitoes are the most suitable target insects with many reasons. Firstly, *Aedes* mosquitoes are major vector insects causing public health problems became a biggest threat to many people, therefore the mosquito control is prerequisite to protect public health. Among *Aedes* mosquitoes, *A. aegypti* and *A. albopictus* are aggressive pests and efficient disease vectors. *A. aegypti* is the main vector of important arboviruses including yellow fever, dengue, and chikungunya virus and *A. albopictus* also transmits devastating pathogens and parasites including malaria, yellow fever, dengue, West Nile, chikungunya, and zika virus (Benelli, 2015; Grard et al., 2014; Gurugama et al., 2010; Reiter et al., 2006; Sang et al., 2015; Tabachnick, 1991; Weaver, 2014). It is also important to choose the proper insecticides for control of mosquito because these mosquitoes are the most important household and urban pests. Secondly, various JHAs have already been used mosquito control. Methoprene and pyriproxyfen have been extensively tested against *Aedes* spp. and shown to be highly effective, both under laboratory and under field conditions (Ramaseshadri et al., 2012). The World Health Organization has even approved the use of methoprene in drinking water for control of mosquitoes (Glare and O'Callaghan, 1999). It means that JH-related IGR insecticides are more effective and safer alternatives than currently used chemical insecticides to control mosquitoes. Finally, the molecular mechanism of JH,

based on a receptor mediated mode of action, is relatively well established. Since the identification of a JH receptor from *Drosophila*, there have been many studies on the interaction between JH and its receptor in *Drosophila*, *Tribolium*, and *Aedes*. Especially in *Aedes*, through a yeast two-hybrid screen, several bHLH-PAS proteins were identified as partner of Met and high-throughput screening of JHAs and JHANs could be performed (Lee et al., 2015; Li et al., 2011; Shin et al., 2012).

In this study, the yeast two-hybrid β-galactosidase assays using the yeast cells transformed with the genes of JH receptor and its partner, Met-FISC of *A. aegypti* were performed to identify novel JHA and JHAN compounds. Through the screening, loreclezole hydrochloride and penfluridol were isolated based on their availability for the strength of JHA and JHAN activity, respectively. These compounds also showed high level of insecticidal activity against 3rd instar larvae of *A. albopictus*. Further studies on the insecticidal activity against *A. albopictus* treated with both JHA and JHAN compounds at the same time would provide important information about its field application.

To find more effective compounds with JH-related IGRs activity for control of mosquitoes, loreclezole hydrochloride and its derivatives were synthesized and their biological characteristics were investigated. Among these derivatives, K21877 demonstrated high level of JHAN activity. Although both loreclezole hydrochloride and K21877 have similar structures, one compound simulated the binding of *A. aegypti* Met-FISC while the other interfered with the pyriproxyfen-mediated binding of *A. aegypti* Met-FISC. Both the JHA and JHAN demonstrated high larvicidal activities, embryonic lethality and adult toxicity against *A. albopictus*, which were due to the modulation of JH-

regulated physiological functions such as expression of JH-responsive genes and follicle development. If more derivatives of loreclezole hydrochloride will be synthesized, it would provide greater insight into the relationship between chemical structure and JH-related IGR activity through the screening system.

To investigate the transcriptional responses of the *A. albopictus* treated with loreclezole hydrochloride and K21877, comprehensive transcriptome sequencing was performed and analyzed. These results suggested that JH-interaction of Met could regulate the expression of genes that related metabolic process, nucleotide binding process, and translation pathway and loreclezole hydrochloride and K21877 could modulate the JH-regulated gene expression. Although the various analyses of related immune, detoxification, and RNAi target suggested the possibility of application of JH-related IGR with currently used chemical and biological insecticides, further studies of molecular and biological aspects need to be designed to provide more information.

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곤충 유약호르몬의 기능을 교란하는

새로운 곤충 생장 조절 물질의 선별 및 특성 구명

서울대학교 대학원

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초록

ABSTRACT IN KOREAN

모기는 인간에게 흡혈을 통해 다양한 질병을 매개하는 주요 위생해충이다. 그 중 흰줄숲모기는 뎅기 바이러스, 치군군야 바이러스, 지카 바이러스 등 다양한 질병을 매개하는, 매우 공격적인 매개 해충이다. 이런 모기를 방제하기 위해 기존에는 화학적 살충제들을 일반적으로 사용해왔다. 하지만 모기를 방제하기 위해 사용하던 기존의 화학 살충제들은 환경에 대한 강한 독성 및 대상 해충에서 저항성이 발달 등의 문제로 인해 지속적으로 사용하는데 한계에 직면했다. 곤충 생장 조절 물질 (Insect Growth Regulators)은 이러한 한계를 효과적으로 극복하기 위한 대안 살충제 중 하나이다. 곤충 생장 조절 물질은 기존의 화학적 살충제와 비교하여 비교적 환경에 독성이 낮을 뿐만 아니라 목

표하는 곤충에 대해 높은 특이성을 보이는 장점이 있다. 하지만 최근 연구결과들에 따르면, 이런 곤충 생장 조절 물질들 또한 저항성이 발달하고 있다는 것이 밝혀졌다. 때문에 새로운 곤충 생장 조절물질의 개발이 절실한 시점이다.

본 연구에서는 이집트 숲모기의 유약호르몬 (Juvenile hormone) 수용체인 Methoprene-tolerant (Met) 단백질과 그 파트너 단백질인 Ftz-F1-interacting steroid receptor coactivator (FISC)를 yeast two-hybrid system에 도입하여, β-galactosidase 검정을 통해 새로운 곤충 유약호르몬 아고니스트 (Juvenile hormone agonist) 및 안타고니스트 (Juvenile hormone antagonist) 물질들을 선별했다. 총 2,349개의 물질에 대한 검정 결과 1개의 곤충 유약호르몬 아고니스트 후보 물질과 17개의 곤충 유약호르몬 안타고니스트 후보 물질을 선별했다. 이들 후보 물질 중에서, 해당 물질들의 곤충 유약호르몬 아고니스트 및 안타고니스트 활성 및 흰줄숲모기 3령 유충에 대한 살충성을 기준으로 Loreclezole hydrochloride와 Penfluridol을 각각 곤충 유약호르몬 아고니스트 및 안타고니스트로 최종 선별했다. Loreclezole hydrochloride와 Penfluridol은 기존에 각각 진정제 (항경련제) 및 향정신성의약품으로 알려져 이었지만, 본 연구를 통해 이들이 곤충 유약호르몬 아고니스트 및 안타고니스트 활성을 나타내는 것을 밝혔다. 또한 이들은 흰줄숲모기 3령 유충에 대해 기존에 모기 방제를 위해 사용되고 있는 곤충 유약호르몬 아고니스트 살충제인 Pyriproxyfen 보다 높은 살충활성을 나타내는 것을 확인했다.

그리고 모기에 대해 보다 높은 살충활성을 나타내는 곤충 유약호르몬 관련 곤충 생장 조절 물질을 선별하기 위해, 곤충 유약호르몬 아고니스트 활성을 나타낸 Loreclezole hydrochloride를 선도물질로 하여 다양한 구조의 유도체들을 확보하고 그들의 곤충 유약호르몬 관련 곤충 생장 조절 활성을 검정했다. Loreclezole hydrochloride를 포함한 유도체들에 대한 곤충 유약호르몬 관련 곤충 생장 조절 활성을 검정한 결과 곤충 유약호르몬 아고니스트 활성 물질로는 선도물질인 Loreclezole hydrochloride, 곤충 유약호르몬 안타고니스트 활성 물질로는 K21877 물질을 선별했다. 선별한 Loreclezole hydrochloride와 K21877 물질은 유사한 화학구조를 가졌음에도 불구하고 곤충 유약호르몬 수용복합체의 형성에 대하여 반대되는 활성을 나타냈다. 또한 이들 물질은 기존에 모기 방제를 위해 사용되고 있는 곤충 유약호르몬 아고니스트 살충제인 Pyriproxyfen에 비해 흰줄숲모기 전 령기에 대해 높은 살충활성을 나타낼 뿐 아니라, 흰줄숲모기 알 및 성충에 대해 높은 배아 치사성 및 살충활성을 나타냈다. 그리고 흰줄숲모기 암컷성충에 대해 선별한 두 물질은 곤충 유약호르몬에 의해 발현이 유도되는 유전자의 전사를 효과적으로 교란하고, 곤충 유약호르몬이 중요한 역할을 하는 난소의 성장을 효과적으로 억제함으로써, 선별한 두 물질이 흰줄숲모기 내부에서 곤충 유약호르몬이 조절하는 생리작용을 효과적으로 교란할 수 있음을 시사했다.

마지막으로 곤충 유약호르몬 아고니스트 활성을 나타내는 Loreclezole hydrochloride와 안타고니스트 활성을 나타내는 K21877 물질을 흰줄숲모기

암컷 성충에 처리하고 RNA-seq을 진행하여, 곤충 유약호르몬 아고니스트 및 안타고니스트에 의한 흰줄숲모기의 전사체 수준의 반응을 분석했다. 그 결과 곤충 유약호르몬과 그 수용체 단백질인 Met에 의해 metabolic process, nucleotide binding process 및 translation pathway에 관련된 유전자들의 전사가 조절되고, 본 연구에서 선별한 곤충 유약호르몬 아고니스트 및 안타고니스트 활성 물질인 Loreclezole hydrochloride와 K21877 물질이 흰줄숲모기 내에서 곤충 유약호르몬이 조절하는 유전자들의 전사를 효과적으로 교란할 수 있음을 시사했다.

검색어: Insect growth regulators, Juvenile hormone, Juvenile hormone receptor complex, Juvenile hormone agonist, Juvenile hormone antagonist

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